UvA-DARE (Digital Academic Repository)

Myocardial microcirculation and mitochondrial energetics in the isolated rat heart

Ashruf, J.F.

Publication date

2015

Document Version

Final published version

[Link to publication](#)

Citation for published version (APA):

Ashruf, J. F. (2015). *Myocardial microcirculation and mitochondrial energetics in the isolated rat heart*. [Thesis, fully internal, Universiteit van Amsterdam].

General rights

It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations

If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: <https://uba.uva.nl/en/contact>, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

MYOCARDIAL MICROCIRCULATION AND MITOCHONDRIAL ENERGETICS IN THE ISOLATED RAT HEART

Jesse Feisal Ashruf

MYOCARDIAL MICROCIRCULATION AND MITOCHONDRIAL ENERGETICS IN THE ISOLATED RAT HEART

Jesse Feisal Ashruf

ISBN: 978-94-91715-07-5

Layout and printing: Netzodruk

Cover design: Talin and Jesse Ashruf

Copyright © Jesse F. Ashruf, Groningen, 2015. All rights reserved. No part of this publication may be reproduced, stored in a retrieval system, or transmitted in any form or by any means mechanically, by photocopy, by recording, or otherwise, without the prior written permission of the holder of copyright.

MYOCARDIAL MICROCIRCULATION AND MITOCHONDRIAL ENERGETICS IN THE ISOLATED RAT HEART

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor

aan de Universiteit van Amsterdam

op gezag van de Rector Magnificus

prof. dr. D.C. van den Boom

ten overstaan van een door het College voor Promoties ingestelde commissie,

in het openbaar te verdedigen in de Aula der Universiteit

op woensdag 4 november 2015, te 11:00 uur

door

Jesse Feisal Ashruf

geboren te Paramaribo, Suriname

Promotiecommissie

Promotor:	prof. dr. ir. C. Ince	Universiteit van Amsterdam
Copromotores:	dr. ir. C.J. Zuurbier	Universiteit van Amsterdam
	dr. D.M.J. Milstein	Universiteit van Amsterdam
Overige leden:	prof. dr. T.W.L. Scheeren	Rijksuniversiteit Groningen
	dr. E.G. Mik	Erasmus Universiteit Rotterdam
	prof. dr. R.J.A. Wanders	Universiteit van Amsterdam
	prof. dr. ir. J.A.E. Spaan	Universiteit van Amsterdam
	prof. dr. R.J. de Winter	Universiteit van Amsterdam

Faculteit der Geneeskunde

Ter nagedachtenis aan mijn dierbare moeder

TABLE OF CONTENTS

1	General introduction; myocardial microcirculation and mitochondrial energetics in the isolated rat heart	9
2	Heterogeneity of the hypoxic state in rat heart is determined at capillary level	17
3	Regional ischemia in hypertrophic Langendorff-perfused rat hearts	33
4	Imminent ischemia in normal and hypertrophic Langendorff rat hearts; effects of fatty acids and superoxide dismutase monitored by NADH surface fluorescence	49
5	Increase of cardiac work is associated with decrease of mitochondrial NADH	61
6	In vivo NADH- and Pd-porphyrin-videofluori-/phosphorimetry	77
7	Optical spectroscopic imaging for non-invasive evaluation of tissue oxygenation	86
8	Epicardial NADH fluorescence of the isolated perfused rat heart reveals mitochondrial dysfunction in early endotoxic shock	97
9	New insights into the pathophysiology of cardiogenic shock: the role of the microcirculation	114
10	Summary and conclusions	125
	References	133
	Portfolio	149
	List of publications	150
	Dankwoord	152

1

GENERAL INTRODUCTION;

MYOCARDIAL MICROCIRCULATION AND MITOCHONDRIAL ENERGETICS IN THE ISOLATED RAT HEART

Ashruf JF¹, Ince C¹

¹Department of Translational Physiology, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands

Accepted for publication in Adv Exp Med Biol, 2015

ABSTRACT

Normal functioning of myocardium requires adequate oxygenation, which in turn is dependent on an adequate (micro-)circulation. NADH-fluorimetry enables a direct evaluation of the adequacy of tissue oxygenation while the measurement of quenching of Pd-porphyrin phosphorescence enables quantitative measurement of microvascular pO_2 . Combination of these two techniques provides information about the relation between microvascular oxygen content and parenchymal oxygen availability in Langendorff hearts. In normal myocardium there is heterogeneity at the microcirculatory level resulting in the existence of microcirculatory weak units, originating at the capillary level, which reoxygenate the slowest upon reoxygenation after an episode of ischemia. Sepsis and myocardial hypertrophia alter the pattern of oxygen transport whereby the microcirculation is disturbed at the arteriolar/arterial level. NADH-fluorimetry also reveals a disturbance of mitochondrial oxygen availability in sepsis. Furthermore it is shown that these techniques can also be applied to various organs and tissues in vivo.

INTRODUCTION

Oxygen is transported to the cells via the microcirculation, which is composed of terminal arterioles, capillaries and venules. Cellular ischemia or hypoxia results in an increase of the mitochondrial NADH/NAD⁺ ratio (redox state), which can be measured using NADH-video-fluorimetry [1]. Using this technique, it was found that there are areas in the healthy myocardium that are more vulnerable to dysoxia; these areas are reproducible in time and place and originate at the capillary level and are therefore closely associated with the architecture of the microcirculation. These weak units show the largest relative reduction in flow (independent of absolute flow levels) during compromising conditions, with dysoxia initially developing at the venous end of the capillary [2, 3]. Myocardial hypertrophy, work and sepsis have a profound effect upon the distribution of oxygen in the microcirculation as is shown by measuring the mitochondrial redox state under these conditions.

Another factor influencing NADH fluorescence intensity is photo-bleaching. Earlier studies showed a gradual decline in fluorescence intensity over time; it was suggested that this was caused by a steady decline of endogenous substrate eventually making the heart totally dependent on substrate in the perfusate. We showed however that this decline is not actually caused by an altered ratio, but by photo-bleaching caused by continuous illumination of the organ surface with UV-light. Applying discontinuous illumination nearly completely eliminated this effect [4, 5].

In myocardial hypertrophy a dysregulation of oxygen distribution can be found at the arteriolar level. Alleviation of ischemia at the arteriolar level can be induced by vasodilation but probably also by preventing acidification of the interstitium by fatty-acid oxidation (thereby preventing lactic acid formation). Scavenging of oxygen free radicals by SOD also improves oxygenation of critical areas in hypertrophic myocardium.

Increase in work results in a homogeneous decrease in NADH/NAD⁺ ratio when precautions are taken to avoid development of ischemia/dysoxia. This is of importance because the development of ischemia can cause one to assume that increase in work actually increases the NADH/NAD⁺ ratio.

The NADH/NAD⁺ ratio can also be measured in vivo in heart and gut of rats and it is shown that changes in the ratio are associated with changes in oxygen content of parenchyma and circulation using Pd-phorphyrine phosphorescence measurements.

Sepsis has a complex effect on the redox state: on the one hand it is known that sepsis induces ischemia through microcirculatory changes; on the other hand there is a strong suggestion that sepsis also changes mitochondrial functioning resulting in a decreased rate of oxygen consumption.

DISTRIBUTION OF OXYGEN TO MYOCARDIUM IN NORMAL AND HYPERTROPHIC RAT HEARTS

Measurement of the intracellular autofluorescence of reduced pyridine nucleotide on myocardial tissue surfaces (NADH; 360-nm excitation, 460-nm emission), pioneered by Barlow et al. [1], allows identification of areas of cellular hypoxia, because reduction of NADH is dependent on an adequate supply of oxygen. Using NADH fluorescence photography of the surface of Tyrode-perfused rat hearts, Barlow et al. [1] found that

lowering coronary perfusion results in patchy NADH fluorescence. Steenbergen et al. [6] found that this heterogeneity in the hypoxic state occurred under ischemic as well as during high-flow hypoxic conditions.

Ince et al. found that this heterogeneity originates at the capillary level and that the ischemic patches occurring during reperfusion after hypoxia represent microcirculatory units furthest away from the oxygen supply. These microcirculatory units also were the first areas to become ischemic during increased cardiac work in Langendorff hearts; it was also shown that these areas were identical to those occurring during reperfusion and during embolization with microspheres roughly having the size of capillaries [2, 7, 8].

Hypertrophia is known to be associated with a decreased muscle mass to vascularization ratio and thus a change of myocardial perfusion when compared to normal hearts [9-12]. Hulsmann et al. [13] found that hypertrophic Langendorff rat hearts displayed ischemic areas during normoxic perfusion whereas normal heart under the same circumstances did not. The relative hypoperfusion in these areas was shown to be caused by acidosis and oxygen free radicals (OFR), since measures taken to prevent acidosis (by stimulating fatty acid oxidation) and OFR production (by adding super oxide dismutase to the perfusate) dramatically improved local hypoxia. In a further study [7] it was shown that perfusion is altered at the arteriolar and arterial level in hypertrophia.

An important observation in experiments with Langendorff hearts using NADH fluorescence imaging is that the Langendorff heart is borderline hypoxic, and in case of higher workloads, even partly hypoxic. This can seriously affect the interpretation of the results and can even render the experiment useless [2, 5, 7, 8, 12].

The NADH/NAD^+ ratio is an effective parameter for the evaluation of tissue hypoxia. It was also known that this ratio can be influenced by other factors besides the lack of oxygen. For instance, earlier studies showed that increased cardiac work was associated with an increased NADH/NAD^+ ratio: it was suggested that this increased ratio actually drives the electron chain and thus ATP production. However, in our studies [2, 4, 5] we found that this increase was caused by development of ischemia in borderline normoxic Langendorff hearts which became hypoxic during increased workload. Instead we found that during increased workload, when securing adequate oxygen supply to the myocardium, the NADH/NAD^+ ratio actually decreased, making it very unlikely that during increased workload NADH drives the electron chain of oxidative phosphorylation. Several studies, for example the study by Territo et al. [13], have shown that the changing extramitochondrial $[\text{Ca}^{2+}]$ during changes in workload probably is the most important signal to cause an increased oxidative phosphorylation.

Another factor influencing this ratio is the type of substrate used in the perfusate: pyruvate increases the ratio more than glucose [4]. In our study of the effect of cardiac work on the NADH/NAD^+ ratio, we observed that during perfusion with glucose as a substrate, the work-related decrease of the ratio was much slower than during perfusion with pyruvate as a substrate. An explanation for this might be that pyruvate enters the Krebs cycle directly, thus reducing NAD^+ relatively quickly, whereas glucose first enters the glycolysis, which is a slower process when compared to the Krebs cycle and speeds up more slowly in case of increased workload. However, more investigations are needed to explain this observation.

DISTRIBUTION OF OXYGEN TO MYOCARDIUM IN SEPTIC RAT HEARTS

Avontuur et al. [14] showed that hearts of septic rats during Langendorff perfusion develop regional ischemia when coronary flow is reduced with N^G -nitro-L-arginine (NNLA) or Methylene blue (MB). NNLA and MB both inhibit the effects of nitric oxide (NO). This finding suggests that endotoxemia promotes myocardial ischemia in vulnerable areas of the heart after inhibition of the NO pathway or direct vasoconstriction. A further study by Avontuur et al. showed that sepsis induces massive coronary vasodilation due to increased myocardial NO synthesis, resulting in autoregulatory dysfunction.

Indeed, in a recent study by us (unpublished data, to be submitted) Langendorff perfused hearts from septic rats appear to develop hypoxic areas which are larger than the heterogeneous areas during reperfusion and embolization with microspheres of 5.9 μm diameter [2, 8]. This agrees with the finding that autoregulation is disturbed [14]; autoregulation is situated at the arterial and arteriolar level and these hypoxic areas were larger than those elicited by embolization of capillaries and are comparable in size to those found in hypoperfused areas in hypertrophy and during embolization of arterioles and arteries [7, 8]. The result of these effects of sepsis on the microcirculation can be described as shunting of the microcirculation [16], which means that certain portions of the myocardial capillary network remain hypoperfused and other portions receive a higher than needed (for adequate oxygenation) capillary flow. We also found that mitochondrial function in septic hearts was diminished, as the development of hypoxia during interrupted perfusion was slower than in normal hearts.

IN VIVO EVALUATION OF HYPOXIA AND ISCHEMIA

NADH-videofluorimetry has been shown to be an effective method to assess tissue hypoxia in numerous studies *ex vivo*. Clinical applicability however requires *in vivo* application of this measurement and therefore we performed several studies to assess the possibility of *in vivo* application of NADH-videofluorimetry on various organs (heart, intestines, skeletal muscle) [16-21]. It was shown, for instance, that with this technique it was possible to detect local hypoxia *in vivo* in myocardium induced by selective ligation of coronary arteries in rat heart [18]. However, *in vivo* NADH fluorescence is disturbed by movement, hemodynamic and oximetric effects. A method was developed to compensate for these factors by means of utilizing the NADH-fluorescence/UV-reflectance ratio, making it possible to monitor the mitochondrial redox state of intact blood-perfused myocardium [20].

Furthermore, measurement of quenching of Pd-porphyrin-phosphorescence enabled us to evaluate microvascular pO_2 . Combined with NADH fluorescence measurements this enabled us to correlate the mitochondrial energy state to the microvascular pO_2 [17-19].

Van der Laan et al. [21] explored the possibility to evaluate ischemia and reperfusion injury in skeletal muscle (in rat) and found a clear correlation between tissue hypoxia and NADH fluorescence intensity.

RECENT DEVELOPMENTS

A new technique for evaluation of the microcirculation is sidestream dark-field (SDF) imaging, mainly applied to observe the sublingual microcirculation. It has provided great insight into the importance of this physiological compartment in (perioperative) medicine and could prove to become a useful tool in treatment of shock, based on evaluation of the microcirculation [22].

A major disadvantage of the use of NADH-videofluorimetry for obtaining information about mitochondrial bioenergetics and oxygenation is that it is a non-quantitative technique relying on relative changes in fluorescence signals. Recently we introduced a new method for a quantitative measurement of mitochondrial pO_2 (mit pO_2) values. The method is based on the oxygen dependent decay of delayed fluorescence of endogenously present mitochondrial protoporphyrin IX (P_pIX) [24]. Proof of concept measurements were initially performed by Mik et al. in single cells and later validated in vivo in rat liver and recently also in the heart [24, 25]. Deconvolution of the decay curves allowed mit pO_2 histograms to be generated and showed a heterogeneous distribution of mit pO_2 values. Ischemia-reperfusion injury was shown to induce mit pO_2 values with hypoxic as well as hyperoxic values [24].

One of the remarkable measurements of the mit pO_2 values was the finding of mit pO_2 values much higher (20-30 mmHg) than previously thought (less than 5 mmHg) and secondly the finding of markedly heterogeneous mit pO_2 values, similar to the distributions of microvascular pO_2 measurements using P_pIX quenching of phosphorescence we had measured in heart [24]. Indeed (early) observations of the heterogeneous state of myocardial and mitochondrial energy states underscores the importance of heterogeneity of oxygen delivery and utilization [3]. It is expected that the combination of NADH-fluorimetry and mit pO_2 measured by P_pIX -phosphorescence will provide new information concerning the transport and consumption of oxygen by the heart.

REFERENCES

- 1 Barlow CH, Chance B (1976). Ischemic areas in perfused rat hearts: measurement by NADH fluorescence photography. *Science*; 193: 909-910
- 2 Ince C, Ashruf JF, Avontuur JAM et al. (1993). Heterogeneity of the hypoxic state in rat heart is determined at capillary level. *Am J Physiol*; 264: H294-H301
- 3 Zuurbier CJ, van Iterson M, Ince C (1999). Functional heterogeneity of oxygen supply-consumption ratio in the heart. *Cardiovasc Res*; 44(3): 488-497
- 4 Ashruf JF, Coremans JM, Bruining HA et al. (1995). Increase of cardiac work is associated with decrease of mitochondrial NADH. *Am J Physiol*; 269: H856-H862
- 5 Ashruf JF, Coremans JM, Bruining HA et al. (1996). Mitochondrial NADH in the Langendorff rat heart decreases in response to increases in work: increase of cardiac work is associated with decrease of mitochondrial NADH. *Adv Exp Med Biol*; 388: 275-282
- 6 Steenbergen CG, Deleeuw C, Barlow B et al. (1977). Heterogeneity of the hypoxic state in perfused rat heart. *Circ Res*; 4: 606-615
- 7 Ashruf JF, Ince C, Bruining HA et al. (1994). Ischemic areas in hypertrophic Langendorff rat hearts visualized by NADH-videofluorimetry. *Adv Exp Med Biol*; 345: 259-262
- 8 Ashruf JF, Ince C, Bruining HA (1999). Regional ischemia in hypertrophic Langendorff-perfused rat hearts. *Am J Physiol*; 277: H1532-H1539
- 9 Anderson PG, Bishop SP, Digerness SB (1987). Transmural progression of morphologic changes during ischemic contracture and reperfusion in the normal and hypertrophied rat heart. *Am J Pathol*; 129: 152-167
- 10 Dellsperger KC, Marcus LM (1990). Effects of left ventricular hypertrophy on the coronary circulation. *Am J Cardiol*; 65: 1504-1510
- 11 Einzig SJ, Leonard J, Tripp MR et al. (1981). Changes in regional myocardial blood flow and variable development of hypertrophy after aortic banding in puppies. *Cardiovasc Res*; 15: 711-723
- 12 Hulsmann WC, Ashruf JF, Bruining HA et al. (1993). Imminent ischemia in normal and hypertrophic Langendorff rat hearts: effects of fatty acids and superoxide dismutase monitored by NADH surface fluorescence. *Biochim Biophys Acta*; 1181: 273-278
- 13 Territo PR, French SA, Dunleavy MC et al. (2001). Calcium activation of heart mitochondrial oxidative phosphorylation: rapid kinetics of mVO_2 , NADH, and light scattering. *J Biol Chem*; 276(4): 2586-2599
- 14 Avontuur JA, Bruining HA, Ince C. (1995). Inhibition of nitric oxide synthesis causes myocardial ischemia in endotoxemic rats. *Circ Res*; 76(3): 418-425
- 15 Ince C, Sinaasappel M (1999). Microcirculatory oxygenation and shunting in sepsis and shock. *Crit Care Med*; 27(7): 1369-1377
- 16 Ince C, Coremans JMCC, Bruining HA (1992). In vivo NADH fluorescence. *Adv Exp Med Biol*; 317: 277-296
- 17 Bruining HA, Pierik GJ, Ince C et al. (1992). Optical spectroscopic imaging for non-invasive evaluation of tissue oxygenation. *Chirurgie*; 118(5): 317-322; discussion 323
- 18 Ince C, Ashruf JF, Sanderse EA et al. (1992). In vivo NADH- and Pd-porphyrin-videofluorimetry. *Adv Exp Med Biol*; 317: 267-275
- 19 Ince C, van der Sluijs JP, Sinaasappel M et al. (1994). Intestinal ischemia during hypoxia and experimental sepsis as observed by NADH-videofluorimetry and quenching of Pd-porphyrin phosphorescence. *Adv Exp Med Biol*; 36: 105-110

- 20 Coremans JMCC, Ince C, Bruining HA (1997). (Semi-)quantitative analysis of reduced nicotinamide adenine dinucleotide fluorescence images of blood-perfused rat heart. *Biophys J*; 72(4): 1849-1860
- 21 van der Laan L, Coremans JMCC, Ince C et al. (1998). NADH-videofluorimetry to monitor the energy state of skeletal muscle in vivo. *J Surg Res*; 74(2): 155-160
- 22 Ashruf JF, Ince C, Bruining HA (2013). New insights into the pathophysiology of cardiogenic shock: the role of the microcirculation. *Curr Opin Crit Care*; 19(5): 381-386
- 23 Mik EG, Stap J, Sinaasappel M et al. (2006). Mitochondrial PO₂ measured by delayed fluorescence of endogenous protoporphyrin IX. *Nature Methods*; 3(11): 939-945
- 24 Mik EG, Johannes T, Zuurbier C et al. (2008). In vivo mitochondrial oxygen tension measured by a delayed fluorescence lifetime technique. *Biophys J*; 95(8): 3977-3990
- 25 Mik EG, Ince C, Eerbeek O et al. (2009). Mitochondrial oxygen tension within the heart. *J Mol Cell Cardiol*; 46(6): 943-951

2

HETEROGENEITY OF THE HYPOXIC STATE IN RAT HEART IS DETERMINED AT CAPILLARY LEVEL

Ince C¹, Ashruf JF¹, Avontuur JAM¹, Wieringa PA², Spaan JAE³, Bruining HA¹

¹Department of Surgery, Erasmus University of Rotterdam, Rotterdam

²Department of Mechanical Engineering, University of Technology Delft, Delft

³Department of Medical Physics, University of Amsterdam, Amsterdam, the Netherlands

Published in: Am J Physiol; 264: H294-H301, 1993

SUMMARY

Heterogeneity in the hypoxic state of Tyrode-perfused rat hearts was studied using NADH- and Pd-porphyrin-videofluorimetry. Ischemic as well as high-flow anoxia resulted in a homogeneous rise of tissue NADH fluorescence, whereas normoxic recovery from both types of anoxia caused transiently persisting patchy fluorescent areas. Patterns were always the same for a given heart. pO_2 distribution in the vasculature measured by Pd-porphyrin phosphorescence showed patterns similar to the NADH fluorescence patterns. Microsphere embolization of the capillaries, but not of arterioles, elicited identical NADH fluorescence patterns as seen during recovery from anoxia without microspheres. High heartbeat rates also caused patchy fluorescent areas but not in the presence of adenosine. Patterns corresponded to those seen during normoxic recovery from anoxia under low beat rates. It is concluded that there are circulatory units in the rat heart at the capillary level that result in the temporary persistence of anoxic areas during recovery from anoxia. These vulnerable areas are the first to be compromised during high heartbeat rates.

INTRODUCTION

Measurement of the intracellular autofluorescence of reduced pyridine nucleotide (NADH; 360-nm excitation, 460-nm emission), pioneered by Chance et al. (5, 6), allows evaluation of the mitochondrial energy state, since NADH fluoresces when excited with ultraviolet light and oxidized NAD^+ does not (8, 10, 13). Measurement of the fluorescence of NADH on tissue surfaces thus allows identification of areas of cellular anoxia, because the reduction of pyridine nucleotide is closely related to the presence of available oxygen in the mitochondria (6). Using NADH fluorescence photography of the surface of Tyrode perfused rat hearts, Barlow and Chance (2) found that lowering coronary perfusion results in patchy NADH fluorescence. Steenbergen et al. (17) found that this heterogeneity in the hypoxic state occurred under ischemic as well as during high-flow hypoxic conditions.

The heterogeneous patchy NADH fluorescence patterns could be caused by a heterogeneous availability of oxygen in the myocardium. If this were the case, it would mean that it is either the structure of the coronary supply vessels or the spatial diffusion and capricious flow patterns within the capillary network that determine the patchy areas of tissue anoxia. Two main types of models related to these possible mechanisms can be considered. The first model is based on observations by Bassingthwaighe et al. (3), who found a continuous network of capillaries in the dog heart. This infinite mesh network suggests the presence of numerous pathways between arterioles and venules (19). Main parallel capillaries could be followed over many millimeters with numerous cross capillaries connecting adjacent main capillaries and with arterioles and venules connected to the network. Because of the structure of the network, however, it is difficult to establish on the basis of anatomy alone which arterioles are supplying which capillary segments. The second model is that of the circulatory unit where it is assumed that each arteriole is feeding exclusively a group of capillaries and myocytes. Such an area is then called a circulatory unit. Patchy patterns in NADH fluorescence have been related to both concepts. Steenbergen et al. (17) suggested that the NADH regions were determined by hypoperfusion of microcirculatory units consisting of capillary beds fed by specific arterioles. Rumsey et al. (15), however, argued that the NADH regions might well be explained by a more or less continuous diffusion field not necessarily associated with microcirculatory units, in which steep oxygen gradients occur down capillary vessels. Apart from these two concepts related to oxygen transfer, it has also been suggested that the sharp demarcations in NADH fluorescence are related to the "on or off" condition of myocytes: either normoxic or anoxic (21).

The present study was designed to elucidate the mechanisms underlying the heterogeneous NADH fluorescence patterns. As mentioned above, the patchy patterns of NADH fluorescence could be caused by heterogeneity in oxygen supply. A possible heterogeneity in demand, however, also needs to be considered. This issue is addressed by comparing the appearance and disappearance of the NADH fluorescence induced by periods of flow cessation or anoxic perfusion. Moreover, the tissue NADH fluorescence distribution is compared with the oxygen pressure distribution in the vascular bed as measured by Pd-porphyrin phosphorescence (16, 22). If the patterns of NADH fluorescence are caused by heterogeneous supply of oxygen it is necessary to estimate at which level within the microcirculation, as defined by vessel diameter, these patterns are established. This was

done by comparing fluorescence patterns during recovery from an anoxic episode to those induced by blocking vessels with microspheres of different diameters. It was hypothesized that the microsphere diameter inducing similar patterns would indicate the microcirculatory level at which the inhomogeneities originate. Finally, the effect of an increase in heart rate was studied. Such an intervention has two effects that possibly could influence the distribution of the anoxic regions: an increase in metabolism and also an alteration in oxygen supply caused by vascular compression induced by increased heart rate. Preliminary results of this study have been reported elsewhere (1, 11).

MATERIALS AND METHODS

Experimental setup. Male Wistar rats weighing 250-450 g were anesthetized by ether inhalation. Before the sternotomy, the rats were given 200 IU/kg heparin (Thromboliquine, Organon Teknika Oss, the Netherlands). The hearts were perfused according to Langendorff and were allowed to beat freely except in the enhanced-pace rate experiments, when atrial leads were used to provide electrical pulses. The experimental setup consisted of two independent containers: one with Tyrode equilibrated with 95% O₂-5%CO₂ and the other with Tyrode equilibrated with 95% N₂-5% CO₂. A stopcock switch made it possible to change rapidly from one perfusate to the other. Perfusates were maintained at 37°C and aortic pressure at 80 mmHg. Mean flow rates (expressed as ml·min⁻¹·g⁻¹ ventricle wetwt) were measured with an electromagnetic flow probe (Skalar-Medical BV, Delft, the Netherlands) placed just before the aortic cannula. Above the flow probe an infusion pump allowed drugs and microsphere emulsions to be added to the perfusate. Infusion of Tyrode in the amount needed to supply a substance via this pump did not influence the temperature or pH of the perfusate and did not affect the heart rate. The perfusate consisted of (in mM) 128 NaCl, 4.7 KCl, 1 MgCl₂, 0.4 NaH₂PO₄H₂O, 1.2 Na₂SO₄, 20.2 NaHCO₃, 1.3 CaCl₂, and 11.0 glucose. These chemicals and NaNO₃ were obtained from Merck (Darmstadt, Germany). Sodium adenosine was purchased from Sigma and ethyl hydrogen peroxide from Eli Lilly. Pollen was used for some of the microsphere experiments (20). The species of these pollens were *urtica dioica*, *uttica*, and *phleum pratense* with respective diameters of 15.4 ± 1.1 , 17.6 ± 1.4 and 36.0 ± 3.5 μ m (Diephuis Apotheekers, Groningen, the Netherlands). The other microsphere experiments were performed with polystyrene microspheres with diameters of 98.8 ± 5.5 , 15.8 ± 2.8 , 9.8 ± 0.3 , or 5.9 ± 0.1 μ m (Polyscience, Warrington, PA). All values are expressed as means $\pm$ SD.

Fluorescence spectroscopy. The videofluorimeter (9) was attached to a brace and positioned in front of the left ventricle of the heart. The videofluorimeter consisted of a fluorescence unit B2-RFCA of an Olympus BH2 microscope, a 100-W mercury arc lamp, and a video camera with a Micro-Nikkor 105-mm macrolens (Fig. 1). The B2-RFCA unit housed a dichroic mirror with a UG-1 barrier filter providing the 360-nm light needed for NADH excitation. A band pass filter allowed transmission of the NADH fluorescence centered around 460 ± 20 nm. The left ventricle of the heart was illuminated by the Hg arc lamp. A 1 mm thick piece of 3·3 mm uranyl fluorescence calibration glass (14) was placed next to the heart within the excitation field. This constant source of fluorescence allowed correction of the images for

changes in the sensitivity of the videofluorimeter or the intensity of the light source. Fluorescence images were recorded with an MXRi second-generation CCD video camera (HCS Vision, Eindhoven, the Netherlands) equipped with an ultraviolet blue sensitive S20 photocathode (Phillips, the Netherlands). Images were recorded on a Panasonic S-VHS professional video recorder and computer-analyzed off-line. Prints of images were made with a Mitsubishi video copy processor and analogue signals such as mean flow and pressure were recorded on a DAT instrumentation recorder (Biologic) and a strip-chart recorder (type BD111, Kipp en Zonen, Delft, the Netherlands). For presentation, the flow signals were read into the computer and printed on a laser printer (Hewlett-Packard Laser Jet III).

Fig. 1. Experimental setup used for measurements consisted of a Langendorff setup with 2 reservoirs for Tyrode. Infusion pump allows infusion of substances into the perfusate. Small piece of uranyl fluorescence calibration glass is placed next to heart as a fluorescence reference. Hg arc lamp provides the 360-nm light needed for NADH excitation and the 545-nm light for Pd-porphyrin phosphorescence. CCD video camera detects 460-nm NADH fluorescence image or 610-nm long pass Pd-porphyrin phosphorescence. Dichroic mirror (DM) separated excitation and emission light at appropriate wavelengths. Signals were recorded on video recorder and analyzed off-line.

Pd-porphyrin phosphorescence measurements. Phosphorescence measurements were made with the use of meso-tetra(4-carboxyphenyl)porphyrin (Porphyrin Products, Logan, UT) coupled to bovine serum albumin according to Wilson et al. (22). The dye was added to the perfusion medium at a final concentration of 30 μM . Pd-porphyrin phosphorescence was evoked at 545-nm excitation and with a 610-nm long pass filter. A dichroic mirror (type DM400, Olympus) was used for separation of excitation and emission light.

Analysis. The video recordings of the fluorescence and phosphorescence images were analyzed by image processing software (TCL Image, Multihouse, Amsterdam, the Netherlands) running on an Olivetti M380-XP5-386 computer. Fluorescence and phosphorescence video images were read from the video recorder frame by frame via a digitizer (Digital Production Mixer type WJ-MX10, Panasonic) into the frame grabber (Data Translation type DT2801, Marlboro, MA) of the computer. The image processing software allowed definition of areas of interest for the measurement of gray levels. The image area analyzed corresponded to 1 cm^2 of heart surface tissue. For quantification of fluorescence

level changes, fluorescence measurements were corrected for fluctuations in the camera sensitivity and of the light source intensity by 1:1 correction for changes in the uranyl glass fluorescence. Only in the Pd-porphyrin experiments was it necessary to enhance the contrast of the images. To accomplish this, background subtraction and gray level stretching routines were used. The Pd-porphyrin images are fuzzier than the NADH images because 545-nm excitation light (associated with the Pd-porphyrin) penetrates deeper into the tissue than does the 360-nm excitation light of NADH, and the emission light of Pd-porphyrin comes from deeper layers than the NADH fluorescence. Both effects result in more light scatter. The high sensitivity of the second generation video camera meant that no averaging of the video frames was needed.

Experimental protocols. The rat hearts were mounted in the setup shown in Fig. 1 and allowed to stabilize for at least 15 min before the following types of experiments were performed. **1)** The control protocol for eliciting heterogeneous anoxic areas commenced with normoxic perfusion. High-flow anoxia was induced by switching the perfusate from oxygen- to nitrogen-saturated Tyrode with 5% CO₂ for 2 min. After this period of anoxia, normoxic perfusion was restored. Once control flow level was established, the stopcock was closed for 2 min to create an ischemic anoxia. Recovery from this episode of anoxia was accomplished by restoring normoxic perfusion. This induces a reactive hyperemic response followed by a return to control flow levels. **2)** To assess the vasodilatory effects of adenosine on NADH fluorescence, the control protocol was carried out in the presence of 10 μ M adenosine. **3)** To study the relation between the anoxic tissue areas identified by NADH fluorescence and the oxygen pressure distribution in the coronary vasculature, Pd-porphyrin bound to albumin was used. After the control protocol was carried out to record the heterogeneous NADH fluorescence patterns, Pd-porphyrin-albumin compound was added to the perfusate (final concentration 30 μ M), excitation and emission wavelengths were changed to that for Pd-porphyrin, and the control protocol was repeated once more. **4)** In these experiments, heterogeneous anoxic areas identified by control protocol were compared with those evoked by micro-embolization of the vasculature by addition of microspheres to the perfusate. This protocol was carried out with batches of microspheres that had different diameters. First, during normoxic perfusion, adenosine at a final concentration of 10 μ M was added to the perfusate to maximally dilate the vasculature. Perfusion was then switched to nitrogen-saturated Tyrode for 2 min and then returned to oxygen-saturated Tyrode. During this procedure the heterogeneous NADH fluorescence patterns were identified. Then, microspheres of fixed diameters emulsified in Tyrode were infused at a rate of $\approx$ 1000 microspheres/min into the perfusate mainstream. The microsphere solution was stirred constantly by a small magnet stirrer placed in the infusion pump. Addition of microspheres led to a progressive decrease of the flow as microspheres constricted the vasculature. Eventually, the flow was reduced to zero. **5)** The nature of NADH fluorescence changes during increased heartbeat rates was investigated. First, the control protocol was carried out at a pace rate of 300 beats/min for identification of the anoxic tissue areas during transition from anoxia to normoxia. After control flow was restored, the pace rate was increased to 600 beats/min until a new steady state set in. Adenosine was then added to the perfusate to a final concentration of 10 μ M until a new flow level was reached. Then the pace rate was reduced to 300 beats/min.

RESULTS

Stepwise transitions between normoxia and anoxia. To establish how the myocardium recovers from an episode of anoxia, stepwise changes between normoxia and anoxia were applied to Tyrode-perfused rat hearts according to the control protocol described in **MATERIALS AND METHODS**. A typical flow trace of such a protocol is shown in the bottom trace of Fig. 2. The mean normoxic flow was $14.6 \pm 1.4 \text{ ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ ($n = 6$). Maximum flow achieved during perfusion with deoxygenated Tyrode was $28.3 \pm 2.2 \text{ ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ ($n = 6$), and peak reactive hyperemic flow after a 2 min episode of ischemic anoxia was $27.8 \pm 2.4 \text{ ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ ($n = 6$). Addition of $10 \mu\text{M}$ adenosine led to an enhanced flow reaching a maximum steady-state level of $27.9 \pm 1.9 \text{ ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ ($n = 6$). In the presence of adenosine, normoxic perfusion followed by anoxic perfusion had little effect on flow. Under the conditions of the above protocol, NADH fluorescence images were recorded and the mean NADH fluorescence of consecutive images was measured. The mean fluorescence-level changes concomitant to the bottom flow trace in Fig. 2 are shown in the top trace of Fig. 2. Strong NADH fluorescence was associated with anoxia irrespective of whether anoxia was ischemic or high flow. Ischemic anoxia led to a 3.9 ± 1.1 -fold increase ($n = 6$) in fluorescence and high-flow anoxia to a 4.8 ± 1.1 -fold increase ($n = 6$). The time needed to reach 50% of maximum fluorescence during ischemic anoxia was $49.0 \pm 5.1 \text{ s}$ ($n = 6$), and during high-flow anoxia this interval was $28.5 \pm 6.2 \text{ s}$ ($n = 6$). The time needed for 50% reduction of fluorescence during recovery from ischemic anoxia, however, was $2.8 \pm 1.0 \text{ s}$ ($n = 6$) and from high-flow anoxia this interval was $3.9 \pm 0.7 \text{ s}$ ($n = 6$). Even though in the presence of adenosine little difference in flow was seen between normoxic and anoxic perfusion (Fig. 2), changes in mean fluorescence levels were comparable to those seen in the absence of adenosine with a 5.2 ± 1.2 -fold increase ($n = 6$) in fluorescence upon replacement of normoxic Tyrode by anoxic Tyrode. The time needed for transitions was also comparable to that without adenosine, $24.5 \pm 5.6 \text{ s}$ ($n = 6$) needed for the development of high-flow anoxia and a half-life of $3.4 \pm 1.1 \text{ s}$ ($n = 6$) needed for normoxic recovery.

Fig. 2. Flow and NADH fluorescence changes in rat heart during stepwise changes in oxygenation. Bottom trace: flow during control protocol with and without adenosine. After a period of normoxic perfusion, perfusate is switched to nitrogen-saturated Tyrode (N_2) and after 2 min back to oxygenated Tyrode (O_2). Then flow is reduced to zero by occlusion (occl) of the aortic cannula. Recovery from this ischemia is accomplished by normoxic perfusion (O_2). Addition of $10 \mu\text{M}$ adenosine increases flow. Changing perfusate from oxygenated (O_2) to deoxygenated Tyrode (N_2) does not now affect flow. Top trace: concomitant changes in mean NADH fluorescence [in arbitrary units (AU)]. Video images at moments indicated by A-F are shown in Fig. 3.

NADH fluorescence images recorded during the control protocol showed a dull, evenly spread, weak fluorescence during normoxia and a bright, evenly spread, strong fluorescence during anoxia. Stepwise transition from anoxia to normoxia was accompanied by a transition to weak fluorescence with patchy, strong fluorescent anoxic areas lagging behind normoxic areas. This changes the NADH fluorescence from a strong to a weak fluorescence level in a transient, patchy manner. This effect was not seen during transitions from normoxia to anoxia, where the enhancement in NADH fluorescence progresses evenly over the surface of the whole heart. Video images taken at the moments indicated by Fig. 2, A-F, are shown in Fig. 3, A-F. These image sequences give rise to the following observations. **1)** During stepwise transition from anoxia to normoxia, some tissue areas are slower in returning to normoxia than others. Of particular interest is the finding that these anoxic areas appear during reactive hyperemia. **2)** For a given heart, the size and location of these patchy high fluorescent areas are the same, irrespective of whether the transition to normoxia is from ischemic anoxia or from high-flow anoxia. **3)** Also, for both conditions, the transition from normoxia to anoxia progresses homogeneously across the surface of the heart. **4)** In the same heart, the location of the high NADH fluorescence areas is the same as that seen before the addition of adenosine, thus indicating that autoregulation probably does not affect these anoxic zones.

To find out whether myoglobin plays a role in formation of the observed NADH fluorescence patterns, experiments were performed in the presence of myoglobin inhibitors. Neither addition of NaNO_3 , ($n = 2$ hearts) at a concentration of 0.1 mM (7) or of 0.15 μM ethyl hydrogen peroxide (18; $n = 2$) affected the observed NADH fluorescence patterns (data not shown). Observation under normal light, however, showed that addition of these myoglobin blockers led to a distinct dark discoloration of the myocardium, indicating the efficacy of these substances. To see whether fluorescence heterogeneity is prevalent throughout the heart, experiments were also performed after resection of the heart parallel to the plane of observation, thereby exposing the endocardium and the myocardium for measurement of NADH fluorescence. Transitions from anoxia to normoxia under these conditions revealed heterogeneous anoxic areas also persisting in the endocardium (data not shown).

Pd-porphyrin phosphorescence. To establish whether it is the heterogeneity in oxygen pressures in the vasculature instead of a heterogeneity in the oxygen demand of the myocytes that determines the location of the anoxic tissue areas identified by NADH fluorescence, the newly developed oxygen-sensitive Pd-porphyrin phosphorescent dye (22) was applied. When this dye is coupled to albumin, it remains intravascular. Its phosphorescence can then be used to image the distribution of oxygen pressures in the perfusate flowing within the vasculature. The progress of the phosphorescence intensity of the images under the conditions of the control protocol is shown in the top trace of Fig. 44 with strong phosphorescence occurring during ischemic perfusion and high-flow anoxia and weak phosphorescence during normoxic perfusion. It can also be seen from Fig. 4A that the phosphorescence changes associated with ischemic and high-flow anoxia do not occur if the perfusate does not contain the dye (bottom trace, Fig. 44). Recordings made in the presence of Pd-porphyrin revealed that during the transition between anoxia and normoxia, heterogeneous areas of phosphorescence persist in the circulation (Fig. 4B) in the same

manner as those identified in the tissues by NADH fluorescence (Fig. 4C; n = 4). Comparison of Fig. 4, B and C, shows that anoxic tissue areas correspond to the high Pd-porphyrin phosphorescence patterns in the vasculature. The way in which the change in Pd-porphyrin phosphorescence occurred during the transition from normoxia to anoxia, i.e., evenly across the heart surface, was also similar in manner as found for NADH fluorescence.

Fig. 3. NADH fluorescence images associated with Fig. 2. Times at which images A-F were recorded correspond to same letter shown in top trace in Fig. 2. A: weak fluorescence associated with normoxic perfusion. B: homogeneous enhancement of fluorescence during ischemic hypoxia. C: high fluorescence associated with full anoxia. D: patchy heterogeneous fluorescent images during restoration of normoxic perfusion after an ischemic episode. E: patchy fluorescence occurring after restoration of normoxic perfusion following an episode of anoxic perfusion. F: same state as in E but in the presence of 10 μ M adenosine. Note similarity of patterns in D, E, and F.

Microsphere measurements. To find out which type of vasculature determines the patchy anoxic tissue areas found during transition from anoxia to normoxia, experiments were carried out in which different-diameter vessels were embolized by the introduction of different-size microspheres. Subsequent NADH images were compared with the NADH fluorescence patterns occurring normally during a transition from anoxia to normoxia. A representative flow trace of such an experiment can be seen in Fig. 5A. To confirm that

Fig. 4. Pd-porphyrin phosphorescence and NADH fluorescence in same heart during transitions between normoxia and anoxia. A: changes in mean Pd-porphyrin phosphorescence were recorded during transitions between normoxia and anoxia. Changes in Pd-porphyrin phosphorescence are parallel to those seen during NADH fluorescence with occlusion as well as perfusion with deoxygenated Tyrode (N2) resulting in strong phosphorescence relative to that seen during normoxic perfusion (O2). With no Pd-porphyrin in perfusate, there was no change in phosphorescence during same protocol (bottom trace). B: before addition of dye to perfusate, heterogeneous tissue NADH fluorescence areas, occurring during transition from high-flow anoxia to normoxia, were recorded. C: filters were changed to detect phosphorescence of Pd-porphyrin compound, which was then added to perfusate. Heterogeneous phosphorescent patterns are also seen during transition from high-flow anoxia to normoxia. Both images were contrast-enhanced using same procedure. To aid comparison of the 2 images, corresponding areas in the 2 images are numbered.

single microspheres were embolizing vessels of corresponding diameters, sections were made of formaldehyde-fixed hearts stained with hematoxylin eosin and studied microscopically. Figure 5B shows such a section of a capillary embolized by a 5.9- μ m microsphere. The effect on the development of NADH fluorescence after coronary embolization by pollen [(in μ m diameter) 15.4, n = 3 hearts; 17.6, n = 2; and 36.0, n = 2] and of polystyrene microspheres [(in μ m diameter) 98.8, n = 1; 15.8, n = 4; 9.8, n = 3; and 5.9, n = 5] was investigated. Infusion of microspheres elicited areas of strong fluorescence whose size was dependent on the diameter of the microspheres. Eventually, after perfusion with the microspheres, the whole surface of the myocardium showed an evenly high level of fluorescence as the flow declined to zero. Anoxic zones elicited by microspheres with diameters corresponding to those of arterioles (i.e., 17.6, 15.8, and 9.8 μ m) were all larger than those elicited by the transition from anoxia to normoxia before the addition of the microspheres. This can be seen in Fig. 5C, where anoxic areas elicited by an anoxia-normoxia transition are shown, and later in the same heart (Fig. 5D), where the anoxic zones elicited by infusion of 9.8- μ m microspheres are shown. Embolization of capillaries with 5.9- μ m microspheres, however, not only elicited anoxic areas (Fig. 5F) similar in size to those found during transition from anoxia to normoxia (Fig. 5E), but the sites of these areas also corresponded to those of the NADH fluorescence patterns seen during transition from anoxia to normoxia before microsphere addition. The images in Fig. 5, E and F, were taken at

the moments indicated by the corresponding letters in the flow trace of Fig. 5A. These results suggest that an anoxic area lagging behind during transition from anoxia to normoxia is determined at capillary level.

High work rates. Enhanced heartbeat rates cause not only enhanced oxygen consumption but also result in vascular compression. To investigate the effect of such enhanced heartbeat rates on fluorescence images, experiments were performed on paced hearts according to the protocol outlined in **MATERIALS AND METHODS**. No difference in NADH fluorescence or in flow was seen between freely beating hearts ($13.5 \pm 1.4 \text{ ml}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$, $n = 10$) and hearts paced at 300 beats/min ($13.5 \pm 2.7 \text{ ml}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$, $n = 10$; Fig. 6A). Increasing the heartbeat rate to 600 beats/min led to a slight increase in flow ($14.9 \pm 2.9 \text{ ml}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$, $n = 10$) and in 7 of 10 hearts to an increase of NADH fluorescence. The increased fluorescence was distributed heterogeneously across the surface of the heart (Fig. 6C). Moreover, the areas of high NADH fluorescence seen during enhanced beat rates corresponded to those elicited during transition from anoxia to normoxia during periods of low heartbeat rates (Fig. 6B). These high beat rate-evoked anoxic areas persisted as long as the enhanced rate was maintained and returned to normoxic levels as soon as control conditions were restored (not shown). Addition of adenosine during a period with a 600 beats/min pace rate led to an enhancement of flow ($19.2 \pm 2.7 \text{ ml}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$, $n = 10$) and also to a reduction in fluorescence (Fig. 6D). Returning the pace rate to 300 beats/min led to further increase of the flow ($28.6 \pm 1.8 \text{ ml}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$, $n = 10$). These findings suggest that the anoxic circulatory units identified during transition from anoxia to normoxia are the areas where the oxygen supply first becomes limiting during circulatory stress associated with increased heartbeat rate.

DISCUSSION

This study was performed to find out the origin of the heterogeneous NADH fluorescence patterns occurring under low oxygen conditions in the Tyrode-perfused rat heart. First identified by Barlow and Chance (2) and later described in more detail by Steenbergen et al. (17), the origin of these hypoxia-induced heterogeneous NADH fluorescence patterns has remained a source of uncertainty. In the presence of sufficient substrate and phosphates, high NADH fluorescent areas indicate a negative balance between oxygen supply and demand occurring when either metabolism exceeds supply or supply falls short of demand (5). In these terms, the heterogeneity in NADH fluorescence described in the present study can be caused either by heterogeneity in metabolic demand with homogeneous perfusion of all capillaries or by homogeneous metabolic demand with heterogeneous supply of oxygen. Obviously the combination of the two is possible as well.

To distinguish between the effects of oxygen consumption and of oxygen supply, flow cessation and high-flow anoxia were used to identify the consumption distribution, whereas restoration of oxygenated Tyrode after a period of ischemia or high-flow anoxia was used to identify the supply heterogeneity. NADH fluorescence always, regardless of the intervention, started and grew homogeneously, whereas recovery from anoxia was

Fig. 5. Heterogeneous NADH fluorescence is evoked by embolization of heart with microspheres (ms) of different diameter. A: flow trace associated with ms embolization (in this case 5.9- μm ms) in presence of 10 μM adenosine. Heterogeneous NADH fluorescence patterns are recorded at E during a transition from high-flow anoxia (N_2) to normoxia (O_2); ms are then infused at a constant rate which leads to a progressive reduction of flow to zero. B: stained section of a fixed heart showing a 5.9- μm ms embolizing a capillary. C: heterogeneous NADH fluorescence of a heart during transition from high-flow anoxia to normoxia next to that elicited in same heart (D) by embolization with 9.8- μm ms. E: NADH fluorescence patterns elicited during transition from anoxia to normoxia in flow trace in A are, as shown in F, the same as those elicited by embolization with 5.9- μm ms.

heterogeneous. Homogeneous increases in fluorescence occurred about 10 times as slowly as heterogeneous transitions. For a given heart, the location of the anoxic sites was fixed and always reappeared at the same sites irrespective of the manner in which anoxia was created. Furthermore, the location or occurrence of these anoxic areas was independent of autoregulatory mechanisms, since addition of adenosine, exerting the expected increase of flow, had no effect on the fluorescence patterns observed without adenosine. The results from these experiments suggest that there is a fairly homogeneous consumption of oxygen, whereas during maximal dilation, there is a marked heterogeneity in oxygen supply. To provide extra evidence for this explanation, the oxygen-dependent phosphorescence of Pd-porphyrin was used. Bound to albumin, the dye remains in the vasculature and provides a means of assessing the distribution of oxygen pressures in the coronary vasculature. The finding that the phosphorescence patterns corresponded closely to those of NADH

Fig. 6. Effect of increased heart rates on myocardial NADH fluorescence. A: NADH fluorescence image of heart paced at 300 beats/min. B: heterogeneous fluorescence patterns evoked during transition from high-flow anoxia to normoxia during 300 beats/min. C: heterogeneous fluorescence patterns seen in same heart when pace rate was increased from 300 to 600 beats/min. Note the similarity in patterns between B and C. D: the 600 beats/min-induced heterogeneous fluorescence diminished when 10 μ M adenosine is added.

fluorescence further supports the explanation that it is the structure of the vasculature that underlies the heterogeneous anoxic patterns seen during transition from anoxia to normoxia.

Given that it is oxygen gradients in the vasculature that cause the heterogeneity in NADH fluorescence, the question follows as to which type of vessel is responsible for limitation of oxygen supply to the anoxic tissue areas. This issue was addressed by microembolization of different diameter vessels with microspheres. Occlusion of vessels with microspheres differing in diameter elicited areas of fluorescence whose size was dependent on microsphere diameter. Steenbergen et al. (17) had suggested that an anoxic area seen during changes in the oxygenation of the perfusate represented the capillary bed of single arterioles. Embolization of arterioles with microspheres of corresponding diameter (9.8 μ m and larger), however, always elicited anoxic areas much larger than those found during transitions from anoxia to normoxia. The finding in this study that the anoxic areas seen during recovery from anoxia could only be reproduced by embolization of much smaller vessels (5.9 μ m) strongly suggests that the circulatory units responsible for the anoxic areas are capillaries.

The results from the present study are consistent with a model in which the pattern of the heterogeneity in tissue fluorescence is determined by oxygen gradients in the capillaries (15). These gradients affect large areas of tissue. The anatomic origin of the anoxic sites allows them to be considered as functional circulatory units. Some circulatory units are more limited in oxygen supply than others, and this manifests itself in heterogeneous anoxic tissue areas persisting during recovery from an episode of anoxia. In this way, the supply heterogeneity is determined by circulatory units where a continuous oxygen supply gradient,

dominated by diffusion, determines the vulnerable high NADH fluorescence areas with oxygen becoming severely limited at capillary level. Nevertheless, whatever the source of the hypoxia defining the anoxic areas, our findings underscore the original observations reported by Barlow and Chance (2) and Steenbergen et al. (17) that quite steep oxygen gradients must occur in the tissues. The possibility raised by Williamson et al. (21) that at the border zones between the normoxic and anoxic areas, or possibly also in homogeneously progressing NADH fluorescence enhancement during transition from normoxia to anoxia, there is a mixed population of normoxic and anoxic cells, with cells being either normoxic or fully anoxic, although unlikely, cannot be excluded on the basis of the present results. Repetition of the same measurements under higher magnification should answer this question.

Our finding that the same heterogeneous fluorescence patterns occurring during recovery from anoxia can be evoked during enhanced heartbeat rates supports the idea that these anoxic areas represent vulnerable units where oxygen first becomes limiting during circulatory distress. Katz et al. (12) reported a rise in NADH fluorescence during high heartbeat rates and attributed this rise in NADH levels to a regulatory reaction of oxidative phosphorylation for the maintenance of a higher turnover of ATP. Our results suggest, however, that a further factor that needs to be taken into account is the enhanced NADH fluorescence level associated with the evocation of local anoxic areas caused by the enhanced heart rates. These could be caused by local hypoperfusion, since more flow is required to maintain the higher heart rates in the areas of preference. This in turn would lead to an imbalance between oxygen supply and oxygen demand. That the high fluorescent patchy areas seen during the high beat rates are caused by hypoperfusion is supported by our finding that adenosine provides relief to these anoxic areas by vasodilation. Bjornsson et al. (4) investigated the effect of leukotriene D₄, a potent vasoconstrictor, in beating guinea pig hearts in a study using NADH fluorescence flash photography, and found that leukotriene D₄ caused heterogeneous NADH fluorescence patterns. They also found that this enhanced NADH fluorescence effect was abolished by the addition of adenosine. These findings, in combination with ours, indicate that during circulatory stress the vulnerable high-fluorescent circulatory areas seem to be the first to suffer distress, an effect that can be partially reversed by the addition of adenosine. It is concluded that further investigation into the behavior of the fluorescence and phosphorescence patterns as presented in this study have the potential to gain new insight into the functional interactions between oxygen demand, supply, and control at the level of the coronary microcirculation.

REFERENCES

- 1 Ashruf JF, Avontuur JAM, Van Bavel E et al. (1990). Local ischemia created by microspheres in the rat heart induces heterogeneous epicardial NADH fluorescence (Abstract). *Pfluegers Arch*; 416: S2
- 2 Barlow CH, Chance B (1976). Ischemic areas in perfused rat hearts: measurement by NADH fluorescence photography. *Science*; 193: 909-910
- 3 Bassingthwaighe JB, Yipintsoi T, Harvey RB (1974). Microvasculature of the dog left ventricular myocardium. *Microvasc Res*; 7: 229-249
- 4 Björnsson OG, Kobayashi K, Williamson JR (1987). Inducers of adenylate cyclase reverse the effect of leukotriene D4 in isolated working guinea pig heart. *Am J Physiol*; 252: H1235-H1242
- 5 Chance B (1976). Pyridine nucleotide as an indicator of the oxygen requirements for energy-linked functions of mitochondria. *Circ Res*; 38, Suppl I: 31-38
- 6 Chance B, Cohen P, Jobsis F et al. (1962). Localized fluorimetry of oxidation-reduction states of intracellular pyridine nucleotide in brain and kidney cortex of the anesthetized rat. *Science*; 136: 325
- 7 Doeller JE, Wittenberg BA (1991). Myoglobin function and energy metabolism of isolated cardiac myocytes: effect of sodium nitrate. *Am J Physiol*; 261: H53-H62
- 8 Eng J, Lynch RM, Balaban RS (1989). Nicotinamide adenine dinucleotide fluorescence, spectroscopy and imaging of isolated cardiac myocytes. *Biophys J*; 55: 621-630
- 9 Ince C, Bruining HA (1991). Optical spectroscopy for the measurement of tissue hypoxia. In: *Update in Intensive Care and Emergency Medicine: Update 1991*, edited by Vincent JL. New York: Springer Verlag, 161-171
- 10 Ince C, Coremans JMCC, Bruining HA (1992). In vivo NADH fluorescence. *Adv Exp Med Biol*; 317: 277-296
- 11 Ince C, Vink H, Wieringa PA et al. (1990). Heterogeneous NADH fluorescence during post-anoxic reactive hyperemia in saline perfused rat heart. *Adv Exp Med Biol*; 277: 477-482
- 12 Katz LA, Koretsky AP, Balaban RS (1987). Respiratory control in the glucose perfused heart. A ³¹P NMR and NADH fluorescence study. *FEBS Lett*; 221: 270-276
- 13 Nuutinen EM (1984). Subcellular origin of the surface fluorescence of reduced nicotinamide nucleotide in the isolated perfused rat heart. *Basic Res Cardiol*; 79: 49-58
- 14 Ploem JS (1990). *Standardization in Immunofluorescence*, edited by E. J. Holborow. Oxford, UK: Blackwell, 1970, 137-153
- 15 Rumsey WL, Schlosser C, Nuutinen EM et al. (1990). Cellular energetics and the oxygen dependence of respiration in cardiac myocytes isolated from adult rat. *J Biol Chem*; 265: 15392-15399
- 16 Rumsey WL, Vanderkooi JM, Wilson DF (1988). Imaging of phosphorescence: a novel method for measuring oxygen distribution in perfused tissue. *Science*; 241: 1649
- 17 Steenbergen C, Deleuw G, Barlow C et al. (1977). Heterogeneity of the hypoxic state in perfused rat heart. *Circ Res*; 41: 606-615
- 18 Tamura M, Oshino MN, Chance B et al. (1978). Optical measurements of intracellular oxygen concentration of rat heart in vitro. *Arch Biochem Biophys*; 191: 8-22

- 19 Wieringa PA, Spaan JAE, Stassen HG et al. (1982). Heterogeneous flow distribution in a three dimensional network simulation of the myocardial microcirculation: a hypothesis. *Microcirculation*; 2: 195-216
- 20 Wieringa PA, Stassen HG, Laird JD et al. (1988). Quantification of arteriolar density and embolization by microspheres in rat myocardium. *Am J Physiol*; 254: H636-H650
- 21 Williamson JR, Davis KN, Medine-Ramirez G (1982). Quantitative analysis of heterogeneous NADH fluorescence in perfused rat hearts during hypoxia and ischaemia. *J Mol Cell Cardio*; 14, Suppl 3: 29-35
- 22 Wilson DF, Pastuszko A, DiGiacomo JE et al. (1991). Effect of hyperventilation on the oxygenation of the brain cortex of newborn piglets. *J Appl Physiol*; 70: 2691-2696

3

REGIONAL ISCHEMIA IN HYPERTROPHIC LANGENDORFF-PERFUSED RAT HEARTS

Ashruf JF¹, Ince C², Bruining HA¹

¹Department of Surgery, Erasmus University of Rotterdam, Rotterdam

²Department of Anesthesiology, University of Amsterdam, Amsterdam, the Netherlands

Published in: Am J Physiol; 277: H1532-H1539, 1999

SUMMARY

Myocardial hypertrophy decreases the muscle mass-to-vascularization ratio, thereby changing myocardial perfusion. The effect of these changes on myocardial oxygenation in hypertrophic Langendorff-perfused rat hearts was measured using epimyocardial NADH-videofluorimetry, whereby ischemic myocardium displays high fluorescence intensity. Hypertrophic hearts, in contrast to control hearts, developed ischemic areas during oxygen-saturated Langendorff perfusion. Reoxygenation of control hearts after a hypoxic episode resulted in a swift decrease of fluorescence in a heterogeneous pattern of small, evenly dispersed, highly fluorescent patches. Identical patterns could be evoked by occluding capillaries with microspheres 5.9 μm in diameter. Ten seconds after reoxygenation there were no more dysoxic areas, whereas reoxygenation in hypertrophic hearts showed larger ischemic areas that took significantly longer to return to normoxic fluorescence intensities. Hypothesizing that the larger areas originate at a vascular level proximal to the capillary network, we induced hypoxic patterns by embolizing control hearts with microspheres 9.8 and 15 μm in diameter. The frequency distribution histograms of these dysoxic surface areas matched those of hypertrophic hearts and differed significantly from those of hearts embolized with 5.9- μm microspheres. These results suggest the existence of areas in hypertrophic Langendorff-perfused hearts with suboptimal vascularization originating at the arteriolar and/or arterial level.

INTRODUCTION

Hypertrophic myocardium is more susceptible to ischemic damage than normal myocardium because of, among other factors, changes in its vascularization (7, 21). Morphological studies of vasculature in hypertrophic myocardium have demonstrated profound changes in anatomy and function. In hypertrophic myocardium, capillary density and coronary vascular reserve decrease, whereas minimal coronary vascular resistance increases (11, 19, 20). Hypertrophy also increases diffusion distances from capillaries to myocytes (22, 24). These changes are not distributed evenly throughout the myocardium, resulting in a diversion of coronary flow from subendocardial to subepicardial layers (1, 8). Many studies have shown this transmural redistribution of coronary flow in hypertrophy with changes in the heterogeneity of flow and vascularization between subsequent transmural myocardial layers (1, 22, 23). Less is known, however, of the effect of hypertrophy on the local distribution of oxygen through the myocardial microcirculation. For instance, measurements of local oxygen consumption and supply did not show any difference between normal and hypertrophic *in situ* hearts at rest (25) and during stress (5, 28), whereas a significant functional impairment in hypertrophic hearts was demonstrated. An explanation may be that these hearts were blood-perfused *in situ* hearts with a physiological oxygen supply-to-demand ratio as compared with Langendorff-perfused rat hearts, which are known to have a marginal oxygen supply, resulting in the development of ischemia when the oxygen supply-to-demand ratio decreases slightly (13). In a previous study we found that hypertrophic Langendorff-perfused hearts spontaneously developed hypoxic areas that could be alleviated by factors influencing oxygen free radical concentrations through the addition of fatty acids or superoxide dismutase to the perfusate (12).

The purpose of the present study was to investigate the nature of the spatial distribution of the hypoxic state in myocardial regions and the vascular level at which perfusion is disturbed in myocardial hypertrophy by using epicardial NADH videofluorescence to monitor the mitochondrial energy state (15). Measurement of the mitochondrial autofluorescence of reduced pyridine nucleotide (NADH) of the epimyocardium allows the identification of hypoxic regions because NADH (which accumulates during hypoxia) fluoresces when excited with ultraviolet (UV) light and oxidized NAD^+ does not (6). There is also a fairly linear relationship between the oxygen concentration available to mitochondria and the NADH fluorescence intensity, as has been shown in isolated mitochondria (6). In this way the local mitochondrial energy state of the myocardium can be visualized (2). In normal Langendorff-perfused rat hearts a stepwise transition from anoxic to normoxic perfusion was accompanied by a transition from high epicardial NADH fluorescence intensity to low fluorescence intensity with a reproducible patchy pattern of high-fluorescence anoxic areas lagging behind low-fluorescence normoxic areas (15). These areas are microcirculatory weak units, because in a given heart they were always at the same location and were the first to become hypoxic during tachycardia (2, 15). These weak units were also the first to become hypoxic during inhibition of nitric oxide synthesis in endotoxemic rat hearts (4) and could be the cause of shunting pathways during sepsis (17). Hypoxic areas of identical size and pattern could also be elicited by embolization of the capillaries by microspheres whose diameter corresponded to the capillary diameter (5.9 μm), whereas embolization of arterioles and arteries by larger microspheres produced

larger hypoxic areas not corresponding to the heterogeneous areas seen during recovery from hypoxia (15). We hypothesized that in hypertrophic myocardium this pattern would be altered because of the formerly mentioned circulatory changes, resulting in suboptimal perfused myocardial regions. Furthermore, the size of the hypoxic areas in hypertrophic myocardium could elucidate at which vascular level the impaired perfusion originated. In this study we analyzed the anatomic substrate responsible for the appearance of these dysoxic areas in hypertrophic hearts. Preliminary results were reported elsewhere (3).

MATERIALS AND METHODS

Experimental setup. Male Wistar rats weighing 200-250 g were operated on, undergoing subdiaphragmatic and suprarenal aortic narrowing to induce left ventricular hypertrophy (18), and were killed 6 wk later when they weighed 250-300 g. For control measurements, rats of similar weight were used. Hearts were removed and perfused according to Langendorff and paced via aortic and right ventricular leads. Mean coronary flow rates (in $\text{ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ ventricle wetwt) were measured with an electromagnetic flow probe (Skalar Medical, Delft, the Netherlands) placed immediately proximal to the aortic cannula. Perfusion pressure was measured with a pressure transducer (Hewlett-Packard 8805B corner amplifier) connected to the aortic cannula. The perfusate was a modified Tyrode solution (128 mM NaCl, 4.7 mM KCl, 1 mM MgCl_2 , 0.4 mM NaH_2PO_4 , 1.2 mM Na_2SO_4 , 20.2 mM NaHCO_3 , and 1.3 mM CaCl_2) containing 11.0 mM glucose. Perfusate temperature was kept at 37°C and equilibrated with either 95% O_2 -5% CO_2 or 95% N_2 -5% CO_2 . Perfusate pH was kept between 7.35 and 7.45. Hearts were paced at 5 Hz. The left ventricle was cannulated and communicated with the atmosphere via its apex to prevent generation of left ventricular pressure (2). In some experiments 10 μM nitroprusside, an endothelium-independent vasodilator was added to the perfusate. In other experiments microspheres (Polyscience, Warrington, PA) of different diameters were infused into the coronary circulation. When indicated, the oxygen-saturated perfusate contained 10% (vol/vol) fluorocarbon F-43 emulsion (an artificial oxygen carrier) and 1% (wt/vol) fatty acid-free BSA. The stock fluorocarbon emulsion was obtained by sonifying 24 ml of F-43 and 5.6 g of pluronic F-68 in 150 ml of water at 4°C for 1 h under continuous bubbling of CO_2 through the solution (9). Chemicals were obtained from Merck (Darmstadt, Germany).

Fluorescence measurements. The NADH-videofluorimeter used has been described previously (2, 15). The UV light from a 100-W mercury arc lamp (Olympus, Tokyo, Japan) was selected by means of a UG-1 barrier filter, centered on 365 nm, to provide the UV light needed for NADH excitation. The NADH fluorescence signal was selected by means of a band-pass filter centered around 470 ± 20 nm and was placed in front of the camera. An image-intensified video camera (MXRi, Adimec, Eindhoven, the Netherlands) with a Micro-Nikkor 105-mm macrolens was used to detect NADH fluorescence images of the left ventricle of the heart. To enable correction of images for changes in the sensitivity of the videofluorimeter and fluctuations in the intensity of the light source, a small piece of uranyl calibration glass was placed next to the heart within the excitation field. Images were recorded on a video recorder and were computer analyzed off-line with the use of image-

processing software (TCLImage, Multihouse, Amsterdam, the Netherlands). Arbitrary units of NADH fluorescence intensities relative to uranyl fluorescence intensity are expressed as percentages, where 100% is taken as the intensity of epicardial NADH fluorescence at the end of a 2-min period of perfusion with nitrogen-saturated medium.

Experimental protocols. Hearts were allowed at least 15 min of stabilization after the start of the Langendorff perfusion at a perfusion pressure of 80 mmHg with oxygen-saturated perfusate before the following experimental protocols were performed.

To examine the fluorescence patterns in hypertrophic left ventricle during oxygen-saturated Langendorff perfusion and posthypoxic recovery, protocol 1 was carried out. After the stabilization of flow during normoxic perfusion, perfusion was switched to nitrogen-saturated perfusate for 2 min and then restored to normoxic perfusion. Because large ischemic areas already existed during normoxic perfusion at a perfusion pressure of 80 mmHg, perfusion pressure was increased to 100 and then 120 mmHg for 5 min each to increase coronary flow and oxygen transport. This did not cause the ischemic areas to disappear, and perfusion pressure was restored to 80 mmHg. Finally, to ascertain that the ischemic areas already visible at the beginning of perfusion were reversible [and did not represent infarcted (fibrotic) highly fluorescent myocardium (16)], oxygen transport to the myocardium was increased by either adding 10 μ M nitroprusside to the oxygen-saturated perfusate or switching perfusion to fluorocarbon-containing oxygen-saturated medium. Protocol 2 was performed to examine fluorescence patterns in control left ventricle during posthypoxic recovery and progressive hypoperfusion. After the stabilization of flow during normoxic perfusion, perfusion pressure was decreased to 60 mmHg to obtain coronary flows comparable between control hearts and hypertrophic hearts. As in protocol 1, control hearts were subjected to a 2-min period of nitrogen-saturated perfusion, after which perfusion was restored to oxygen-saturated perfusate. Hereafter, each heart was subjected to stepwise reduction of perfusion pressure from 60 mmHg to 10 mmHg in steps of 10 mmHg, with each step lasting 3.5 min.

To determine which vascular level determined the appearance of the ischemic areas in hypertrophic hearts, control hearts were embolized with microspheres of different diameters in protocol 3. The frequency distributions of ischemic surface areas in embolized control hearts were compared with those of hypertrophic hearts. After the stabilization of flow in control hearts, heterogeneous fluorescence patterns were elicited by switching perfusion from oxygen-saturated perfusate to nitrogen-saturated perfusate (for 2 min) and back. When flow had returned to baseline values, microspheres emulsified in Tyrode solution were infused at a rate of 500-1000 microspheres $\cdot$ min⁻¹ into the coronary flow (15). Microspheres had fixed diameters of 5.9, 9.8, and 15 μ m. Each heart was perfused with microspheres of one diameter, and infusion was stopped when flow was decreased to $\sim$ 75% of the baseline value. The surfaces of the ischemic areas caused by embolization in control hearts and the surfaces of ischemic areas in hypertrophic hearts during normoxic perfusion were measured in the fluorescence images and expressed in pixels (1 pixel = 670 μ m² of myocardial surface). The transition from a normoxic area to an ischemic area is characterized by a small transitional zone (26) where fluorescence intensity increases gradually from normoxic to hypoxic intensity. The surface of an ischemic area was defined as the number of pixels with a fluorescence intensity larger than or equal to the intensity halfway between the

intensity of the normoxic surrounding area and the peak intensity of the ischemic area. Relative frequency distributions were calculated for six intervals of surface areas (see Fig. 7), and the proportion of total ischemic epicardial surface and the number of ischemic areas were measured (see Fig. 8).

Data are presented as means $\pm$ SE. Mean relative frequencies of surface areas per interval were tested for differences between groups using unpaired t-tests. Group means of other data were also tested for differences, also using unpaired t-tests. The criterion for significance was taken to be $P < 0.05$ for all comparisons.

RESULTS

Five hypertrophic Langendorff-perfused hearts (total ventricle wetwt 1.6 ± 0.2 g) were subjected to protocol 1. Hearts instantly developed ischemic areas, indicated by patches of highly fluorescent myocardium (Fig. 1A). These ischemic areas were stable in size and time during the entire period of normoxic perfusion, lasting up to 30 min. After stabilization, the perfusate was switched to nitrogen-saturated medium for 2 min, which caused an increase of coronary flow from 13.8 ± 0.4 (at 2 min in Fig. 2A) to 24.4 ± 0.4 $\text{ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ (at 4 min in Fig. 2A) and an increase of NADH fluorescence intensity of the total epicardium from $32 \pm 3\%$

Fig. 1. NADH fluorescence images of a hypertrophic heart at the start of oxygen-saturated perfusion (A), at the end of nitrogen-saturated perfusion (B), 20 s after reoxygenation (C), and during perfusion with oxygen-saturated nitroprusside-containing medium (D).

(Fig. 1A; at 2 min in Fig. 2B) to 100% (Fig. 1B; at 4 min in Fig. 2B). Restoration to oxygen-saturated perfusion decreased coronary flow and epicardial fluorescence intensity (Figs. 1C and 2A). The decrease of NADH fluorescence intensity was spread in uneven patterns across the epimyocardial surface with larger areas having high fluorescence intensity, persisting even after 20 s of reperfusion (Fig. 1C). Eventually fluorescence intensity decreased to the baseline level at the start of the experiment; this took longer than 5 min. To increase oxygen transport to the myocardium after coronary flow had reached baseline levels, perfusion pressure was increased to 100 and then 120 mmHg. Coronary flow increased from 14.1 ± 0.3 to 17.0 ± 0.5 and then 19.8 ± 0.7 $\text{ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ total ventricle wetwt, respectively, but this did not cause ischemic areas to disappear (data not shown). Perfusion pressure was then restored to 80 mmHg. The perfusate was switched to oxygen-saturated medium containing nitroprusside to increase oxygen transport by vasodilatation. Flow increased from 13.5 ± 0.5

Fig. 2. A: coronary flow changes in rat hearts during stepwise changes in oxygenation. Thin line, mean coronary flow of control heart during protocol 2 ($n = 5$); thick line, mean coronary flow of hypertrophic heart during protocol 1 ($n = 5$); dashed lines, $\pm$ SE. Horizontal bar indicates period of statistically significant ($P < 0.05$) difference in coronary flow between control and hypertrophic hearts. B: mean relative NADH fluorescence in rat hearts during stepwise changes in oxygenation. $\square$, NADH fluorescence of control heart during protocol 2; $\bullet$, NADH fluorescence of hypertrophic heart during protocol 1. Values are means $\pm$ SE. Steps: 0–2 min, perfusion with oxygen-saturated medium; 2–4 min, perfusion with nitrogen-saturated medium; 4–6 min, reoxygenation with oxygen-saturated medium. *Statistically significant ($P < 0.05$) difference in mean relative fluorescence between control and hypertrophic hearts.

Fig. 3. NADH fluorescence images of a hypertrophic heart during perfusion with oxygen-saturated medium containing no fluorocarbons (A) and 3 min after perfusion with oxygen-saturated medium containing fluorocarbons (B).

to $27.8 \pm 1.2 \text{ ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ total ventricle wetwt, and ischemic areas completely disappeared (Fig. 1D). To increase oxygen transport in a different manner, in three additional hypertrophic hearts (total ventricle wetwt $1.5 \pm 0.1 \text{ g}$) not subjected to this protocol but which also displayed ischemic areas on commencement of Langendorff perfusion, the perfusate was switched from oxygen-saturated medium to fluorocarbon-containing oxygen-

saturated medium. Flow decreased from 14.5 ± 0.7 to 12.1 ± 0.6 $\text{ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ total ventricle wetwt (not significant), and the size of ischemic areas dramatically decreased (Fig. 3).

Control hearts ($n = 5$, total ventricle wetwt 1.1 ± 0.1 g) were subjected to protocol 1, which differed from protocol 2 (hypertrophic hearts) in that, at the start of the protocol, perfusion pressure was decreased to 60 mmHg to obtain coronary flows comparable to those of the hypertrophic hearts. Coronary flows of control hearts 2 min before the start of nitrogen-saturated perfusion did not differ significantly from those of hypertrophic hearts (Fig. 2A). At the start of perfusion there were no ischemic areas (Fig. 4A). When the perfusate was switched from oxygen- to nitrogen-saturated medium, NADH fluorescence homogeneously increased over the entire epicardial surface (Figs. 2B and 4B). Also, coronary flow increased from 14.3 ± 0.8 (at 2 min in Fig. 2A) to 28.3 ± 2.0 $\text{ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ (at 4 min in Fig. 2A). During reperfusion with oxygen-saturated medium, NADH fluorescence rapidly decreased in a heterogeneous pattern (Fig. 4C) of small areas with fluorescence intensities ranging from a low (normoxic) to high (hypoxic) fluorescence intensity, as described previously (15). Ten seconds after reperfusion, control hearts no longer displayed ischemic areas (data not shown), whereas in hypertrophic hearts postreperfusion ischemia persists much longer (compare Fig. 1, A and C; 20 s after reperfusion the hypertrophic hearts have ischemic areas that are still larger than they were at the start of perfusion). This is also shown in Fig. 2B; after reperfusion, mean NADH fluorescence intensity decreases in both control and hypertrophic hearts but remains significantly higher in hypertrophic hearts for at least 2 min. Fig. 2A also shows that after reperfusion, coronary flow decreases faster in hypertrophic hearts than in control hearts (for instance, 1 min after reperfusion, coronary flow in the hypertrophic hearts is 15.2 ± 0.7 $\text{ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$, whereas in control hearts it is 24.1 ± 1.0 $\text{ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$).

These results suggest that Langendorff-perfused rat hearts that are hypertrophic are hypoperfused, causing ischemic areas to appear. To investigate whether hypoperfusion of control hearts causes similar ischemic areas, perfusion pressure was decreased in a stepwise manner from 60 to 10 mmHg, with each step lasting 3.5 min (protocol 2 continued) after flow returned to baseline levels after the reperfusion experiment. Flow concomitantly decreased (Fig. 5E) and stabilized in <2 min after each drop in perfusion pressure. Ischemic areas grew progressively larger during perfusion pressures of <30 mmHg (Fig. 5, B-D). The highly fluorescent ischemic areas were identical in pattern to those seen after switching from nitrogen- to oxygen-saturated perfusion and were evenly distributed over the epicardium (compare Figs. 4C and Fig. 5, B-D). In these experiments, however, patterns with relatively large ischemic areas coexisting with large normoxic areas as seen in hypertrophic hearts did not appear.

To identify at which level of arteriolar and/or arterial vasculature the ischemic areas originate in hypertrophic myocardium during oxygen-saturated perfusion, control hearts were embolized with microspheres of different diameters according to protocol 3; five hearts were embolized with 5.9- μm microspheres, three with 9.8- μm microspheres, and three with 15- μm microspheres. The larger the diameter, the larger the ischemic areas, as shown in Figs. 6 and 7. Fig. 7 shows the frequency distribution of ischemic surface areas of hearts embolized with 5.9-, 9.8- and 15- μm microspheres and of the hypertrophic hearts of protocol 1. The mean relative frequency distribution of ischemic surface areas in hypertrophic hearts did not differ significantly from those in control hearts embolized with

Fig. 4. NADH fluorescence images of a control heart (protocol 2) at the start of oxygen-saturated perfusion (A), at the end of nitrogen-saturated perfusion (B), and 5 s after reoxygenation with oxygen-saturated medium (C).

9.8- and 15- μm microspheres (except for the interval from 600 to 800 pixels, where there was a significant difference between hypertrophic hearts and control hearts embolized with 9.8- μm microspheres), whereas it did differ significantly from the frequency distribution of hearts embolized with 5.9- μm microspheres (Fig. 7). The proportion of ischemic epicardium and number of ischemic areas of hearts embolized with the different microspheres and of hypertrophic hearts are shown in Fig. 8.

Fig. 5. NADH fluorescence images of a control heart during oxygen-saturated perfusion (protocol 2) at a perfusion pressure of 40 (A), 30 (B), 20 (C), and 10 mmHg (D). E: coronary flow in control hearts ($n = 5$) during stepwise decreases of perfusion pressure.

Fig. 6. NADH fluorescence images of control hearts during embolization of vasculature with microspheres of different diameters: 5.9 μm (A), 9.8 μm (B and C), and 15 μm (D).

DISCUSSION

Earlier work identified hypoperfused ischemic areas in hypertrophic Langendorff-perfused hearts (12). The present study was performed to locate the vascular level at which these hypoperfused areas originate. The main findings of this study were that the ischemic areas in hypertrophic Langendorff-perfused rat hearts were significantly larger than ischemic areas evoked by capillary embolization or by normoxic recovery from nitrogen-saturated perfusion. The ischemic surface areas had a frequency distribution closely resembling that of normal hearts embolized at the arteriolar and/or arterial level, suggesting the existence of hypoperfusion originating at the arteriolar and/or arterial level.

In this study we used epicardial NADH fluorescence to measure epicardial ischemia. The definition of ischemia in terms of fluorescence intensity requires further elaboration. Anoxia induced in Langendorff-perfused rat hearts leads to a four- to fivefold increase in NADH fluorescence intensity relative to the normoxic NADH fluorescence intensity (15). In this study we measured the surface areas of ischemic epicardial zones with NADH fluorescence intensity greater than the intensity halfway between the intensities of the normoxic surrounding area and those of the ischemic epicardial zones. The NADH fluorescence intensities of the ischemic zones were at least threefold greater than the NADH fluorescence intensities of normoxic epicardium. Because fluorescence measurements were made in beating hearts, we minimized motion artifacts by selecting images from the hearts in identical positions in the cardiac cycle (2).

The NADH fluorescence intensity is also dependent on the amount of work output and the mitochondrial state (2, 6). High work output induces a decrease of the basal normoxic fluorescence intensity. The left ventricle was cannulated and communicated with the atmosphere in all experiments to prevent the development of left ventricular pressure buildup, resulting in similar mitochondrial states in both hypertrophic and control hearts. This prevented confounding of the fluorescence measurements by variations in work output (2). Still, differences in ADP substrate levels between control hearts and hypertrophic hearts could cause differences in NADH fluorescence intensities. However, as can be deduced from Refs. 2 and 6, differences in fluorescence induced by ADP substrate-level variation are not as large as differences induced by ischemia.

Another factor influencing NADH fluorescence intensity is substrate availability. It was shown earlier that the normoxic NADH fluorescence intensity is dependent on the

substrate: pyruvate increases normoxic fluorescence the most, oleate less, and glucose even less (2, 12). In this study, however, all experiments were performed with 5.5 mM glucose. Normoxic fluorescence intensity did not differ between hypertrophic and control hearts (data not shown), and highly fluorescent areas in hypertrophic hearts disappeared on improvement of myocardial oxygenation (Figs. 1D and 3B), thereby excluding the confounding of NADH fluorescence measurements by differences in substrate availability between control and hypertrophic hearts.

In this study we decreased perfusion pressure in control hearts to obtain comparable coronary flow rates between control and hypertrophic hearts. This did not result in qualitatively different NADH fluorescence patterns in control hearts (data not shown). We did not increase perfusion pressure in hypertrophic hearts to this end, because this would have resulted in nonphysiologically high perfusion pressures in hypertrophic hearts. In all experiments in this study the left ventricular cavity communicated with the atmosphere via a cannula inserted through the apex of the left ventricle to ensure the lowest possible cardiac work output in all hearts, thereby reducing the confounding of NADH fluorescence measurements by differences in oxygen consumption (2).

Normal rat hearts Langendorff perfused with oxygen-saturated medium continuously release lactate in their effluent, indicating that they are borderline aerobic (13). However, epicardial NADH fluorescence measurements under these circumstances do not reveal dysoxic areas (Fig. 4A) (15). Hypertrophic hearts are known to have a decreased myocardium-to-vascularization ratio (8, 22). Because of this less optimal vascularization, we expected hypertrophic Langendorff-perfused hearts to develop highly NADH-fluorescent ischemic areas during normoxic perfusion, as shown earlier (Fig. 1A) (3, 12). That study had suggested that the development of ischemia was related to the production of oxygen free radicals and acidosis in hypoperfused areas of hypertrophic hearts because of the finding that these ischemic areas could be relieved by perfusion with superoxide dismutase, a scavenger of oxygen free radicals, or by perfusion with fatty acids, resulting in a protection from acidosis-initiated loss of capillary flow. Steenbergen et al. (26) also observed the development of relatively large ischemic areas induced by acidosis in normal hearts and suggested that acidosis-induced coronary (arteriolar and/or arterial) changes were responsible.

Ischemic areas in hypertrophic hearts were elicited at flow rates that did not cause local ischemia in control hearts, suggesting the existence of local hypoperfused areas in hypertrophic Langendorff-perfused rat hearts. To ascertain that these highly fluorescent areas were still viable areas of myocardium and were dysoxic because of impaired oxygenation, oxygen transport was increased in several ways. Adding fluorocarbons or nitroprusside to the perfusate resulted in either complete disappearance (nitroprusside, Fig. 1A) or significant reduction of ischemia (fluorocarbons, Fig. 3). Furthermore, histological analysis in formaldehyde-fixed hypertrophic hearts stained with hematoxylin-eosin revealed no evidence of infarction (data not shown). Increasing coronary flow by increasing perfusion pressure, however, did not produce a significant decrease in ischemic areas (data not shown) in hypertrophic hearts. An explanation for this observation could be that because of the increase in coronary perfusion pressure, oxygen consumption increases, thereby more or less keeping the ratio between oxygen supply and demand unchanged (10). Alternatively,

enhancing flow by increasing perfusion pressure could only affect shunting flow, thereby leaving the hypoperfused areas still dysoxic.

In a previous study (15) we had shown with NADH fluorescence measurements in normal hearts that occlusion of vessels of increasing diameter induced patchy ischemic areas of increasing surface area. Furthermore, normoxic recovery in control hearts from perfusion with nitrogen-saturated medium was accompanied by a heterogeneous NADH fluorescence pattern with small, highly fluorescent patches lagging behind areas with a much faster decrease of fluorescence. These ischemic areas were shown to be microcirculatory units originating at the capillary level, which were the last to be reoxygenated during reperfusion and could also be elicited by occluding capillaries with microspheres 5.9 μm in diameter (Fig. 6 A) (15). A recent histological study by Vetterlein et al. (27) in normal rat hearts *in vivo* found that tissue located within the capillary bed in proximity to the draining venule is more prone to the development of hypoxia in critical oxygen supply conditions. Hypoxia develops during hypoperfusion because of a combination of disturbances of perfusion in feeding areas of arterioles and the loss of oxygen lengthwise along capillaries. Apparently the microcirculatory units described by Ince et al. (15) are located in capillary beds close to venules. Ischemic areas in hypertrophic hearts at the start of perfusion are significantly larger than those elicited by capillary occlusion in normal hearts (compare Figs. 1A and 6A), suggesting that they originate at a vascular level proximal to the capillaries. This is also supported by the finding that the embolization of increasingly larger vessels (protocol 3) produces increasingly larger ischemic areas that resemble those of hypertrophic hearts (compare Figs. 6, B-D, and 1A). One could argue, taking into account the study of Vetterlein et al. (27), that in hypertrophy the disturbances of perfusion of certain individual arterioles predominate over the loss of oxygen lengthwise along capillaries, thereby inducing larger ischemic areas. Another argument in favor of this hypothesis is the close resemblance of the frequency distribution histogram of ischemic surface areas of hypertrophic hearts to that of control hearts with embolized arterioles and/or arteries (Fig. 7).

Reperfusion with oxygen-saturated medium after nitrogen-saturated perfusion in hypertrophic hearts revealed a significantly different fluorescence pattern from that of control hearts. Mean fluorescence intensity decreased significantly slower with, 1 min after reperfusion, a decline in mean fluorescence intensity of $52 \pm 2\%$ in hypertrophic hearts compared with $40 \pm 2\%$ in control hearts (Fig. 2B). This was accompanied by a significantly faster decrease of flow and thus less oxygen transport after reperfusion in hypertrophic hearts ($15.2 \pm 0.7 \text{ ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ 1 min after reperfusion) compared with control hearts ($24.1 \pm 1.0 \text{ ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ 1 min after reperfusion) (Fig. 2B). Peak flow during nitrogen-saturated perfusion was significantly higher in control hearts compared with that in hypertrophic hearts (Fig. 2B). These findings indicate a decreased coronary flow reserve in hypertrophic hearts, as was found in earlier studies (11, 19, 20). The accompanying fluorescence images during the reperfusion phase show the persistence of larger ischemic areas (Fig. 1C) with high fluorescence even 20 s after reperfusion, whereas in control hearts fluorescence images reveal no ischemia even before that time. The ischemic areas persisting in hypertrophic hearts after reperfusion are also larger than those in control hearts (compare Figs. 1C and 4C) and resemble those after occlusion of arterioles and/or arteries with microspheres (Fig. 6). This finding suggests the existence of hypoperfused areas of myocardium in hypertrophy,

Fig. 7. Frequency distribution histogram of ischemic surface area (1 pixel = 670 μm^2) in hearts embolized with microspheres 5.9 ($n = 5$), 9.8 ($n = 3$), and 15 μm ($n = 3$) in diameter and in hypertrophic hearts ($n = 5$). Columns represent mean frequency and bars represent SE. *Statistically significant ($P < 0.05$) different from 5.9- μm microspheres.

originating at the arteriolar and/or arterial level. To examine the effect of hypoperfusion on control hearts, perfusion pressure was progressively decreased (protocol 2). This induced ischemic areas that match the pattern elicited by occluding capillaries (15) and are much smaller in size than the dysoxic areas seen in hypertrophic hearts. We were never able to induce patterns of ischemia in control hearts resembling those seen in the hypertrophic hearts. Apparently hypertrophy produced by the method used in this study induces changes in arteriolar and/or arterial vascular control that result in ischemia of myocardial areas during suboptimal coronary perfusion. Earlier work (12, 14) has shown that a possible mechanism accounting for such an effect could be scavenging of nitric oxide (endothelium-derived relaxing factor) by increased oxygen free radical production in ischemic myocardial areas in hypertrophic hearts.

In conclusion, in this study we found that the pattern of oxygen transport to the epimyocardium is profoundly altered in hypertrophy. These changes are the result of dysregulation of flow at the arteriolar and/or arterial level. The areas with the slowest reoxygenation after ischemia in hypertrophy are larger than the microcirculatory units described by Ince et al. (15) and have a surface area distribution matching that of the ischemic areas evoked by arteriolar and/or arterial embolization.

Fig. 8. Proportion of ischemic epicardium (open bars) and number of ischemic areas (filled bars) of hearts embolized with microspheres 5.9 ($n = 5$), 9.8 ($n = 3$), and 15 μm ($n = 3$) in diameter and in hypertrophic (hyp) hearts ($n = 5$). Columns represent mean values and bars represent SE.

REFERENCES

- 1 Anderson PG, Bishop SP, Digerness SB (1987). Transmural progression of morphologic changes during ischemic contracture and reperfusion in the normal and hypertrophied rat heart. *Am J Pathol*; 129: 152-167
- 2 Ashruf JF, Coremans JMCC, Bruining HA et al. (1995). Increase of cardiac work is associated with decrease of mitochondrial NADH. *Am J Physiol*; 269: H856-H862
- 3 Ashruf JF, Ince C, Bruining HA et al. (1994). Ischemic areas in hypertrophic Langendorff rat hearts visualized by NADH-videofluorimetry. *Adv Exp Med Biol*; 345: 259-262
- 4 Avontuur JAM, Bruining HA, Ince C (1996). Inhibition of nitric oxide synthesis causes myocardial ischemia in endotoxemic rats. *Circ Res*; 76: 418-425
- 5 Cimini CM, Weiss HR (1990). Isoproterenol and myocardial O₂ supply/consumption in hypertension-induced myocardial hypertrophy. *Am J Physiol*; 259: H346-H351
- 6 Chance B (1976). Pyridine nucleotide as an indicator of the oxygen requirements for energy linked functions of mitochondria. *Circ Res*; 38, Suppl 1: 31-38
- 7 Dellsperger KC, Marcus ML (1990). Effects of left ventricular hypertrophy on the coronary circulation. *Am J Cardiol*; 65: 1504-1510
- 8 Einzig S, Leonard JJ, Tripp MR et al. (1981). Changes in regional myocardial blood flow and variable development of hypertrophy after aortic banding in puppies. *Cardiovasc Res*; 15: 711-723
- 9 Geyer RP (1975). "Bloodless" rats through the use of artificial blood substitutes. *Federation Proc*; 34: 1499-1505
- 10 Gregg DE (1963). Effect of coronary perfusion pressure or coronary flow on oxygen usage of the myocardium. *Circ Res*; 13: 497-500
- 11 Harrison DG, Barnes DH, Marcus ML (1985). The effect of cardiac hypertrophy on the coronary circulation. *Circulation*; 71: 1135-1145
- 12 Hulsmann WC, Ashruf JF, Bruining HA et al. (1993). Imminent ischemia in normal and hypertrophic Langendorff rat hearts: effects of fatty acids and superoxide dismutase monitored by NADH surface fluorescence. *Biochim Biophys Acta*; 1181: 273-278
- 13 Hulsmann WC, de Wit LEA (1990). Acidosis, cardiac stunning and its prevention by oxygen. *Cell Biol Int Rep*; 14: 311-315
- 14 Hulsmann WC, Dubelaar ML (1987). Early damage of vascular endothelium during cardiac ischemia. *Cardiovasc Res*; 21: 674-677
- 15 Ince C, Ashruf JF, Avontuur JAM et al. (1993). Heterogeneity of the hypoxic state in rat heart is determined at capillary level. *Am J Physiol*; 264: H294-H301
- 16 Ince C, Coremans JMCC, Bruining HA (1992). In vivo NADH fluorescence. *Adv Exp Med Biol*; 317: 277-296
- 17 Ince C, Sinaasappel M (1999). Microcirculatory oxygenation and shunting in sepsis and shock. *Crit Care Med*; 27(7): 1369-1377
- 18 Malik AB, Shapiro JM, Yanics J et al. (1974). A simplified method for producing rapid ventricular hypertrophy in rats. *Cardiovasc Res*; 8: 805-801
- 19 Marcus ML, Koyanagi S, Harrison DG et al. (1983). Abnormalities in the coronary circulation that occur as a consequence of cardiac hypertrophy. *Am J Med*; 75, Suppl 1: 62-66

- 20 Marcus ML, Mueller TM, Gascho JA et al. (1979). Effects of cardiac hypertrophy secondary to hypertension on the coronary circulation. *Am J Cardiol*; 44: 1023-1028
- 21 Rakusan K, Flanagan MF, Geva T et al. (1992). Morphometry of human coronary capillaries during normal growth and the effect of age in left ventricular pressure-overload hypertrophy. *Circulation*; 86: 38-46
- 22 Rakusan K, Moravec J, Hatt PY (1980). Regional capillary supply in the normal and hypertrophied rat heart. *Microvasc Res*; 20: 319-326
- 23 Rakusan K, Wicker P (1990). Morphometry of the small arteries and arterioles in the rat heart: effects of chronic hypertension and exercise. *Cardiovasc Res*; 24: 278-284
- 24 Roberts JT, Wearn JT (1941). Quantitative changes in the capillary-muscle relationship in human hearts during normal growth and hypertrophy. *Am J Heart*; 21: 617-633
- 25 Scholz PM, Grover GJ, Mackenzie JW et al. (1990). Regional oxygen supply and consumption balance in experimental left ventricular hypertrophy. *Basic Res Cardiol*; 85: 575-584
- 26 Steenbergen C, Deleeuw G, Barlow C et al. (1977). Heterogeneity of the hypoxic state in perfused rat heart. *Circ Res*; 41: 606-615
- 27 Vetterlein F, Prange M, Lubrich D et al. (1995). Capillary perfusion pattern and microvascular geometry in heterogeneous hypoxic areas of hypoperfused rat myocardium. *Am J Physiol*; 268: H2183-H2194
- 28 Wright C, Weiss HR, Kedem J et al. (1991). Effect of dopamine on regional myocardial function and oxygen consumption in experimental left ventricular hypertrophy. *Basic Res Cardiol*; 86: 449-460

4

IMMINENT ISCHEMIA IN NORMAL AND HYPERTROPHIC LANGENDORFF RAT HEARTS; EFFECTS OF FATTY ACIDS AND SUPEROXYDE DISMUTASE MONITORED BY NADH SURFACE FLUORESCENCE

Hulsmann WC², Ashruf JF¹, Bruining HA¹, Ince C¹

¹Department of Surgery, Erasmus University of Rotterdam, Rotterdam

²Thorax center, Erasmus University of Rotterdam, Rotterdam, the Netherlands

Published in: Biochimica et Biophysica Acta; 1181: 273-278, 1993

SUMMARY

Hypertrophic hearts contain areas of hypoperfusion which can be visualized by increased NADH surface fluorescence during in vitro perfusion without oxygen-carrying particles under constant pressure and pacing. By contrast, fluorescence remained low when non-hypertrophic hearts were used instead. When during perfusion of normal hearts the pH of the medium was lowered from 7.5 to 7.0, areas of high fluorescence appeared in a few minutes. The high fluorescent areas under conditions of cardiac hypertrophy or pH 7.0 perfusion could be reduced by addition of superoxide dismutase. It indicates that oxygen free radicals interfere with proper flow regulation in areas of low pH. Fluorescence in hypertrophic hearts also diminished during addition of albumin-bound oleate to the standard, glucose-containing, medium. This is in agreement with our earlier finding of fatty acid protection from acidosis-initiated loss of capillary flow (Biochim Biophys Acta; 1033: (1990) 214-218). In contrast to low concentrations of free fatty acids, high concentrations interfere with tissue oxygenation. This has been illustrated by the use of 1 mM octanoate, which after a few min caused the appearance of high fluorescent areas. We conclude that decompensation of flow in hypoperfused areas of heart, as occurs in hypertrophy, may be stimulated by acidosis and oxygen free radicals.

INTRODUCTION

Previous work has shown that the Langendorff rat heart, when paced at a rate of 300 beats/min, but perfused with neither erythrocytes nor with fluorocarbon as oxygen carriers, is borderline aerobic, as concluded from the continuous release of lactate. Glyco(geno)lysis is inhibited by increase of O₂ supply by fluorocarbon addition [1, 2]. Lowering of the pH of the perfusion medium results in loss of high affinity binding of Ca²⁺ to membranes [3-5]. This results in a brief period of positive inotropy, accompanied by an increase of flow rate, followed by progressive decline of inotropy and flow so that ischemia follows [1, 6, 7]. This phenomenon may be delayed by fatty acids [6, 7], which are known to have vasodilatory properties (compare ref. 8). Development of cardiac ischemia may be studied by NADH fluorescence [6, 9-11]. It allows the detection of small areas in which the ischemia begins so that early intervention of imminent ischemia may be studied. We have selected to study the effect of superoxide dismutase (SOD) as we found earlier that cardiac function, lost by hypoperfusion of Langendorff hearts, improves by SOD addition by the removal of oxygen free radicals (OFR) from the interstitium [12]. In the present investigation we also studied hypoperfusion in normal hearts by lowering the perfusion pH, in order to induce interstitial acidosis which may be involved in the progression of cardiac hypertrophy. These hearts contain hypoperfused areas [13] in which local acidosis, due to enhanced glycolysis, may be present. As the heterogeneous distribution of (imminent) ischemia make data of overall metabolic changes difficult to interpret, we refrained from determining metabolic parameters in the present investigation.

MATERIALS AND METHODS

Male Wistar rats weighing between 220 and 320 g were used. The hearts were removed, cooled in cold perfusion medium and rapidly arranged for perfusion at 37°C and a pressure of 8.3 kPa according to the Langendorff technique with a medium consisting of 128 mM NaCl, 4.7 mM KCl, 1 mM MgCl₂, 0.4 mM NaH₂PO₄, 20.2 mM NaHCO₃, 1.3 mM CaCl₂, and 11 mM glucose [8]. Other additions are described under **RESULTS**. All hearts were paced at 5 Hz. NADH surface fluorescence was done after excitation of about 1 cm² of the left ventricle of Langendorff rat hearts with light of 360 nm and the emitted light (460-490 nm) registered as described [10]. Images were recorded with a S-VHS Panasonic video recorder and computer-analyzed [10]. Normal hearts had a total ventricle weight of 1.00 ± 0.04 g (n = 10) and hypertrophic hearts (obtained after suprarenal abdominal aorta banding; 15) of 1.52 ± 0.06 g (n = 10). The values are mean values ± Standard Error of the Mean (S.E.M.). Oleate-containing medium was used when indicated and prepared by sonicating oleic acid with an equivalent amount of NaOH. The warm soap was added to fatty acid-free bovine serum albumin (molar ratio fatty acid to albumin 4:1). The freshly prepared stock solution was added to the other components of the perfusion buffer and filtered through Millipore filter (0.45 µm) and 0.1 mM L-carnitine (a gift from Sigma Tau, Pomezia, Italy) was added. Most chemicals were from E. Merck (Darmstadt, Germany); SOD was from Boehringer (Mannheim, Germany).

RESULTS

‘Reducing power’ of various substrates in Langendorff hearts. During perfusion of the paced rat Langendorff heart with standard perfusion medium (pH 7.5), containing 11 mM glucose as substrate, NADH fluorescence of a representative area of the left ventricle was measured, and the acute change by adding various substrates was registered. Fig. 1 shows that the increase of NADH fluorescence by additional substrates tested (1-2 mM) decreased in the order: pyruvate > oleate > octanoate > lactate > beta-hydroxybutyrate/acetoacetate. Average values of $n = 3$ are presented. This led us to select pyruvate, octanoate and oleate in the present studies, as we assumed that an optimal supply of reducing equivalents to cardiomyocytes could limit breakdown of ATP and generation of (hypo)xanthine, substrate for (endothelial) xanthine oxidase-dependent O_2 -generation. That the addition of pyruvate increases NADH fluorescence confirms that the NADH fluorescence observed is of mitochondrial and not of cytosolic origin [9], as in the latter case it would have declined instead, due to stimulation of lactate dehydrogenase activity.

Sufficient O_2 supply disrupted by acidosis; effects of respiratory substrates and superoxide dismutase (SOD). As mentioned earlier, changing the pH of the standard perfusion medium from 7.5 to 7.0 rapidly results in a decrease of coronary flow and contractility of the heart (after an initial increase of both parameters (compare ref. 7)). This is accompanied by the appearance of areas of high fluorescence (Fig. 2A, B). Addition of pyruvate was not able to prevent pH 7.0 induced ischemia (not shown; $n = 4$). When SOD had been present in the pH 7.5 medium, the change to the pH 7.0 medium still led to immediate and strong increase of NADH fluorescence in large parts of the heart, which persisted. However, it gradually decreased when the SOD supply was also contained in the pH 7.0 medium (Fig. 3). When after 30 min SOD in pH 7.0 medium, pH 7.0 perfusion without SOD was continued, no fluorescence increase was observed after 15 min, suggestive for continued SOD activity in the interstitium when SOD supply was discontinued (not shown). The flow, that had decreased from about 8 ml/min per g (after 20 min perfusion in pH 7.5 medium) to 1.9 ± 0.7 ml/min per g ($n = 6$) after 15 min pH 7.0 perfusion without SOD, generally did not drop when SOD was present (flow: 8.6 ± 2.5 ml/min per g; $n = 4$). The large variation may be due to the overall heterogeneity of the phenomena. We interpreted the absence of SOD effect when it was only supplied during pH 7.5 perfusion by its inability to penetrate into the interstitium when interendothelial junctions are tight. During pH 7.0 perfusion, SOD entry into the interstitium must have occurred, perhaps due to increased permeability by pH lowering, as will be discussed.

Octanoate addition and NADH fluorescence. Our previous experience [7], that albumin-bound oleate can prevent the strong decrease of flow by changing the pH of the perfusion medium from 7.5 to 7.0, led us to use another fatty acid, octanoate, in the present study. This fatty acid as well as acetate has been shown by others to protect the energy state of glucose-perfused working rat hearts after respiratory acidosis [6]. Under our conditions,

Fig. 1. Comparison of the NADH fluorescence of normal Langendorff hearts perfused with the substrates indicated (in addition to glucose), with the NADH fluorescence when perfused with glucose as the sole substrate ($n = 3$; mean $\pm$ S.D.).

changing of the standard perfusion medium of pH 7.5 (with glucose) to one containing in addition 1 mM octanoate caused an immediate overall increase of fluorescence (cf. Fig. 1). After a few min, areas of high NADH fluorescence became visible, which gradually increased in size (Fig. 4). This phenomenon appeared to be reversible by continued perfusion without octanoate, requiring at least 30 min. This experiment was repeated thrice with similar result.

Fig. 2. Effect of pH 7.0 perfusion on the NADH fluorescence of a normal heart: (A) a heart during pH 7.5 perfusion; no ischemia is present as witnessed by the low fluorescence of the displayed left ventricle surface and (B) the same heart 3 min after switching from pH 7.5 to pH 7.0 perfusion; the white areas represent high NADH fluorescent ischemic areas.

Fig. 3. Effect of SOD addition to the medium on ischemic areas induced by pH 7.0 perfusion: (A) a heart after 5 min pH 7.0 perfusion and (B) the same heart 15 min after pH 7.0 perfusion with 10 mg/l SOD in the perfusate.

Fig. 4. Effect of octanoate addition to the medium on NADH fluorescence of a normal heart: (A) prior to octanoate addition, (B) 1 min after octanoate addition; there is an overall increase of NADH fluorescence, (C) 10 min after octanoate addition; large ischemic areas are present and (D) 10 min after switching back to perfusion without octanoate.

Therefore 1 mM final concentration of octanoate probably resulted in redistribution of flow, which we had never seen when using oleate or pyruvate at pH 7.5 instead. In the latter cases a moderate, but evenly distributed fluorescence increase was seen, which served to construct Fig. 1. When during octanoate addition the pH of the medium was switched from 7.5 to 7.0, the flow rapidly deteriorated, which resulted in increase of NADH fluorescence (Fig. 5).

Fig. 5. Effect of pH change upon a normal heart perfused with octanoate containing medium: (A) a heart during pH 7.5 perfusion with octanoate; note the ischemic areas and (B) the same heart 1 min after switching to pH 7.0 perfusion; the ischemic area has rapidly increased in size.

Hypertrophic hearts and NADH fluorescence. From the start of Langendorff perfusion (pH 7.5 and glucose as the only substrate), hypertrophic hearts show areas of high NADH

fluorescence, that gradually increase in size and intensity. Fig. 6 shows that hypertrophy is not a homogeneous phenomenon, probably due to different sarcomere/capillary ratios in different areas [13]. The increase of fluorescence can be stopped and acutely reversed by the addition of vasodilators, like nitroprusside or adenosine, or by improvement of tissue oxygenation by fluorocarbon [11]. Addition of 0.8 mM oleate (containing 0.2 mM bovine serum albumin and 0.1 mM carnitine) resulted in a gradual decrease of the fluorescent areas (Fig. 7; two examples of four experiments with similar effect). SOD (final concentration 10 mg/l) added instead of oleate/albumin/carnitine also gradually diminished NADH fluorescence. Whereas the disappearance of ischemia by nitroprusside, adenosine or fluorocarbon was a matter of seconds, the effects of oleate/albumin/carnitine or SOD was a matter of minutes. The effect of SOD required a lag phase of about 15 min. Fig. 8 shows two examples out of four with similar effect (in a fifth experiment no effect was observed after 30 min SOD perfusion). When SOD addition was stopped after 30 min, fluorescence remained low ($n = 4$) for at least 30 min, suggesting persistent SOD activity in the interstitium.

Fig. 6. Three examples of hypertrophic Langendorff hearts. The white areas on the left ventricle surface are ischemic areas which developed within 5 min after the start of perfusion.

DISCUSSION

The present work indicates that in the absence of oxygen-carrying particles, the O_2 -saturated perfusion medium only provides borderline aerobiosis in paced normal Langendorff hearts. This is reflected by a low production rate of lactate [1, 2], while NADH fluorescence is low. Fluorescence rapidly increases when the pH of the medium is lowered to 7.0 or less, indicative of decreased flow in affected areas, as may have been expected from previous investigations in which either respiratory acidosis was effected in working hearts [6, 14] or metabolic acidosis in Langendorff hearts [1, 7]. The heterogeneous pattern of fluorescence increase by acidosis, induced by changing the pH of the medium from 7.5 to 7.0 during Langendorff perfusion in the present work (Fig. 2), could be due to differences in the regulation of flow in different areas of the heart. Also, in hypertrophic hearts perfused with standard medium (pH 7.5) the appearance of heterogeneously distributed high fluorescent areas became visible [11]. They rapidly disappear when the coronary flow is increased by the addition of nitroprusside or adenosine [11]. Apparently, in those areas flow decreases progressively in the absence of added vasodilators so that a critical O_2 level is quickly

reached in which NADH of the mitochondrial respiratory chain accumulates. This is in agreement with the disappearance of NADH fluorescence by increasing the O₂ supply by addition of fluorocarbon to the perfusion medium [11]. This also limits lactic acid formation during pH 7.0 perfusion [1]. These observations suggest that local acidosis is a common factor involved in the decline of flow in certain areas of pH 7.0-perfused control hearts or pH 7.5-perfused hypertrophic hearts. Steenbergen et al. [14] observed that “acidosis-induced ischemia is characterized by relatively large areas of high fluorescence as opposed to the mottled appearance of high flow hypoxia”. They concluded from this that changes of coronary circulation are important for the development of the anoxic zones [14]. Interestingly, mild acidosis may result in vasodilation, but severe acidosis constricts vessels. As for acidosis and vasodilation, we observed that initially, after changing pH 7.5 to pH 7.0 perfusion, the contractility of non-working Langendorff hearts increased together with coronary flow [7]. Only after contractility had started to fall, did the increased flow gradually diminish and finally resulted in low flow ischemia, probably due to further decline of the pH in the tissue by lactic acid formation and ATP breakdown (1, 7). Fatty acids also have dual effects on flow, depending on their free concentration. Low levels of long-chain fatty acids, bound to albumin, are vasodilators [8]. The initial effect of 1 mM octanoate on overall flow during pH 7.5 perfusion was also an increase ($45 \pm 8\%$, $n = 4$, present study; compare also ref. 8). However, after 5 min the overall flow had started to fall, while ischemic areas were formed (Fig. 4). When the octanoate-containing medium of pH 7.5 was changed into one of pH 7.0, the ischemic area largely increased within 5 min (Fig. 5), while the overall flow dropped to low values (1-3 ml/min per g). It suggests that the degree of fatty acid dissociation plays a role in their effectiveness to penetrate membranes. It has been

Fig. 7. Effect of oleate addition to the perfusion medium on ischemic areas of hypertrophic hearts: (A) and (B) are images of 2 different hearts prior to oleate addition; (C) and (D) display the same hearts as (A) and (B) respectively, 2 min after switching to perfusion containing oleate.

Fig. 8. Effect of SOD addition to the perfusion medium on ischemic areas of hypertrophic hearts: (A) and (B) are images of 2 different hearts prior to SOD addition; (C) and (D) display the same hearts as (A) and (B) respectively, 15 min after perfusion with SOD (10 mg/l) containing medium.

shown that the effect of amphiphiles depends on the charge of these membrane-active agents [16]. Fatty acids and lysophospholipids have (indirect) ionophoric properties (compare refs. 8 and 16-18) which can affect the Ca^{2+} level in cells. In smooth muscle- and endothelial cells of blood vessels, amphiphiles affect the contractile state of these cells and influence capillary flow. They may also cause edema, as had been seen earlier by employing 5 mM octanoate during pH 7.5 Langendorff perfusion. After an initial flow increase, the flow rate progressively decreased, which was accompanied by the release of lactate, adenosine, inosine and hypoxanthine, suggestive of insufficient oxygenation [8]. In those experiments macroscopic edema appeared, judged by the shiny appearance of the heart and increase of its size. In the present experiments in which 1 mM octanoate was used instead, macroscopic edema did not occur during 30 min perfusion. Yet, extravasation of fluid may have been present as restoration of low fluorescence by octanoate-free perfusion only slowly occurred during a 30-min further observation.

The interstitial space plays an important role in the initiation of cardiac hypertrophy [19]. Increase of the permeability of endothelium by fatty acids and/or acidosis has been described by many authors. A relation between protons and fatty acids on permeability of capillaries may be the natural presence of fatty acids in membranes, which at pH 7.4 are mainly in ionized form and as such part of the bilayer structure. However, upon acidification, unionized species may escape from lamellar structures [20, 21] and activate membrane processes that result in increased intracellular Ca^{2+} levels. The increased penetration of vascular constituents into the interstitial space may also be the reason why SOD was able to enter the interstitium during pH 7.0 perfusion and not during the previous pH 7.5 perfusion in the experiments shown in Fig. 3. These illustrate improvement of flow during acidosis by SOD. The beneficial effect of SOD on tissue oxygenation also applies to pH 7.5-perfused hypertrophic hearts (Fig. 8). It indicates also that in hypertrophic hearts extracellular OFR interferes with the regulation of capillary flow. This could be due to the

ability of OFR to destroy endothelium-derived relaxing factor [22]. A low rate of secretion and/or adequate removal of OFR from the outside of endothelial cells are most important to prevent ischemia. This may also be the basis of the inhibitory effect of albumin-complexed oleate upon the progression of flow decrease during pH 7.0 perfusion of normal hearts [7] and to pH 7.5-perfused hypertrophic hearts, in which hypoperfusion may even disappear under the conditions of the tests (Fig. 7). The explanation may be that during fatty acid oxidation in the (peri)vascular area, in which O_2 is still present, lactic acid formation is inhibited and endothelium-derived relaxing factor is increased [23].

REFERENCES

1. Hulsmann WC, De Wit LEA (1990). Acidosis, cardiac stunning and its prevention by oxygen. *Cell Biol Int Rep*; 14: 311-315
2. Hulsmann WC, De Wit LEA, Stam H et al. (1990). Hormonal control of cardiac lipolysis by glyco(geno)lysis. *Biochim Biophys Acta*; 1055: 189-192
3. Williamson JR, Safer B, Rich T et al. (1976). Effects of acidosis on myocardial contractility and metabolism. *Acta Med Scand Suppl*; 587: 95-112
4. Vandeplassche G, Borgers M (1990). Ultrastructure and Ca^{2+} reallocation during ischemia, the Ca^{2+} paradox and metabolic acidosis. *Cell Biol Int Rep*; 14: 317-334
5. Kwan CY, Daniel EE (1989). In: Calcium in essential hypertension (Aoli K, Frohlich ED, eds.), 201-230, Academic Press Japan
6. Schaffer SW, Safer B, Ford C et al. (1978). Respiratory acidosis and its reversibility in perfused rat heart: regulation of citric acid cycle activity. *Am J Physiol*; 234: H40-H51
7. Hulsmann WC, De Wit LEA, Schneydenberg C et al. (1990). Loss of cardiac contractility and severe morphologic changes by acutely lowering the pH of the perfusion medium: protection by fatty acids. *Biochim Biophys Acta*; 1033: 214-218
8. Hulsmann WC (1976). Coronary vasodilation by fatty acids. *Basic Res Cardiol*; 71: 179-191
9. Chance B (1976). Pyridine nucleotide as an indicator of the oxygen requirements for energy-linked functions of mitochondria. *Circ Res*; 38: suppl I, 31-38
10. Ince C, Ashruf JF, Avontuur JAM et al. (1993). Heterogeneity of the hypoxic state in rat heart is determined at capillary level. *Am J Physiol*; 264: H294-H301
11. Ashruf JF, Ince C, Bruining HA et al. (1994). Ischemic areas in hypertrophic Langendorff rat hearts visualized by NADH videofluorimetry. *Adv Exp Med Biol*; 345: 259-262
12. Hulsmann WC, Dubelaar ML (1987). Early damage of vascular endothelium during cardiac ischaemia. *Cardiovasc Res*; 21: 674-677
13. Katz AM (1990). Cardiomyopathy of overload. A major determinant of prognosis in congestive heart failure. *N Engl J Med*; 323: 100-110
14. Steenbergen C, Deleeuw G, Barlow C et al. (1977). Heterogeneity of the hypoxic state in perfused rat heart. *Circ Res*; 41: 606-615
15. Malik AB, Shapiro JM, Yanics J et al. (1974). A simplified method for producing rapid ventricular hypertrophy in rats. *Cardiovasc Res*; 8: 801-805
16. Epand RM, Lester DS (1990). The role of membrane biophysical properties in the regulation of protein kinase C activity. *TIPS*; 11: 317-321
17. Kirber MT, Ordway RW, Clapp LH et al. (1992). Both membrane stretch and fatty acids directly activate large conductance Ca^{2+} -activated K^{+} channels in vascular smooth muscle cells. *FEBS Lett*; 297: 24-28
18. Locher R, Weissner B, Mengden L et al. (1992). Lysolecithin actions on vascular smooth muscle cells. *Biochim Biophys Res Commun*; 183: 156-162
19. Allen GA, Laine SJ (1991). Left ventricular myocardial edema. Lymph flow, interstitial fibrosis, and cardiac function. *Circ Res*; 68: 1713-1721
20. Amende LM, Blanchette-Mackie JJ, Chernick SS et al. (1985). Effect of pH on visualization of fatty acids as myelin figures in mouse adipose tissue by freeze-fracture electron microscopy. *Biochim Biophys Acta*; 837: 94-102

21. Cistola DP, Hamilton JA, Jackson D et al. (1988). Ionization and phase behavior of fatty acids in water: application of the Gibbs phase rule. *Biochemistry*; 27: 1881-1888
22. Moncada S, Palmer RMJ, Higgs EA (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev*; 43: 109-142
23. Hulsmann WC, Dubelaar ML (1992). Carnitine requirement of vascular endothelial and smooth muscle cells in imminent ischemia. *Mol Cell Biochemistry*; 116: 125-129

5

INCREASE OF CARDIAC WORK IS ASSOCIATED WITH DECREASE OF MITOCHONDRIAL NADH

Ashruf JF¹, Coremans JMCC¹, Bruining HA¹, Ince C^{1,2}

¹Department of Surgery, Erasmus University of Rotterdam, Rotterdam

²Department of Anesthesiology, University of Amsterdam, Amsterdam, the Netherlands

Published in: Am J Physiol; 269: H856-H862, 1995

SUMMARY

In this study we investigated the effect of work and substrate supply on mitochondrial NADH/NAD⁺ using epicardial autofluorescence in rat hearts perfused according to Langendorff. To avoid vasoconstrictor effects during high work output, nitroprusside-containing Tyrode solution was used. Photobleaching was avoided by using discontinuous ultraviolet excitation for NADH fluorescence measurements. To increase work, heartbeat rate was raised from 5 to 7 Hz, and concomitantly left ventricular pressure was raised stepwise from 0 to ± 90 mmHg. During substrate-limited (5.5 mM glucose) perfusions, increase in O₂ consumption ($3.5 \pm 0.4 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$, mean $\pm$ SE, n = 6) caused by increase of heartbeat rate was associated with a significant decrease of NADH fluorescence ($-31 \pm 2.5\%$, mean $\pm$ SE, n = 6). During perfusions with 10 mM pyruvate increase of O₂ consumption ($3.6 \pm 0.7 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$, mean $\pm$ SE, n = 6) was associated with significant decrease of NADH fluorescence ($-20 \pm 2.6\%$, mean $\pm$ SE, n = 6). These results suggest that a rise in mitochondrial NADH/NAD⁺ is not the primary stimulus for increase in respiration and that changes of mitochondrial NADH/NAD⁺ are secondary to changes in O₂ consumption.

INTRODUCTION

The mechanism that tunes the rate of respiration to a changing ATP demand during alterations in work of the intact myocardium is still a source of uncertainty (2-4, 13). Thermodynamically the rate of respiration is described by the difference in substrate oxidation potential and the proton motive force, which is in near equilibrium with the phosphate potential. Hence, the phosphate potential (ATP/ADP) and the substrate oxidation potential (e.g., redox potential difference of NADH/NAD⁺ couple and O₂/H₂O couple) have been proposed as potential regulatory factors of oxidative phosphorylation. Although studies of isolated mitochondria have demonstrated that the rate of respiration can be controlled by changes in the concentration of ADP and P_i (6), in intact myocardium no significant change in the phosphorylation potential has been found (2, 3). Whereas elevation of the NADH concentration can increase the maximum rate of oxidative phosphorylation and stimulate ATP synthesis in isolated mitochondria (20), conflicting data on the NADH redox state during increase of work in intact myocardium have been found. In isolated rabbit heart no change was found in the NAD(P)H redox state during changes in afterload and heart rate (13). An increase of mitochondrial NADH/NAD⁺ was found in response to increased heart rate in the rat heart (18). In contrast a decrease of intramitochondrial NADH/NAD⁺ was reported for isolated papillary rabbit muscle in response to twitches (8). Methodological differences could explain these contradictory results. Measurement of the intracellular autofluorescence of NADH (365-nm excitation, 470-nm emission) allows qualitative evaluation of mitochondrial NADH/NAD⁺ redox state, since NADH fluoresces when excited with ultraviolet (UV) light, and NAD⁺ does not (5). The NADH fluorescence measurement can be disturbed by ischemia (10, 13), oximetric effect (which is the metabolic state related change in absorption of fluorescence excitation and emission light caused by spectral characteristics of cytochromes and myoglobin) (10, 13), endogenous substrate depletion (13, 18), movement (10, 13, 15, 18), and photobleaching (13).

The present study was conducted to investigate whether changes in work of the myocardium are associated with changes in mitochondrial NADH/NAD⁺, taking into account the above-mentioned factors influencing NADH fluorescence measurements. A vasodilator was added to the perfusate to avoid the development of ischemia by increasing coronary flow (15). To detect a possible oximetric effect in this study, diffuse reflectance spectra were acquired during low and high cardiac work. Substrate conditions were used which either tend to limit (5.5 mM glucose) or provide abundant (10 mM pyruvate) reducing equivalent delivery to the mitochondria. NADH fluorescence images were measured, enabling the recognition of heterogeneously distributed ischemic areas and the reduction of motion artifacts. Photobleaching was diminished by using discontinuous UV illumination. Preliminary results of this study have been reported elsewhere (1).

MATERIALS AND METHODS

Experimental Setup. Male Wistar rats weighing 275-375 g were anesthetized by ether inhalation. Before sternotomy, the rats were given 200 IU/kg heparin intravenously (Thromboliquine, Organon Teknika Oss, Netherlands). The hearts were perfused according

to Langendorff and were paced via aortic and right ventricular leads. Mean flow rates (in $\text{ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ ventricle wetwt) were measured with an electromagnetic flow probe (Skalar Medical, Delft, Netherlands) placed just before the aortic cannula. Left ventricular pressure was measured as described elsewhere (21), using a 22-gauge cannula inserted through the apex in the left ventricle and connected to a pressure transducer (Hewlett-Packard 8805B corner amplifier). In case the cannula communicated with the atmosphere, no pressure was developed by the left ventricle, and the work output of the heart was low. To increase the amount of work performed by the heart, the apex cannula was closed, and simultaneously the heartbeat frequency was increased from 5 to 7 Hz. To decrease the work output, the apex cannula was opened, and the heartbeat frequency was decreased from 7 to 5 Hz according to the method of Kobayashi and Neely (19). One pulmonary artery was cannulated and its effluent was diverted with a constant flow to an O_2 probe (YSI biological oxygen monitor, model 5300, Yellow Springs Instruments, Yellow Springs, OH). O_2 tensions in both the aortic influent and pulmonary effluent were measured with an YSI biologic oxygen monitor. From these measurements O_2 consumption was calculated (expressed as $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ ventricle wetwt). The perfusate was a modified Tyrode solution (128 mM NaCl, 4.7 mM KCl, 1 mM MgCl_2 , 0.4 mM NaH_2PO_4 , 1.2 mM Na_2SO_4 , 20.2 mM NaHCO_3 , 1.3 mM CaCl_2) containing either glucose (5.5 mM) or pyruvate (10 mM). The perfusate was thermostatted at 37 °C and equilibrated with either 95% O_2 -5% CO_2 or 95% N_2 -5% CO_2 . In some experiments 10 μM sodium-nitroprusside, an endothelium independent vasodilator was added to the perfusate. Aortic pressure was kept at 80 mmHg. Chemicals were obtained from Merck (Darmstadt, Germany).

NADH Fluorescence Measurements. The videofluorimeter used has been described in Ref. 15. The 365-nm line from a 100-W mercury arc lamp (Olympus, Tokyo) was selected by means of an UG-1 barrier filter to provide the UV light needed for NADH excitation (Fig. 1). An image intensified video camera (MXRi second generation charge-coupled device video camera, HCS vision, Eindhoven, Netherlands) with a Micro-Nikkor 105-mm macrolens was used to record NADH fluorescence images of the left ventricle of the heart. The NADH fluorescence signal was selected by means of a band-pass filter centered around 470 ± 20 nm and was placed in front of the camera. To enable correction of images for changes in the sensitivity of the videofluorimeter and fluctuations in the intensity of the light source, a small piece of uranyl calibration glass (15, 22) was placed next to the heart within the excitation field. Images were recorded on a video recorder and computer-analyzed off-line. To minimize motion artifacts, pacemaker signals and fluorescence images were recorded simultaneously on videotape, thus enabling selection of fluorescence images recorded in the same geometrical configuration (15). In a metabolically stable preparation, these precautions restricted variations in NADH fluorescence intensity due to motion to the 5% margin. We also evaluated the stability of the fluorescence signal with an intracellular fluorescence standard 5(6)-carboxy-2',7'-dichlorofluorescein diacetate (CDCF), as described by Heineman and Balaban (13), and found no work load dependence of the CDCF fluorescence intensity with our experimental setup. NADH fluorescence intensities are expressed in arbitrary units (AU), which are defined as the ratio of the NADH fluorescence intensity of myocardium and the fluorescence intensity of the uranyl calibration glass.

Changes in intensity of NADH fluorescence are expressed in percent, taking initial NADH fluorescence intensity (i.e., just before change) as 100%.

Fig. 1. Experimental setup used for NADH fluorescence measurements consists of a Langendorff setup with 2 perfusate containers. A small piece of uranyl calibration glass is placed next to heart as a fluorescence reference. Hg arc lamp provides 360-nm light needed for NADH excitation. Dichroic mirror (DM) separated excitation and emission light. Charge-coupled device (CCD) video camera detects 470-nm NADH fluorescence image. Signals were recorded on a video recorder and analyzed off-line. LVP cannula, left ventricular pressure cannula.

Fig. 2. Experimental setup used for measurements of diffuse reflectance spectra. Heart is illuminated by a Xe (Hg) lamp. Reflected light is collected (under an angle of 45° with respect to incident UV light) with a fiber. Reflected light is dispersed in spectrograph and a CCD detector registered resulting reflectance spectra that were later analyzed on computer

Diffuse Reflectance Spectroscopy. Diffuse reflectance spectra of the heart surface were measured with an OMA Vision System (EG&G Princeton Applied Research), essentially as previously described (10) (Fig. 2). The heart was illuminated by a Xe (Hg) lamp (100 W, Oriel). Remitted light from a small area of the left ventricle was transmitted to a spectrograph (model 1228, EG&G Princeton Applied Research; focal length 0.32 m, 25 μm entrance slit, grating 147.5 $\text{g}\cdot\text{mm}^{-1}$ blazed at 300 nm) by a quartz fiber (KOI655) positioned at 45° with respect to the heart surface at a working distance of 1.5 mm. In this configuration the field under observation was $\sim 3 \text{ mm}^2$. The diffuse reflectance spectra (450-650 nm, 240-ms scans) were measured with a 1024-element silicon photodiode array (model 1453A, EG&G Princeton Applied Research; thermoelectrically cooled to 10°C).

Experimental Protocols. After the hearts were arranged for Langendorff perfusion, they were allowed to stabilize for ± 15 min before the following experimental protocols were performed:

Protocol 1. Illumination procedure and substrate utilization. To assess the possible contribution of photobleaching on the gradual decrease of NADH fluorescence over time, hearts were subjected to different illumination procedures for a period of 30 min. During the first 10 min, hearts were exposed to UV excitation light 5 s of every min. From 10 to 20 min, the hearts were illuminated continuously, and in the last 10 min, 5 s of every minute of exposure were resumed again. To elucidate the possible role of endogenous substrate depletion upon the gradual decrease of NADH fluorescence over time, hearts were perfused with Tyrode solution containing either pyruvate or glucose. Pyruvate was added to the perfusate to saturate the reducing equivalent supply to the mitochondria, thereby preventing depletion of endogenous substrates (19). With glucose added to the perfusate, substrate-limited perfusion was achieved (19), thus inducing usage of endogenous substrates. Vasodilation was achieved by addition of 10 μ M nitroprusside.

Protocol 2. Cardiac work at limited O₂ supply and limited or abundant substrate supply. Perfusion with O₂ carrier-free medium renders the Langendorff heart borderline aerobic (9, 14). Under these conditions ischemia easily develops during increase in O₂ demand (15). To study the effect of increased work on the mitochondrial NADH concentration under conditions that tend to limit O₂ supply and either limit or maximize substrate supply, the heartbeat rate of the Langendorff hearts was increased, and pressure development by the left ventricle was induced. The work was changed according to a fixed protocol of 30 min duration. During the first 10 min the hearts were paced at 5 Hz with an open apex cannula (no pressure development by left ventricle, low cardiac work). Then the apex cannula was closed and the frequency was increased to 7 Hz for 10 min (pressure development by left ventricle, high cardiac work), during which period flow and O₂ consumption were stabilized. The apex cannula was then reopened, and the frequency was decreased to 5 Hz (again low cardiac work) for another period of 10 min to stabilize flow and O₂ consumption. This work protocol was performed twice on each heart under conditions of limited (5.5 mM glucose) and abundant (10 mM pyruvate) substrate supply.

Protocol 3. Cardiac work at ample O₂ supply and limited or abundant substrate supply. To study the effect of increased work without development of hypoxic areas, 10 μ M nitroprusside was added to the perfusate, and protocol 2 was performed. Some hearts underwent protocol 3 twice, the first time during registration of NADH fluorescence and the second time during registration of diffuse reflectance spectra. During protocols 2 and 3 the hearts were illuminated with UV light 5 s every min except during switches in pace frequency or substrate, when the hearts were illuminated continuously for $\pm$ 10 s.

Protocol 4. Diffuse reflectance spectra during perfusion with 95% O₂-95% N₂-saturated Tyrode solution. To demonstrate the effect of limited O₂ supply upon diffuse reflection spectra, diffuse reflectance spectra were recorded when hearts were perfused with 95% O₂-95% N₂-saturated Tyrode solution, respectively. Group means were tested for difference using paired t-tests. The criterion for significance was taken to be $P < 0.01$ for all comparisons. Descriptive data are presented as means $\pm$ SE.

RESULTS

Photobleaching, caused by illumination of tissue with UV light, could be responsible for a gradual decline of the NADH fluorescence intensity, thereby complicating the interpretation of changes of the NADH fluorescence intensity associated with changes in cardiac work. To examine the effect of illumination with UV light on NADH fluorescence intensity, hearts were alternately exposed to continuous and discontinuous illumination according to protocol 1 (Fig. 3). Table 1 summarizes the changes of NADH fluorescence intensity associated with different methods of illumination. Continuous UV illumination is associated with a larger decrease of NADH fluorescence intensity than discontinuous UV illumination. To examine whether endogenous substrate utilization can contribute to the observed time-dependent decrease of the NADH fluorescence intensity, the changes in NADH fluorescence intensity in glucose-perfused hearts were compared with those of pyruvate-perfused hearts (Table 1). No difference was found. Hence, to avoid the photobleaching effect in future experiments, discontinuous UV illumination was applied.

Fig. 3. Influence of UV illumination procedure on NADH-fluorescence intensity. Each point in graph represents one NADH fluorescence measurement in a different video image. 0-10 min: discontinuous illumination, 10-20 min: continuous illumination, 20-30 min: discontinuous illumination. F_{NADH} : mean NADH-fluorescence intensity.

	discontinuous illumination (10 min)	continuous illumination (10 min)	discontinuous illumination (10 min)
$\Delta NADH$ fluorescence, % (5.5 mM glucose)	-2.5 $\pm$ 2.3	-28 $\pm$ 3.4	-2.8 $\pm$ 1.9
$\Delta NADH$ fluorescence, % (10 mM pyruvate)	-5.8 $\pm$ 1.5	-28 $\pm$ 4.6	-4.3 $\pm$ 2.1

Table 1. Changes of NADH fluorescence associated with discontinuous and continuous ultraviolet illumination. Values are means $\pm$ SE; n = 5. $\Delta NADH$ fluorescence, change of NADH fluorescence in % between beginning and end of 10-min period. $\Delta NADH$ fluorescence between glucose and pyruvate perfused hearts did not differ, whereas it did differ for discontinuous and continuous illumination.

To investigate whether redox changes of the mitochondrial respiratory chain and oxygenation changes of myoglobin influence the absorptive and reflective properties of cardiac tissue during the low and high cardiac work period, diffuse reflectance spectra were measured (protocol 3). Diffuse reflectance spectra obtained during the low and high cardiac work period did not differ from each other (Fig. 4A), whereas redox changes and oxygenation changes can easily be deduced from the diffuse reflectance spectra obtained from hearts perfused with O₂- and N₂-saturated Tyrode solution, respectively (protocol 4, Fig. 4B).

Fig. 4. Diffuse reflectance spectra of rat heart perfused with Tyrode solution containing 5.5 mM glucose and 10 μM nitroprusside. A: heart perfused with oxygenated perfusate during low cardiac work (solid line) and during high cardiac work (broken line). B: heart paced at 5 Hz and perfused with Tyrode solution equilibrated with either 95% O₂-5% CO₂ (solid line) or 95% N₂-5% CO₂ (broken line).

To study the effect of changes of work upon epicardial NADH fluorescence during O₂-limited perfusions, six hearts were subjected to periods of low and high cardiac work (Fig. 5). These work changes were applied to each heart during perfusion with glucose- and pyruvate-containing Tyrode solution in the absence of a vasodilator (protocol 2). Figure 5, A and C, show the epicardial NADH fluorescence at low cardiac work during glucose and pyruvate perfusion, respectively. The NADH fluorescence is increased upon switching from glucose- to pyruvate-containing Tyrode solution and is distributed homogeneously over the heart surface. Upon increase in work under both perfusion conditions the NADH fluorescence images revealed the development of very prominent highly fluorescent ischemic areas (Fig. 5, B and D). The ischemic areas were very different in size and distribution pattern from heart to heart. To demonstrate changes in work during the protocol, hemodynamic data are shown in Table 2.

Fig. 5. NADH fluorescence images of heart perfused with Tyrode solution containing 5.5 mM glucose (A and B) or 10 mM pyruvate (C and D) in absence of nitroprusside. A and C: low cardiac work; B and D: high cardiac work.

LVP cannula	glucose(5.5 mM) nitroprusside(0 μ M)			pyruvate (10 mM) nitroprusside (0 μ M)		
	open	closed	open	open	closed	open
Frequency Hz	5	7	7	5	7	5
+dP/dt mmHg/s	2094 $\pm$ 125			1489 $\pm$ 72		
-dP/dt mm Hg/s	1466 $\pm$ 129			961 $\pm$ 74		
Peak LVP mm Hg	87 $\pm$ 5.5			82 $\pm$ 5.7		
Diastolic LVP mm Hg	15 $\pm$ 1.1			32 $\pm$ 6.4		
Flow ml $\cdot$ min ⁻¹ $\cdot$ g ⁻¹	13.3 $\pm$ 0.9	15.4 $\pm$ 0.9	13.3 $\pm$ 0.5	22.2 $\pm$ 1.9	20.6 $\pm$ 0.7	24 $\pm$ 1.8
MVO ₂ μ mol $\cdot$ min ⁻¹ $\cdot$ g ⁻¹	3.2 $\pm$ 0.4	7.9 $\pm$ 0.3	2.9 $\pm$ 0.3	3.9 $\pm$ 0.4	8.3 $\pm$ 0.5	4.1 $\pm$ 0.6

Table 2. Hemodynamic values during changes in work in absence of nitroprusside. Values are steady-state values expressed in means $\pm$ SE; n = 6. Changing pace frequency resulted in changes in MVO₂. During pyruvate perfusion +dP/dt, -dP/dt, diastolic LVP, and coronary flow differed from those values during glucose perfusion. +dP/dt and -dP/dt, 1st time derivative of positive and negative LVP, respectively; LVP, left ventricular pressure; MVO₂, myocardial oxygen consumption.

O₂ consumption increased upon increase of work; it also increased upon switching from glucose- to pyruvate-containing perfusate. Left ventricular end-diastolic pressure was larger

during pyruvate than glucose perfusion. After a work change coronary flow did not alter but flow increased upon switching from glucose to pyruvate perfusion.

To avoid the development of ischemic areas during increase in cardiac work, the experiment was repeated with 10 μM nitroprusside present in the perfusate (protocol 3). Hemodynamic data are presented in Table 3. Due to nitroprusside induced vasodilation, coronary flows were larger than those during protocol 2. Comparison of Tables 2 and 3 reveals that other hemodynamic data were not affected by nitroprusside. O_2 consumption increased in response to increased cardiac work. NADH fluorescence images of a heart recorded in periods of low and high cardiac work (Fig. 6, A and C, and B and D, respectively), all showed an even distribution of the fluorescence intensity. The corresponding time trace of NADH fluorescence intensity changes during protocol 3 is shown in Fig. 6E, where fluorescence images taken at the moments indicated by A-D are depicted in Fig. 6, A-D. On switching from glucose- to pyruvate-containing perfusate, fluorescence increased 2.3 ± 0.09 ($n = 6$) times, and O_2 consumption increased $0.5 \pm 0.05 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ ($n = 6$), whereas coronary flow did not change. Under both substrate regimes increase of work was associated with decrease of NADH fluorescence intensity and vice versa. Grouped data of the changes in NADH fluorescence intensity and the associated changes in O_2 consumption are summarized in Table 4.

Fig. 6. NADH fluorescence images of a heart perfused with Tyrode solution containing 5.5 mM glucose (A and B) or 10 mM pyruvate (C and D) in presence of nitroprusside. A and C: low cardiac work. B and D: high cardiac work. E: time trace of NADH-fluorescence intensity changes during protocol 3. From 0 to 30 min: perfusion with 5.5 mM glucose. From 30 to 60 min: perfusion with 10 mM pyruvate. From 0 to 10, 20 to 30, 30 to 40, and 50 to 60: low cardiac work. From 10 to 20 and 40 to 50 min: high cardiac work. Video images at moments indicated by A-D are shown in panels A-D.

Fig. 7. Time trace of NADH fluorescence intensity changes during a stepwise increase of cardiac work during 5.5 mM glucose (A) and 10 mM pyruvate (B) perfusion. Cardiac work was increased at moments indicated by an arrow.

LVP cannula	glucose(5.5 mM) nitroprusside(10 μ M)			pyruvate (10 mM) nitroprusside (10 μ M)		
	open	closed	open	open	closed	open
Frequency Hz	5	7	7	5	7	5
+dP/dt mmHg/s		2086 $\pm$ 109			1361 $\pm$ 93	
-dP/dt mm Hg/s		1552 $\pm$ 126			904 $\pm$ 93	
Peak LVP mm Hg		89 $\pm$ 1.7			81 $\pm$ 5.9	
diastolic LVP mm Hg		14 $\pm$ 2.9			32 $\pm$ 3.3	
Flow ml $\cdot$ min ⁻¹ $\cdot$ g ⁻¹	28.3 $\pm$ 1.7	27.9 $\pm$ 1.6	28.6 $\pm$ 1.8	29.5 $\pm$ 2.0	25.8 $\pm$ 1.6	31.1 $\pm$ 2.2
MVO ₂ μ mol $\cdot$ min ⁻¹ $\cdot$ g ⁻¹	4.0 $\pm$ 0.2	7.5 $\pm$ 0.4	3.8 $\pm$ 0.1	4.3 $\pm$ 0.1	7.9 $\pm$ 0.6	4.2 $\pm$ 0.1

Table 3. Hemodynamic values during changes in work in presence of 10 μ M nitroprusside. Values are steady-state values expressed in means $\pm$ SE; n = 6. Changing pace frequency resulted in changes in MVO₂. During pyruvate perfusion +dP/dt, -dP/dt, and diastolic LVP differed from those values during glucose perfusion.

LVP cannula	glucose(5.5 mM) nitroprusside(10 μ M)		pyruvate (10 mM) nitroprusside (10 μ M)	
	open $\rightarrow$ closed	closed $\rightarrow$ open	open $\rightarrow$ closed	closed $\rightarrow$ open
frequency change Hz	5 $\rightarrow$ 7	7 $\rightarrow$ 5	5 $\rightarrow$ 7	7 $\rightarrow$ 5
Δ NADH fluorescence %	-31 $\pm$ 2.5	26 $\pm$ 2.4	-20 $\pm$ 2.6	17 $\pm$ 1.9
Δ MVO ₂ μ mol $\cdot$ min ⁻¹ $\cdot$ g ⁻¹	3.5 $\pm$ 0.5	-3.7 $\pm$ 0.5	3.6 $\pm$ 0.7	-3.7 $\pm$ 0.7

Table 4. Changes of NADH fluorescence associated with changes in myocardial oxygen consumption. Values are steady state values expressed in means $\pm$ SE; n = 6. Δ NADH fluorescence during pyruvate perfusion differed from Δ NADH fluorescence during glucose perfusion, whereas associated Δ MVO₂ values did not differ. Δ NADH fluorescence, change of NADH fluorescence relative to the NADH fluorescence just prior to change in frequency from 5 to 7 Hz; Δ MVO₂, difference of oxygen consumption before and after change in cardiac work.

The work-induced NADH fluorescence kinetics during pyruvate perfusion, differ from those during glucose perfusion in that the changes of NADH fluorescence occur faster in pyruvate- than in glucose-perfused hearts (Fig. 7). During perfusion with glucose the T_{1/2} (defined as time passed between onset of work change and moment at which 50% of NADH fluorescence change has been reached) was 68 $\pm$ 6 s for the decrease and 45 $\pm$ 14 s for the increase in fluorescence intensity. T_{1/2} for decrease and increase of NADH fluorescence intensity during perfusion with pyruvate was < 3 s.

DISCUSSION

This study was performed to investigate whether mitochondrial NADH forms a regulatory factor of oxidative phosphorylation, tuning the rate of respiration to a changing ATP demand during alterations in cardiac work. Following the pioneering work of Chance et al. (5, 7) mitochondrial NADH was evaluated using its fluorescent property. Several studies monitoring NADH fluorescence in isolated working heart preparations under conditions of limited substrate supply (glucose alone) showed a decrease in baseline NADH fluorescence intensity in time, thereby complicating interpretation of work-induced NADH fluorescence changes (13, 15, 18, and this study). This decrease could possibly be ascribed to the gradual decline in the availability of intracellular substrate as the heart becomes dependent solely on the perfusate glucose because of the depletion of endogenous substrates (such as fatty acids and glycogen) (13, 18, 19).

Additionally, prolonged illumination of tissue with UV light, necessary to induce NADH fluorescence, may eventually result in decrease in NADH fluorescence intensity due to photobleaching (13, 18). In this study, during continuous UV illumination, we observed a decrease of NADH fluorescence in hearts perfused with pyruvate, which was not different from the decrease observed during perfusion with glucose (Fig. 3, Table 1). Because the

presence of pyruvate prevents use of endogenous substrates (19), we conclude that the observed decrease in NADH fluorescence intensity is not caused by depletion of endogenous substrates. When hearts were exposed to UV illumination discontinuously, the decrease in NADH fluorescence intensity greatly reduced in both glucose- and pyruvate-perfused hearts (trend again did not differ between glucose- and pyruvate-perfused hearts), indicating that photobleaching by UV illumination is probably the principal factor responsible for the time-dependent decline of NADH fluorescence (23).

Alterations in the redox equilibria of respiratory cytochromes and changes in oxygenation of myoglobin alter the absorption spectrum of the myocardium. To prevent this oximetric effect (10) from obscuring the NADH fluorescence measurements, one could measure NADH fluorescence at emission wavelengths isosbestic for tissue oxygenation changes (13). In this study, however, NADH fluorescence was measured in the 460- to 490-nm range, and the tissue absorbance at low and high cardiac work was monitored using diffuse reflectance spectra. When the perfusate was supplemented with 10 μM nitroprusside, we did not observe changes in the reflectance spectra of isolated heart during changes in cardiac work (Fig. 4). This invariability of the reflectance spectra of vasodilated myocardium indicates that the NADH fluorescence was not affected by changes in tissue absorbance during alterations in cardiac work.

A further complication of the NADH fluorescence measurement is movement of the organ surface with respect to the illumination detection system (13, 18). In a recent study Heineman and Balaban (13) accounted for movement artifacts by applying an internal fluorescence standard. In the present study, however, simultaneous registration of NADH fluorescence images and pacemaker signals allowed acquisition of the NADH fluorescence intensities of a well-defined area on the epicardial surface in exactly the same geometrical configuration, thereby eliminating differences in fluorescence intensity due to motion as described before (15).

The importance of image formation is also stressed by the fact that isolated rat hearts all developed ischemic tissue areas when subjected to increase in pace frequency (from 5 to 10 Hz) (15) or to increase in cardiac work (this study) when no vasodilator was added to the perfusate. During ischemia, inadequate O_2 supply will reduce the transfer of reducing equivalents from the respiratory chain and will result in an increase of the mitochondrial NADH concentration. This ischemia is distributed heterogeneously over the myocardium (15) and will be a serious distorting effect in NADH fluorescence measurements which do not take this spatial heterogeneity into account. To be able to assess the effect of changes in cardiac work on mitochondrial NADH without inducing ischemic spots, nitroprusside was added to the perfusate in protocol 3. The finding that, apart from the expected increase in coronary flow, nitroprusside addition did not change the hemodynamic parameters of hearts during increase of cardiac work (Tables 2 and 3) illustrates the superior sensitivity of epicardial NADH-fluorimetry for identification of tissue hypoxia. In vasodilated rat hearts perfused with Tyrode solution containing either glucose or pyruvate, increase of work was associated with decrease in NADH fluorescence intensity (Fig. 6).

These methodological considerations may explain the conflicting data of previous studies on work-related changes in the NADH/ NAD^+ redox state. The reported increase in NADH fluorescence intensity in response to doubling of heart rate in a paced Langendorff rat heart without using a vasodilator could be explained by development of ischemia (18).

Inadequate myocardial oxygenation upon progressive increase in heart rate was recognized in the isolated rabbit heart (13). In this study no significant work-related changes in NADH fluorescence intensity were observed. However, to prevent the development of ischemia in this working heart preparation the maximal allowable increase in cardiac work was probably smaller than in our vasodilated preparation. Because no precautions were taken to reduce photobleaching in this particular study (13), relatively small work-related changes in NADH fluorescence intensity could be obscured.

In accordance with our study (Fig. 7), twitches of isolated papillary muscle of the rabbit resulted in reduction of the mitochondrial NADH fluorescence intensity, and the work-induced fluorescence transients were also strongly dependent on the available substrate (8). The decreased NADH fluorescence intensity of papillary muscle induced by mechanical activity has been attributed to stimulation of mitochondrial respiration by ADP (8). In this classical view on regulation of oxidative phosphorylation the slowed kinetics of the NADH fluorescence transients in the absence of pyruvate can be explained by a combined effect of limited availability of endogenous substrate and a reduced ADP sensitivity of a more oxidized respiratory chain. Alternatively, the kinetics of the work-related NADH fluorescence transients in the presence of pyruvate may be accelerated through activation of cytosolic pyruvate dehydrogenase (12). The substrate dependence of myocardial energy metabolism is also reflected by differences in O_2 consumption, developed tension, and coronary flow (Tables 2 and 3) (12, 17). The increased energy expenditure and coronary flow observed in the presence of pyruvate have been related to an increased cytoplasmic phosphate potential and reduced P_i potential (11, 12). More investigations are required to elucidate these issues.

Pacing of well-oxygenated isolated ventricular myocytes induced a decrease of mitochondrial NADH fluorescence intensity, whereas pacing of inadequately oxygenated isolated ventricular myocytes induced an increase of mitochondrial NADH fluorescence intensity (24). These results agree very well with the findings in this study on intact rat hearts.

In summary, the present findings suggest that the increase or decrease of respiratory activity induced by the increase or decrease of cardiac work in the Langendorff rat heart are associated with the decrease or increase of NADH fluorescence. Under the conditions of this study, an increase of myocardial oxidative phosphorylation is not related to an increase of mitochondrial NADH, making it unlikely that an increase of mitochondrial NADH is a primary stimulus for oxidative phosphorylation. Instead, it seems that changes in mitochondrial NADH concentrations were probably (partially) secondary to changes in respiratory chain activity.

REFERENCES

- 1 Ashruf J F, Ince C, Bruining HA. (1993). Increase of heart beat rate causes decrease of mitochondrial $[NADH]/[NAD^+]$ (Abstract). *Pfluegers Arch*; 422: R2
- 2 Balaban RS (1990). Regulation of oxidative phosphorylation in the mammalian cell. *Am J Physiol*; 258: C377-C389
- 3 Balaban RS, Heineman FW (1989). Interaction of oxidative phosphorylation and work in the heart, in vivo. *News Physiol Sci*; 4: 215-218
- 4 Balaban RS, Kantor HL, Katz LA et al. (1986). Relation between work and phosphate metabolites in the in vivo paced mammalian heart. *Science*; 232: 1121-1123
- 5 Chance B, Cohen P, Jöbsis F et al. (1962). Localized fluorimetry of oxidation-reduction states of intracellular pyridine nucleotide in brain and kidney cortex of the anesthetized rat. *Science*; 136: 325
- 6 Chance B, Williams CM (1956). The respiratory chain and oxidative phosphorylation. *Adv Enzymol*; 17: 65-134
- 7 Chance B, Williamson JR, Jamieson D et al. (1965). Properties and kinetics of reduced pyridine nucleotide fluorescence of the isolated and in vivo rat heart. *Biochem Z*; 341: 357-377
- 8 Chapman JB (1972). Fluorimetric studies of oxidative metabolism in isolated papillary muscle of the rabbit. *J Gen Physiol*; 59: 135-154
- 9 Chemnitz JM, Burger W, Bing RJ. (1985). Crystalloid and perfluorochemical perfusates in an isolated working rabbit heart preparation. *Am J Physiol*; 249: H285-H292
- 10 Coremans JMCC, Ince C, Bruining HA. (1992). NADH-fluorimetry and diffuse reflectance spectroscopy on rat heart. In: *Medical Optical Tomography: Functional Imaging and Monitoring*. Washington DC: SPIE; vol. IS11, 589-617
- 11 Daut J, Elzinga G.(1989). Substrate dependence of energy metabolism in isolated guinea-pig cardiac muscle: a microcalorimetric study. *J Physiol Lond*; 413: 379-397
- 12 Hak JB, van Beek JHGM, Eijgelshoven MHJ et al. (1993). Mitochondrial dehydrogenase activity affects adaptation of cardiac oxygen consumption to demand. *Am J Physiol*; 264: H448-H453
- 13 Heineman, F. W., and R. S. Balaban (1993). Effects of afterload and heart rate on NAD(P)H redox state in the isolated rabbit heart. *Am J Physiol*; 264: H433-H440
- 14 Hulsmann W C, Ashruf JF, Bruining HA et al. (1993). Imminent ischemia in normal and hypertrophic Langendorff rat hearts; effects of fatty acids and superoxide dismutase monitored by NADH surface fluorescence. *Biochim Biophys Acta*; 1181: 273-278
- 15 Ince C, Ashruf JF, Avontuur JAM et al. (1993). Heterogeneity of the hypoxic state in rat heart is determined at capillary level. *Am J Physiol*; 264: H294-H301
- 16 Ince C, Coremans JMCC, Bruining HA (1992). In vivo NADH fluorescence. *Adv Exp Med Biol*; 317: 277-296
- 17 Kang Y, Mallet HRT, Bunger R. (1992). Coronary autoregulation and purine release in normoxic heart at various cytoplasmic phosphorylation potentials: disparate effects of adenosine. *Pfluegers Arch*; 421: 188-199
- 18 Katz LA, Koretsky AP, Balaban RS (1987). Respiratory control in the glucose perfused heart. A ^{31}P NMR and NADH fluorescence study. *FEBS Lett*; 221: 270-276

- 19 Kobayashi K, Neely JR (1979). Control of maximum rates of glycolysis in rat cardiac muscle. *Circ Res*; 44: 166-175
- 20 Koretsky AP, Balaban RS (1987). Changes in pyridine nucleotide levels alter oxygen consumption and extramitochondrial phosphates in isolated mitochondria: a ³¹P NMR and fluorescence study. *Biochim Biophys Acta*; 893: 398-408
- 21 Nishiki K, Erecinska M, Wilson DF (1978). Energy relationships between cytosolic metabolism and mitochondrial respiration in rat heart. *Am J Physiol*; 234: C73-C81
- 22 Ploem JS (1970). In: *Standardization in Immunofluorescence*, edited by E. J. Holborow. Oxford, UK: Blackwell; chapt. 20, Standards for fluorescence microscopy: 137-153
- 23 Udenfriend S (1962). *Fluorescence Assay in Biology and Medicine*. New York: Academic; vol. 1: 104
- 24 White RL, Wittenberg BA (1993). NADH fluorescence of isolated ventricular myocytes: effects of pacing, myoglobin and oxygen supply. *Biophys J*; 65: 196-204

6

IN VIVO NADH- AND Pd-PORPHYRIN-VIDEOFLUORI- /PHOSPHORIMETRY

Ince C¹, Ashruf JF¹, Sanderse EA¹, Pierik EGJM¹, Coremans JMCC¹, Bruining HA¹

¹Department of Surgery, Erasmus University of Rotterdam, Rotterdam, the Netherlands

Published in: Adv Exp Med Biol; 317: 267-275, 1992

INTRODUCTION

Optical spectroscopy can be used to visualize the extent of tissue hypoxia and of blood oxygenation, using either endogenously present chromophores or artificial dyes added to the circulation. Reduced nicotinamide adenine dinucleotide (NADH) is a naturally occurring intracellular fluorophore which plays a key role in the transfer of reducing equivalents from the tricarboxylic acid cycle to the respiratory chain in the mitochondria. Inhibition of the respiratory chain due to inadequate oxygen supply is reflected by increased NADH levels. Upon illumination of tissue with ultraviolet light NADH (and not NAD^+) fluoresces in the blue. In cardiac tissue the blue fluorescence emitted from the organ surface mainly originates from the mitochondrial NADH pool. In this way NADH fluorescence provides direct information about the mitochondrial redox state. Introduced by Chance [Chance et al. 1962, Chance 1976], NADH fluorescence has been applied to a wide range of organs both in vivo and in vitro to investigate the oxygen dependence of mitochondrial oxidative phosphorylation in situ.

Wilson and co-workers developed the phosphorescent properties of Pd-porphyrin compounds as indicators of oxygen concentrations. Not only is the phosphorescence intensity of these Pd-porphyrin compounds oxygen dependent, also the time constant of the quenching of phosphorescence is directly related to the oxygen concentration as described by the Stern-Volmer relation. Hence after excitation of a Pd-porphyrin compound, measurement of the lifetimes of phosphorescence extinction enables quantitative determination of the O_2 concentration [Vanderkooi et al. 1987, Wilson et al. 1991]. When complexes of Pd-porphyrin and bovine serum albumin are injected into the blood, the oxygen probe is confined to the circulation and in vivo measurements of the microcirculatory O_2 concentration can be made [Wilson et al. 1991]. Images of the distribution of O_2 in the vasculature of intact organs can be obtained through continuous registration of the phosphorescence intensity of the Pd-porphyrin dye [Rumsey et al. 1988].

Taking the above into consideration it would be expected that combination of NADH fluorescence (oxygen in tissue) and Pd-porphyrin phosphorescence measurements (oxygen in microcirculation) in vivo would provide additional information about the factors effecting mitochondrial respiration in a continuous and non-invasive way. In this study we present a videofluorimeter based on a second generation charge-coupled-device (CCD) video camera which is suitable for in vivo observation of NADH fluorescence and Pd-porphyrin phosphorescence. Consequently, this instrument allows investigation of both intracellular O_2 demand (as reflected by the mitochondrial redox state) and O_2 distribution within the vascular system of the gut and heart of mechanically ventilated rats during anoxic and ischemic hypoxia created by N_2 ventilation and vascular ligations, respectively.

MATERIALS AND METHODS

The videofluori-/phosphorimeter (Fig. 1A) consists of an image-intensified CCD video camera with storage and processing features, coupled to an optical unit which contains the illumination source and filter combinations for selection of suitable wavelengths of excitation and emission light. The apparatus is attached to an operation

Figure 1. A. Block diagram of the optical system of the videofluori-/phosphorimeter. The illumination unit consists of a 100-W mercury arc lamp. The filter unit consists of two sets of filters to select excitation and emission light for NADH fluorescence measurements (360-nm and 460-nm band pass (BP) filters, respectively) and for Pd-porphyrin phosphorescence measurements (545-nm band pass and 600-nm long pass filters, respectively). Excitation and emission light are separated by a dichroic mirror (DM). A second generation CCD camera fitted with a cathode tube with optimal sensitivity in the ultraviolet/blue spectral region is able to detect *in vivo* organ surface NADH fluorescence and Pd-porphyrin phosphorescence. Images are displayed on a monitor and stored on a S-VHS video recorder. Hard copies of the fluorescence and phosphorescence images can be obtained with a video printer and a computer can be used for image processing and quantification. B. Spectral characteristics of the image intensifier used in the second generation CCD video camera.

microscope stand, allowing flexible positioning of the instrument above an experimental animal. Images are taken with a second generation CCD video camera (MXRi 5051 camera, HCS Vision Technology, Eindhoven, the Netherlands) in which the existing photocathode is replaced with a cathode tube with optimal sensitivity in the ultraviolet/blue spectral region (S20 Philips; Fig. 1B). The camera is fitted with a C-mount to which a 105-mm Micro-Nikkor macrolens is attached. The use of this highly sensitive second generation CCD video camera was a prerequisite for detection of the low intensity fluorescence and phosphorescence images *in vivo*. Images were continuously recorded on a S-VHS video recorder (Panasonic Type AG 7330) and printed on a Mitsubishi video copy processor. Images could also be computer analyzed off-line for quantification of the intensity of fluorescence.

The camera is connected to a B2-RFA optical unit of an Olympus BH-2 fluorescence microscope composed of an illumination unit and a filter unit. The illumination unit consists of a 100-W mercury arc lamp which provides the excitation light for both NADH fluorescence (360 nm) and Pd-porphyrin phosphorescence (545 nm). The filter unit is composed of two switchable filter blocks; one for NADH fluorescence measurements and one for Pd-porphyrin phosphorescence measurements. Each filter block contains a band pass filter to select the desired excitation light, a dichroic mirror to separate excitation and emission light and a filter to select the emission light from the organ surface (i.e. a 460-nm band pass filter for NADH fluorescence and a 600-nm long pass filter for Pd-porphyrin phosphorescence).

Measurements were made of heart and gut surfaces of thoraco-laparotomized Wistar rats. The animals were mechanically ventilated with 98% O₂ and 2% of the anesthetic ethrane. In order to create anoxic hypoxia, N₂ was substituted for O₂ in the respiratory gas. Ischemic hypoxia in the heart was created by ligation of the coronary artery as described by Barlow et al. (1976). NADH fluorescence measurements were performed as described above. The lumiphore Pd-meso-tetra(4-carboxyphenyl)porphyrin (Porphyrin Products, Logan, Utah, USA) was chosen as artificial oxygen probe. This phosphorescent dye was coupled to bovine serum albumin dissolved in physiological saline at pH 7.4 [Wilson et al. 1991], and injected intravenously to a final concentration of about 30 μ M.

RESULTS

The applicability of the developed videofluorimeter for detection of NADH fluorescence and Pd-porphyrin phosphorescence images of organ surfaces *in vivo* was examined in heart and gut of mechanically ventilated rats (Figs. 2, 3). NADH fluorescence images of the organ surfaces during O₂ ventilation were characterized by dull fluorescence of the tissue, interrupted by the dark contours of the strongly absorbing blood vessels (Figs. 2A, 2C). Due to the limited depth of penetration of the excitation light (estimated to range from 300-600 μ m) crisp images of the coronary vasculature could be observed enabling vessel diameter changes to be measured by gray level profile analysis. Substitution of N₂ for O₂ in the respiratory gas resulted in an almost two-fold enhancement of the NADH fluorescence intensity of the myocardial tissue and in enlargement of the coronary blood vessels associated with hypoxia induced vasodilation (Figs. 2A, B). Measurements of the gut in the same animal also revealed enhanced NADH fluorescence during N₂-ventilation (Figs. 2C, 2D) but here constriction of the vasculature is observed due to opposite autoregulation

mechanisms of gut and heart vasculature in response to anoxic hypoxia. Hypovolemic hypoxia induced by bleeding resulted in a slight further increase in NADH fluorescence, demonstrating attenuation of the fluorescence signal due to the absorption of excitation and emission light by blood (not shown).

Figure 2: NADH fluorescence recordings of the heart and gut in the same mechanically ventilated rat before and during anoxic hypoxia created by N₂-ventilation. A. Normoxic heart tissue shows a dull fluorescence indicative of low NADH concentrations in the tissue. Due to the absorption of light by blood, blood vessels appear as dark lines. B. Substitution of N₂ for O₂ in the respiratory gas results in higher tissue NADH fluorescence, accompanied by hypoxia induced coronary vasodilation. C. NADH fluorescence image of the normoxic gut also shows a dull fluorescence. D. During anoxic hypoxia enhanced NADH fluorescence can also be observed in the gut tissues. In contrast to the heart, however, the gut vasculature shows constriction during N₂ ventilation. Stray rat hairs in the abdominal cavity cause the bright fluorescent spots in panels C and D.

To monitor O₂ concentration changes in the coronary microcirculation, Pd-porphyrin bound to bovine serum albumin was injected intravenously into the mechanically ventilated rat. During O₂ ventilation a dull phosphorescence is emitted from the well-oxygenated heart surface (Fig. 3A). Anoxic hypoxia created by N₂ ventilation, however, resulted in a marked increase in phosphorescence intensity, indicative of a decrease in the microcirculatory oxygen concentration (Fig. 3B). These effects did not occur when the dye was excluded from the circulation.

Figure 3. Alteration in Pd-porphyrin phosphorescence associated with microcirculation hypoxia in the mechanically ventilated rat heart. A. During O₂ ventilation low phosphorescence is observed. B. During N₂ ventilation, however, enhanced phosphorescence is observed due to decrease of the microcirculatory O₂ concentration. This effect is not seen in the absence of dye in the circulation.

To investigate whether both the fluorescence and phosphorescence technique could also be applied for detection of ischemic hypoxia in vivo, videofluorimetric measurements were made of the rat heart surface during ligations of coronary arteries. Under normoxic conditions the NADH fluorescence (Fig. 4A) as well the Pd-porphyrin phosphorescence (Fig. 4C) were low in intensity, reflecting high oxygenation in both tissue and microcirculation. Ligation of the coronary artery, however, resulted in an ischemic area in the cardiac tissue with high NADH fluorescence intensity, clearly and abruptly demarcated from the surrounding normoxic tissue (Fig. 4B). Enhanced Pd-porphyrin phosphorescence distal to the ligation also revealed impaired oxygen supply to a specific zone of the myocardium (Fig. 4D). As can be seen by comparison of Fig. 4B with Fig. 4D hypoxic areas indicated by the fluorescent and phosphorescent probes correspond well with each other. In experiments where no Pd-porphyrin dye was injected prior to ligation of coronary arteries, alteration of NADH fluorescence could still be observed following ligation, but observations at wavelengths associated with Pd-porphyrin phosphorescence did not show any difference before and after ligation.

CONCLUSIONS

In this communication we report on a new videofluorimeter based on a second generation CCD camera for simultaneous measurement of NADH fluorescence and Pd-porphyrin phosphorescence in vivo. Use of the described second generation CCD camera provided a

Figure 4. The effect of ischemic hypoxia on NADH fluorescence and Pd-porphyrin phosphorescence in rat heart as a result of coronary ligation in the left ventricle. Prior to the experiment albumin-coupled Pd-porphyrin compound was injected into the blood of the rat (final concentration 30 μ M). A. NADH fluorescence image of the rat heart surface prior to ligation. B. After coronary ligation a sharp region of increased NADH fluorescence is seen, indicative of tissue hypoxia. C. Pd-porphyrin phosphorescence image of the same heart as in (A), prior to ligation. D. After coronary ligation enhanced phosphorescence is observed in the area distal to the ligation, demonstrating decreased O_2 concentrations in the microcirculation. The hypoxic areas as indicated by increased NADH fluorescence (B) and Pd-porphyrin phosphorescence (D) correspond well with each other.

number of technical advantages for photometric imaging of fluorescence and phosphorescence distributions of organ surfaces. CCD cameras are well suited for quantitative imaging as they provide minimal geometric distortion and the light intensity of each pixel is well defined. Moreover, choice of an ultraviolet/blue sensitive phototube in the image-intensified CCD camera provides the extra sensitivity required for detection of the low light intensities associated with fluorimetry and phosphorimetry in vivo. Mounting the whole instrument on an operation microscope stand provides the flexibility to select a convenient optical configuration; e.g. horizontal positioning of the camera for spectrophotometric measurements of saline-perfused organs in vitro [Ashruf et al. 1990] or vertical set-up of the camera for study of organ surfaces in vivo [Ince et al. 1991].

To our knowledge we present the first combined NADH fluorescence and Pd-porphyrin phosphorescence measurements in the rat heart in vivo. Application of these optical techniques enables identification of both anoxic as well as ischemic hypoxia in intact organs. It is expected that the ability to image the distribution of the mitochondrial redox state of tissue as well as the distribution of oxygen concentration in the microcirculation (as described in this study), will provide new insights into the relation between local oxygen supply and demand in cells, tissues and organs in vivo.

REFERENCES

Ashruf JF, Avontuur JAM, van Bavel E et al. (1990). Local ischemia created by microspheres in the rat heart induces heterogeneous epicardial NADH fluorescence. *Pfluegers Arch*; 416: S2

Barlow CH, Chance B (1976). Ischemic areas in perfused rat hearts: Measurement by NADH fluorescence photography. *Science*; 193: 909-910

Chance B, Cohen P, Jöbsis F et al. (1962). Intracellular oxidation-reduction states in vivo. *Science*; 137: 499-508

Chance B (1976). Pyridine nucleotide as an indicator of the oxygen requirements for energy-linked functions of mitochondria. *Circ Res*; 38 (Suppl I): 31-38

Ince C, Bruining HA (1991). Optical Spectroscopy for the measurement of tissue hypoxia. In: *Update in Intensive Care and Emergency Medicine: Update 1991* (Vincent JL, ed.); 161-171, Springer-Verlag, New York

Ince C, Coremans JMCC, Bruining HA (1992). In vivo NADH fluorescence. *Adv Exp Med Biol*; 317: 277-296

Rumsey WL, Vanderkooi JM, Wilson DF (1988). Imaging of phosphorescence: A novel method for measuring oxygen distribution in perfused tissue. *Science*; 241: 1649-1651

Vanderkooi JM, Maniara G, Green TJ et al. (1987). An optical method for measurement of dioxygen concentration based upon quenching of phosphorescence. *J Biol Chem*; 262: 5476-5482

Wilson DF (1992). Oxygen dependent quenching of phosphorescence: A perspective. *Adv Exp Med Biol*; 317: 195-201

Wilson DF, Pastuszko A, DiGiacomo JE et al. (1991). Effect of hyperventilation on oxygenation of the brain cortex of newborn piglets. *J Appl Physiol*; 70(6): 2691-2696

7

OPTICAL SPECTROSCOPIC IMAGING FOR NON-INVASIVE EVALUATION OF TISSUE OXYGENATION

Bruining HA¹, Pierik EGJM¹, Ince C¹, Ashruf JF¹

¹Department of Surgery, Erasmus University of Rotterdam, Rotterdam, the Netherlands

Published in: Chirurgie; 118(5): 317-23, 1992

SUMMARY

Altered rates of oxygen delivery and uptake between and possibly within different organs occur during critical illness. The mechanisms governing this heterogeneity are as yet not fully understood and techniques directed at being able to map the course of oxygen to the mitochondria to produce ATP, the main molecule needed to drive energy requiring processes in the cell, would give valuable information about the mechanisms underlying organ dysfunction during disease.

Oxidative phosphorylation occurring in the mitochondria is the main site for the production of ATP in mammalian cells. Metabolic substrates ADP, P_i , and O_2 are the ingredients needed to produce ATP. Due to the central role of oxidative phosphorylation in the metabolism of the cell, much attention has been directed at developing non-invasive techniques to measure intermediates of the oxidative phosphorylation in tissue as an indication of the metabolic state of tissue. One such method enables mapping the distribution of tissue hypoxia by use of a fluorescence technique based on the measurements reduced nicotinic amide dinucleotide (NADH). NADH is situated at the high-energy side of the respiratory chain and during tissue hypoxia accumulates in concentration because less NADH is oxidized to NAD^+ . Excitation of NADH by 366-nm light produces, unlike NAD^+ , fluorescence at 460-nm light. Previously, however, producing images of NADH fluorescence distribution in tissue has been limited to saline perfused in vitro models. We recently undertook to develop an NADH-videofluorimeter sensitive enough to measure NADH fluorescence in vivo (Ince C, Bruining HA (1991) Optical Spectroscopy for the measurement of tissue hypoxia. In: Update in Intensive Care and Emergency Medicine: Update 1991. Ed. JL Vincent, Publ. Springer-Verlag pp. 161-171). This device enables video images to be made of the spread of tissue hypoxia at video speeds. Recently a new phosphorescent dye has been introduced with which it is possible to quantitatively determine oxygen concentrations in the microcirculation in vivo. This technique makes use of the optical properties of Pd-porphyrin compounds. Using the mentioned videofluorimeter it is also possible to image the distribution of oxygen concentrations in the microcirculation in vivo. With this newly developed videofluorimeter therefore it is now possible to detect hypoxia in tissue (NADH fluorescence) as well as in the microcirculation (Pd-porphyrin phosphorescence) in vivo. It is expected that videofluorimetry will potentially prove to be an important clinical technique.

RÉSUMÉ

Spectroscopie optique pour évaluation non invasive de l'oxygénation des tissus

Par H.A. Bruining, R.G.J.M. Pierik, C. Ince et J.F. Ashruf

Des altérations de la distribution et de la consommation d'oxygène, entre les différents organes et possiblement à l'intérieur de ceux-ci surviennent en cas d'affection grave. Les mécanismes de ces phénomènes ne sont pas encore entièrement connus et des techniques permettant de suivre le cours de l'oxygène jusqu'aux mitochondries et jusqu'à la formation d'ATP, la principale molécule énergétique de la cellule, donneraient des informations utiles sur la physiopathologie des mauvais fonctionnements organiques.

La phosphorylation oxydée survenant dans les mitochondries est le site principal de production d'ATP dans les cellules des mammifères. Des substrats métaboliques, ADP, Pi et O_2 sont nécessaires à cette production. Étant donné le rôle important de la phosphorylation oxydée dans le métabolisme cellulaire, on a porté beaucoup d'attention à la mise au point de techniques non invasives pour mesurer les intermédiaires de la phosphorylation oxydée comme indicateurs de l'état métabolique des tissus. Une de ces méthodes permet de dessiner la distribution de l'hypoxie tissulaire par fluorescence en se basant sur les dosages de la nicotine amide dinucléotide réduite (NADH). Celle-ci est située du côté de haute énergie de la chaîne respiratoire et s'accumule durant l'hypoxie tissulaire parce que moins de NADH est oxydée en NAD^+ . L'excitation de NADH par une lumière de 366 nm produit, contrairement à NAD^+ , une fluorescence à une lumière de 460 nm. Jusqu'ici, cependant, la production d'images de la fluorescence de NADH dans les tissus a été limitée à des modèles perfusés in vitro. Nous avons récemment entrepris de réaliser un vidéofluorimètre assez sensible pour enregistrer la fluorescence de NADH in vivo. (Ince C, Bruining HA (1991) Spectroscopie optique pour la mesure de l'hypoxie tissulaire. In: Update in Intensive Care and Emergency Medicine: Update 1991. Ed. JL Vincent, Publ. Springer-Verlag, pp. 161-171). Cet appareil permet une imagerie vidéo de l'extension de l'hypoxie tissulaire à la vitesse de l'enregistrement vidéo. Plus récemment, un nouveau colorant phosphorescent a rendu possible la détermination quantitative de la concentration d'oxygène dans la microcirculation in vivo. Cette technique utilise les propriétés optiques des composants de la Pd-porphyrine. Le vidéofluorimètre rend possible l'imagerie de la distribution des concentrations d'oxygène dans la microcirculation in vivo. Avec ce nouveau appareil, il est donc possible de déceler l'hypoxie tissulaire (par fluorescence de NADH) et dans la microcirculation (par phosphorescence de Pd-porphyrine) in vivo. Il est permis de penser que la vidéofluorimétrie sera un important outil clinique.

INTRODUCTION

The maintenance of optimal physiological function is mainly dependent on the ability of the circulation to deliver adequate amounts of oxygen to the mitochondria of cells where the respiratory chain produces high-energy phosphate compounds needed for cellular activity. At various steps in this course disturbances can arise with potentially harmful consequences. Clinical evaluation of such disturbances is essential for diagnosis and therapy of patients. Examples specifically relevant to general surgeons include the assessment of tissue damage, ischemia of vascular compromised organs and viability of tissues after restoration of the circulation. Up until now on-site judgment of the viability of tissue is mainly based upon criteria such as color, motility and pulsations. Clinical and experimental studies have shown, however, that the subjective assessment of tissue viability based on standard clinical criteria alone is inaccurate (4) and is strongly dependent on the experience of the surgeon.

Awareness of the importance of adequate tissue oxygenation in the intensive care unit has led among other things to the widespread use of pulse oximeters and the use of thermodilution pulmonary artery catheters to measure oxygen transport, oxygen consumption, and mixed venous oxygen saturation in critically ill patients. Although these measurements can supply important information concerning major changes in the oxygen status of the patient, they do not assess the adequacy of local tissue perfusion and cellular respiration at the level of the microcirculation. Doppler ultrasonography (22), intravenous injection of fluorescein (14), surface oximetry (8) and transcutaneous oxygen electrodes (7) all have been proposed as methods to evaluate tissue oxygenation. Their clinical effectiveness for evaluating local oxygen supply and uptake, however, has been limited (6). Continuing advances in the application of optical spectroscopic techniques may provide this desired non-invasive method of assessing and imaging of tissue viability.

OPTICAL SPECTROSCOPY

Optical spectroscopy studies the interaction between light and matter and can provide detailed information about the metabolic state of blood and tissue, making use of the oxygen dependent optical properties of either endogenously present chromophores or artificial dyes added to the circulation. The amount of absorption (and thus reflection) of light by molecules at specific wavelengths is dependent on the concentration of that molecule and the optical penetration path as described by the law of Beer-Lambert (10). Selection of chromophores whose concentration are oxygen dependent, thus, provides the opportunity to determine the presence and origin of local tissue hypoxia.

The oxygen dependent optical properties of hemoglobin have been used to assess the saturation of blood as far back as 1943 where an ear clip device was used to assess whether pilots were suffering from hypoxemia (15). The later development of pulse oximetry has seen wide spread clinical application and can be considered as one of the success stories of optical spectroscopy. Measurements of tissue oxygenation using optical techniques have focused on intermediates of the respiratory chain since a number of these intermediates possess oxygen dependent optical properties. Monitoring of the redox state of cytochrome aa_3 , for example, can be accomplished by spectroscopic measurements in the

near infrared region of the light spectrum and are applied to assess the metabolic state of cerebral tissue. These measurements also provide important information about blood oxygenation and blood volume (13) and several devices are commercially available. Because of the limited depth of penetration of near infrared light particular use was found in monitoring cerebral function during intensive care of newborn infants where optical measurements are made across the head (23). Although an overall macroscopic measure of the oxygenation of an organ or part of organ is provided by this technique, surgical and endoscopic procedures would favor imaging techniques to locate border zones of compromised tissue. Use of fluorescence spectroscopy provides such a technique. Here, in contrast to oximetry and near infrared spectroscopy, not the overall absorption is being measured, but the fluorescence reflection of tissue components is imaged. The main intermediate of mitochondrial oxidative phosphorylation present in tissue which possesses oxygen dependent fluorescence properties is reduced nicotinamide adenine dinucleotide (NADH).

NADH is an electron carrier which plays an important role in the transfer or reducing equivalents from the tricarboxylic acid cycle to the respiratory chain in the mitochondria. During tissue hypoxia and in the presence of sufficient substrate and phosphates, NADH accumulates in concentration because less NADH is oxidized to NAD^+ . This increase in NADH level can be measured, since NADH and not NAD^+ emits blue fluorescence (broad-band emission centered around 460-nm light) when excited with ultraviolet light (around 360 nm) (5). Because erythrocytes do not harbor mitochondria, only tissues and not blood will contribute to the NADH fluorescence signal. The intrinsic fluorescence signals from NADH in the mitochondrial respiratory chain can thus be used as a sensitive indicator of changes in the oxygen supply-demand ratio (1, 3, 16). First introduced by Britton Chance (5), NADH fluorescence has been mainly applied to cellular organelles, cell suspensions and saline-perfused organs to investigate the oxygen dependence of mitochondrial oxidative phosphorylation.

Application of this technique to blood perfused tissue is complicated by the high absorption of NADH fluorescence by hemoglobin (17). To compensate for this effect a large number of methods have been introduced. [For recent review see Ince et al. (12)]. The resulting reduced fluorescence signal has further limited the application of imaging techniques due to the lack of sensitive imaging technology. With newly introduced ultra-low-level-light video cameras a method was provided to detect these low fluorescence images. We developed such a sensitive video camera equipment specifically for NADH fluorescence (10, 16).

Next to information on the existing oxygen deficit of tissues, information about the oxygen concentration in the microcirculation would be of great additional value for the evaluation of the cause of tissue hypoxia. Wilson and co-workers recently evaluated the phosphorescent properties of Palladium (Pd)-porphyrin compounds as indicator of oxygen concentration (20, 21). Because oxygen is a powerful quenching agent for phosphors, the imaging of phosphorescence provides a method to monitor oxygen distribution. When complexes of Pd-porphyrin and albumin are injected intravenously, this oxygen probe is confined to the circulation and measurements of the microcirculatory oxygen concentration can be made. Besides emitting oxygen dependent phosphorescence, the decay in phosphorescence after a pulse of light is directly proportional to the oxygen concentration,

based on the Stem-Volmer relation (18, 20). A main advantage of this method over others is that direct calculation of the amount of oxygen present can non-invasively be made in vivo by measurement of the decay of phosphorescence. This study describes the applicability of a videofluorimeter suitable for in vivo observation of both the tissue redox state by measuring the NADH fluorescence as well as the circulatory oxygen concentration by measuring the Pd-porphyrin phosphorescence of rat hearts in vivo.

MATERIAL AND METHODS

The videofluori-/phosphorimeter (Fig. 1) consists of an illumination unit and a second generation image-intensified charge-coupled-device (CCD) video camera adapted for fluorimetry with storage and processing features, coupled to an optical unit. The illumination source consists of a 100-W mercury arc lamp with suitable filters for NADH fluorescence (366-nm excitation and 460-nm band pass emission) and for Pd-porphyrin phosphorescence (540-nm excitation and 630-nm long pass emission) (11). Fluorescence emission light and reflected excitation light are separated by a dichroic mirror.

Fig. 1. Block diagram of the optical system of the videofluori-/phosphorimeter. The filter unit consists of two sets of filters to select excitation and emission light for NADH fluorescence measurements and for Pd-porphyrin phosphorescence measurements. A second generation charge-coupled-device (CCD) camera fitted with a cathode tube with optimal sensibility in the ultraviolet/blue spectral region is able to detect in vivo organ surface NADH fluorescence and Pd-porphyrin phosphorescence; images are displayed on a monitor and stored on a S-VHS video recorder. DM = dichroic mirror, BP = band pass.

To monitor oxygen concentration changes in the circulation the lumiphore Pd-meso-tetra(4-carboxy-phenyl)porphyrin (Porphyrin Products, Logan, Utah, USA) coupled to bovine serum albumin (20) is injected intravenously to a final concentration of 30 μ M.

The NADH fluorescence and Pd-porphyrin phosphorescence can alternately be monitored by switching the filters. Images are recorded continuously on a video cassette using a video camera in which the existing photocathode is replaced by a cathode tube with optimal sensitivity in the ultraviolet/blue spectral region (MXRi 5051 camera, HCS Vision Technology, Eindhoven, the Netherlands; S20 Philips cathode tube) and video recorder (Panasonic Type AG 7330). Images are analyzed off-line and quantified by computer-aided processing and grey level analysis.

Male Wistar rats (300 g body weight) were anesthetized and artificially ventilated via intubation of the trachea with 97% oxygen and 3% ethrane. After the chest was opened via a median sternotomy anoxic hypoxia was created by substitution of nitrogen for oxygen in the respiratory gas and ischemic hypoxia imposed by selective ligation of the left coronary artery (19).

RESULTS AND DISCUSSION

NADH fluorescence images of the heart surface during oxygen ventilation were characterized by low fluorescence of the heart tissue (Fig. 2A). Blood vessels could be observed clearly due to the absorbing properties of blood and short wavelength of the excitation light. Substitution of nitrogen for oxygen in the respiratory gas resulted in an almost two-fold enhancement of the NADH fluorescence intensity of the myocardial tissue and in an increase of the coronary blood vessel diameter associated with hypoxia-induced vasodilatation (Fig. 2B). To monitor oxygen concentration changes in the coronary microcirculation, Pd-porphyrin bound to albumin was injected intravenously. During oxygen ventilation a low phosphorescence was emitted from the well-oxygenated heart surface (Fig. 3B). Anoxic hypoxia created by nitrogen ventilation, however, resulted in an increase in phosphorescence intensity, indicating a decrease in the microcirculatory oxygen concentration (Fig. 3B). These effects did not occur when the dye was not added to the circulating blood.

To investigate whether both the fluorescence and phosphorescence technique could also be applied for detection of ischemic hypoxia *in vivo*, videofluoric/phosphoric measurements were made of the rat heart surface during ligation of the left coronary artery. Ligation of this artery resulted in an ischemic area with high NADH fluorescence intensity, clearly and abruptly demarcated from the surrounding normoxic tissue (Fig. 4A). Enhanced Pd-porphyrin phosphorescence distal to the ligation also revealed impaired oxygen supply to a specific zone of the myocardium (Fig. 4B), corresponding well to the area of high fluorescence in figure 4A, depicting microcirculatory hypoxia. Although the present technique measures tissue surface properties, measurements of myocardial surface pO_2 are considered as a sensitive method not only for determination of regional epicardial tissue oxygenation, but also of transmural tissue oxygenation (9).

Fig. 2. - NADH fluorescence images of rat heart surface. A: Normoxic heart tissue shows a dull fluorescence indicative of low NADH concentration in the tissue. Due to the absorption of light by blood, blood vessels appear as dark lines. B: Substitution of nitrogen for oxygen in the respiratory gas results in higher tissue NADH fluorescence, accompanied by hypoxia induced coronary vasodilation.

Fig. 3. Palladium-porphyrin phosphorescence images of rat heart surface. A: During oxygen ventilation a low phosphorescence signal of the rat heart is observed. B: During nitrogen ventilation enhanced phosphorescence is observed due to a decrease of the microcirculatory oxygen concentration.

Fig. 4. NADH fluorescence image of the rat heart after coronary artery ligation. A: sharp region of increased NADH fluorescence is seen in the left ventricle, delineating local ischemic tissue hypoxia. B: Pd-porphyrin phosphorescence of the same heart after coronary artery ligation shows enhanced phosphorescence in the corresponding area distal to the ligation, demonstrating decreased oxygen concentrations in the microcirculation

CONCLUSION

NADH fluorescence can offer an important tool for the objective evaluation of tissue hypoxia and viability. In combination with the complementary information of Pd-porphyrin phosphorescence a differentiation between tissue and blood oxygenation can be made. It is expected that the ability to image, non-invasively, the distribution of the mitochondrial redox state of cells will provide the surgical physiologist and general surgeon a more accurate parameter for the objective evaluation of regional tissue hypoxia and its border zones under various patho(physio)logical conditions.

REFERENCES

- 1 Barlow CH, Chance B (1976). Ischemic areas in perfused rat hearts: measurement by NADH fluorescence photography. *Science*; 193: 909-910
- 2 Barlow CH, Harken AH, Chance B (1977). Evaluation of cardiac ischemia by NADH fluorescence photography. *Ann Surg* 1977; 186: 737-740
- 3 Barlow CH, Harden WR, Harken AH et al. (1979). Fluorescence mapping of mitochondrial redox changes in heart and brain. *Crit Care Med*; 7: 402-406
- 4 Bussemaker JB, Lindeman J (1972). Comparison of methods to determine viability of small intestine. *Ann Surg*; 176: 97- 101
- 5 Chance B, Cohen P, Jobsis F et al. (1962). Intracellular oxidation- reduction states in vivo. *Science*; 137: 499-708
- 6 Dyess DC, Bruner BW, Donnel CA et al. (1991). Intraoperative evaluation of intestinal ischemia: A comparison of methods. *South Med J*; 84: 966-9
- 7 Ehrly AM, Fleckenstein W, Hauss J et al. *Clinical Oxygen Pressure Measurements II, Tissue oxygen pressure and transcutaneous oxygen pressure*, Blackwell Ueberreuter Wissenschaft, Berlin
- 8 Ferrara JJ, Dyess DL, Lasecki M (1988). Surface oximetry: a new method to evaluate intestinal perfusion. *Ann Surg*; 54: 10-14
- 9 Hobbhahn J, Gonzen P, Hansen E et al. (1990). Myocardial surface oxygen tension is an indicator of transmural tissue oxygenation of the in vivo beating pig heart. In: *Clinical Oxygen Pressure Measurements II*; 315-326. Editors: Ehrly AM et al. Blackwell Ueberreuter Wissenschaft, Berlin
- 10 Ince C, Bruining HA (1991). Optical spectroscopy for the measurement of tissue hypoxia. In: *Update in Intensive Care and Emergency Medicine: Update 1991* (Vincent JL, ed.); 161-171, Springer-Verlag, New York
- 11 Ince C, Ashruf JF, Sanderse EA et al. (1992). In vivo NADH- and Pd-porphyrin videofluorimetry. *Adv Exp Med Biol*; 317: 267-275
- 12 Ince C, Coremans JMCC, Bruining HA (1992). In vivo NADH fluorescence. *Adv Exp Med Biol*; 317: 277-296
- 13 Jöbsis FF (1977). Noninvasive, infrared monitoring of cerebral and myocardial oxygen sufficiency and circulatory parameters. *Science*; 198: 1264-7
- 14 Lange K, Boyd IJ (1942). The use of fluorescein to determine the adequacy of the circulation. *Med Clin North Am*; 26: 943-952
- 15 Millikan GA (1943). Oximeter, an instalment for measuring continuously the oxygen saturation of arterial blood in man. *Rev Sei Instrum*; 13: 434
- 16 Pierik EGJM, Ince C, Avontuur JAM et al. (1991). The application of NADH fluorescence to identify noninvasively tissue hypoxia in vivo. *Eur Surg Res* 1991; 23: 12-13
- 17 Renault G, Raynal E, Sinet M et al. (1984). In situ double-beam NADH laser fluorimetry: choice of a reference wavelength. *Am J Physiol*; 246: 491-499
- 18 Rumsey WL, Vanderkooi JM, Wilson DF (1988). Imaging of phosphorescence: a novel method for measuring oxygen distribution in perfused tissue. *Science*; 241: 1649-1651
- 19 Seleye H, Bajusz E, Grasso S et al. (1960). Simple techniques for the surgical occlusion of coronary vessels in the rat. *Angiology*; 11: 398-407

- 20 Wilson DF, Pastuszko A, DiGiacomo JH et al. (1991). Effect of hyperventilation on oxygenation of the brain cortex of newborn piglets. *J Appl Physiol*; 70: 3691-3696
- 21 Wilson DE (1992). Oxygen dependent quenching of phosphorescence: a perspective. *Adv Exp Med Biol*; 317: 195-201
- 22 Wright CB, Hobson RW (1975). Prediction of intestinal viability using Doppler ultrasound techniques. *Am J Surg*; 129: 642-645
- 23 Wyatt JS, Delpy DT, Cope M et al. (1986). Quantification of cerebral oxygenation and hemodynamics in sick newborn infants by near infrared spectrophotometry. *Lancet*; 2: 1063-1066

EPICARDIAL NADH FLUORESCENCE OF THE ISOLATED PERFUSED RAT HEART REVEALS MITOCHONDRIAL DYSFUNCTION IN EARLY ENDOTOXIC SHOCK

Ashruf JF¹, Ergin B¹, Milstein DMJ¹, Zuurbier CJ¹, Eerbeek O¹, Ince C¹

¹Department of Translational Physiology, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands

To be submitted for publication

ABSTRACT

Background: Mitochondrial dysfunction has been proposed as an underlying mechanism of sepsis-induced cardiac dysfunction. However, these observations are mainly derived from isolated mitochondria studies. Here we report on an ex-vivo mitochondrial function evaluation in the intact endotoxemic rat heart, using epifluorescence analysis of mitochondrial NADH.

Methods: Early endotoxemia was induced by intravenous administration of LPS and allowing endotoxemia to develop during 2 (short) or 4 (long) hours in the intact rat. Subsequently, hearts were isolated and Langendorff-perfused and mitochondrial NADH monitored using epifluorescence analysis. Mitochondrial function was evaluated by measuring the rate of mitochondrial NADH accumulation upon ischemia reflecting the kinetics of mitochondrial oxygen consumption. Oxygen delivery and microcirculatory oxygen distribution were evaluated by measuring the rate of mitochondrial NADH reduction and the size and distribution of myocardial areas of long-lasting NADH elevation in the reperfusion phase.

Results: Intravenous administration of LPS resulted in a significant decrease of the mean arterial pressure and the development of acidosis as expected during endotoxic shock. The half-time of NADH accumulation during acute ischemia in the endotoxemia Langendorff heart increased from 20.2 ± 2.9 s to 27.1 ± 4.2 s and 35.8 ± 12.2 s, for control, short- and long LPS treatment, respectively. This increase in half-time by endotoxemia was mimicked by complex I inhibition with rotenone. These data suggest impaired mitochondrial function present in early endotoxemia. In contrast, the half-time for NADH decrease upon reperfusion was similar for all three groups: 3.3 ± 1.9 s, 4.1 ± 1.2 s and 3.9 ± 1.8 s, respectively, for control, short- and long LPS treatment. Rotenone, a complex I inhibitor did not affect the half-time of NADH decrease. In addition, heterogeneity of areas of high NADH fluorescence upon reperfusion was also similar among groups. These data suggest unaltered oxygen supply in early endotoxemia despite mitochondrial impairment.

Conclusion: These data suggest that early endotoxemia results in cardiac mitochondrial dysfunction, without apparent disturbances in oxygen supply or microcirculatory oxygenation, within the intact heart perfused with a crystalloid perfusate. The experimental model presented here employed non-invasive epifluorescence of the beating heart and can be useful in further studies concerning the effects of sepsis (and effects of therapeutic interventions in sepsis) on metabolism and microcirculation of the myocardium and thereby present an alternative to the less physiological conditions in isolated mitochondria.

INTRODUCTION

Sepsis, a syndrome characterized by a potentially fatal whole-body inflammation, is today still one of the leading causes of death in the intensive-care patient population worldwide. There is a wealth of literature concerning the pathophysiology of sepsis, but despite all this research the mainstay of management of this syndrome is still focused at optimizing macrocirculatory hemodynamics, antimicrobial treatment and anti-inflammatory interventions [1]. The ongoing search for understanding the mechanisms that ultimately induce the septic syndrome has not yet yielded treatment strategies that radically alter clinical outcome. There remains therefore a need for experimental sepsis models enabling the examination of the pathophysiology of sepsis (and potential evaluation of treatment strategies).

Cardiovascular collapse represents one of the mainstays of sepsis. Cardiac dysfunction in septic shock, characterized by decreased contractility, resistance to fluid therapy and ventricular dilatation has a complex underlying pathophysiology [2]. Two key factors are a disturbed mitochondrial metabolism [3] and microvascular dysfunction [4, 5, 6]. Mitochondrial dysfunction is characterized by attenuated oxidative phosphorylation resulting in an inability to utilize molecular oxygen for ATP synthesis [3, 7, 8, 9, 17, 18]. Even though mitochondrial dysfunction has been shown to exist in sepsis, it is still unresolved if this is a consequence of microcirculatory alterations or if this is a consequence of inherent mitochondrial alterations. The majority of studies on mitochondrial function in sepsis have been performed on isolated mitochondria. NADH-videofluorimetry, however, is a validated technique for observing the mitochondrial energy state and as such can be used to directly monitor mitochondrial function as well as microcirculatory perfusion properties in intact working isolated perfused hearts.

Earlier studies with NADH-videofluorimetry [10] enabled us to evaluate mitochondrial myocardial metabolism during both physiological (increased cardiac work) [13] and pathophysiological (hypoperfused areas in hypertrophy) [14] conditions. Hypoxic myocardium displays high NADH fluorescence intensity while normoxic myocardium emits a low level of fluorescence, since endogenous NADH emits blue fluorescence upon 360-nm excitation whereas NAD^+ , its oxidized counterpart, does not. This allows imaging of the mitochondrial energy state in intact heart [22]. In this study we evaluated mitochondrial function by measuring the rate of NADH accumulation during ischemia (OFF-KINETICS). In the reperfusion phase the ON-KINETICS (the rate of NADH oxidation after an ischemic episode) represent the patency of the myocardial microcirculation. This model therefore allows us to discriminate between mitochondrial and microcirculatory (dys)function.

Directly after reperfusion, after an ischemic episode, a relatively fast decrease of NADH fluorescence (ON-KINETICS), indicating a fast reoxygenation of the myocardium is always observed (10, 11, 12, 13). The decrease of NADH fluorescence is much faster as the increase during ischemia because the mitochondrial redox balance shifts to NAD^+ at relatively low myocardial oxygen content as is also known from studies in isolated mitochondria. However, during this swift decrease small areas of the myocardium are slower to re-oxygenate after reperfusion with oxygen-saturated medium following an ischemic period, resulting in a patchy epicardial NADH fluorescence pattern. This heterogeneous reoxygenation is caused by the heterogeneity of the patency of the microcirculation of the

myocardium and originates at the level of capillary microcirculatory units [10]. The areas last to recover are also those that first become compromised when the heart is stressed such as during tachycardia. We termed these vulnerable areas as weak microcirculatory units (WMU) [10]. In endotoxemic hearts we showed in a NADH-videofluorimetry study that these WMU's develop ischemia earlier under stress than control hearts [5].

During induction of ischemia in endotoxemic hearts, due to attenuated mitochondrial oxygen metabolism, we hypothesized to find a decreased rate of development of ischemia with a slower increase of NADH fluorescence, when compared with healthy hearts (OFF-KINETICS: the rate of increase of NADH fluorescence emerging during the development of ischemia). The main purpose of the present study therefore was to test the hypothesis that endotoxemic-induced changes cause mitochondrial dysfunction and that this occurs independent of microcirculatory dysfunction.

MATERIALS AND METHODS

The study design and experimental protocols for this study were reviewed and approved by the institutional Animal Experimentation Committee of the Academic Medical Center of the University of Amsterdam. Care and handling of the animals was in accordance with the European Institutional Animal Care and Use Committee Guidelines.

Animals. The experiments in this study were conducted on 15 male specific-pathogen free Wistar rats (Harlan, the Netherlands) with mean body weight of 330 ± 30 g. Before the start of the study, all animals were housed individually in large conventional cages in a light-controlled room (12 hours light: 12 hours dark) kept at 22 ± 1 °C with a relative humidity of $55 \pm 10\%$ and were given a minimum of 1 week to acclimatize to their new environment with access to standard food pellets and water for consumption ad libitum. Prior to surgical procedures all animals were anesthetized with an intraperitoneal injection of a mixture of 100 mg.kg⁻¹ ketamine (Nimatek®; Eurovet, Bladel, the Netherlands), 0.5 mg.kg⁻¹ medetomidine (Domitor; Pfizer, New York), and 0.05 mg.kg⁻¹ atropine-sulfate (Centrafarm, Etten-Leur, the Netherlands).

Surgical instrumentation. A tracheotomy was prepared and the animals were mechanically ventilated with a FiO₂ of 40%. Body temperature was maintained at 37 ± 0.5 °C during the entire experiment by external warming (padded heating mat and a heating lamp). The ventilator settings were adjusted to maintain end-tidal pCO₂ between 30-35 mmHg and arterial pCO₂ between 35-40 mmHg. Blood vessels were cannulated with polyethylene catheters (outer diameter = 0.9 mm; Braun, Melsungen, Germany) for maintenance anesthesia, endotoxin, drug, fluid administration, blood gas and hemodynamic monitoring. A catheter in the right carotid artery was connected to a pressure transducer to monitor mean arterial blood pressure (MAP), heart rate (HR), and to obtain blood samples for blood gas measurements. The right jugular vein was cannulated for continuous infusion of Ringer Lactate (Baxter, Utrecht, the Netherlands) at a rate of 15 ml.kg⁻¹.h⁻¹ and eventual administration of the experimental interventions endotoxin (lipopolysaccharide (LPS;

Escherichia coli 0127: B8; Sigma, Paris, France) or 0.9% NaCl. The surgical field was covered with a humidified gauze compress throughout the entire experiment to prevent drying of the exposed tissues.

Langendorff perfusion. Before excorporation of the hearts, all subjects were given 200 IU·kg⁻¹ heparin (Thromboliquine, Organon Teknika BV, Oss, the Netherlands). Hearts were removed and immediately perfused according to Langendorff. Hearts were perfused at a constant flow of 15 ml·min⁻¹ and were allowed a period of 15 min perfusion with oxygenated Tyrode to stabilize at baseline conditions before performing the experimental protocols listed below. Perfusion pressure was measured with a pressure transducer connected to the aortic cannula. Mean coronary flow rates (in ml·min⁻¹·g⁻¹ ventricle wetwt) were calculated. The perfusate was a modified Tyrode solution (128 mM NaCl, 4.7 mM KCl, 1 mM MgCl₂, 0.4 mM NaH₂PO₄, 1.2 mM Na₂SO₄, 20.2 mM NaHCO₃, and 1.3 mM CaCl₂) containing 11.0 mM glucose. Perfusate temperature was kept at 37°C and equilibrated with 95% O₂ and 5% CO₂. The pH of the perfusate was kept between 7.35 and 7.45. The main determinant of oxygen consumption in myocardium is myocardial work; therefore the left ventricle was cannulated in all Langendorff hearts to ascertain that cardiac work was nearly completely prevented [13]. To this end the left ventricle was cannulated and communicated with the atmosphere via its apex to prevent generation of left ventricular pressure [13]. 10 µM adenosine (Sigma, St. Louis, USA), an endothelium-independent vasodilator, was added to the perfusate. Chemicals were obtained from Merck (Darmstadt, Germany).

Experimental protocols. In all experiments the animals were ventilated as described above for 2 hours or 4 hours. Arterial blood samples (0.5 ml) were drawn at two time points, 30 min after start of ventilation and at the end of the ventilation period. To evaluate biochemical and hemodynamic effects of endotoxemia we performed arterial blood gas analysis (pO₂, pCO₂, pH, and serum lactate (LAC)) and measured MAP and HR. The animals were randomly allocated into three different research protocols:

Protocol 1: Control group (CON). The animals were given a bolus of 0.9% NaCl (saline) before excorporating the heart for Langendorff perfusion. After the hearts were stabilized, a period of ischemia (no flow) was induced for 2 min (OFF-KINETICS), after which reperfusion with oxygenated Tyrode followed (n = 5) (ON-KINETICS). 3 hearts in this group were subsequently perfused with rotenone 5 µM (Sigma, Paris, France) for 2 min. Perfusion was subsequently switched to oxygenated Tyrode for 2 min, when another episode of 2 min of ischemia was induced (OFF-KINETICS) followed by reperfusion with oxygenated Tyrode (ON-KINETICS) (n = 3).

Protocol 2: LPS group (LPS1). Endotoxemic shock was induced by infusion of 10 mg.kg⁻¹ LPS over a period of 30 min. The course of endotoxemia was allowed to develop for 2 hours. Thereafter, hearts were excorporated for Langendorff perfusion (n = 4; 1 subject died during the ventilation period due to severe sepsis and was excluded from the study). The same procedures as presented in Protocol 1 were performed with the exception of rotenone perfusion.

Protocol 3: extended LPS group (LPS2), similar to LPS Protocol 2, however, the course of endotoxemia was allowed to develop for 4 hours before excorporating the hearts for perfusion (severe sepsis model). The same procedures presented in Protocol 1 were performed with the exception of rotenone perfusion (n = 5).

Analysis of NADH fluorescence images

NADH-videofluorimetry set-up. The NADH-videofluorimeter technique used in this study has been described previously [10-13]. The UV light from a 100-W mercury arc lamp (Olympus, Tokyo, Japan) was selected by means of a UG-1 barrier filter, centered on 365 nm, to provide the UV light needed for NADH excitation. The NADH fluorescence signal was set by means of a band-pass filter centered on 470 ± 20 nm and was placed in front of the camera. An image-intensified video camera (LI- μ CAM, Lambert instruments BV, the Netherlands) with a Micro-Nikkor 105-mm macrolens was used to detect NADH fluorescence images of the left ventricle of the heart. To enable correction of images for changes in the sensitivity of the videofluorimeter and fluctuations in the intensity of the light source, a small piece of uranyl calibration glass was placed next to the heart within the excitation field. Images were recorded on a video recorder and were analyzed off-line with the use of an image-processing software package (Matlab version R210B). Arbitrary units of NADH fluorescence intensities relative to uranyl fluorescence intensity (to correct for excitation light fluctuations) were expressed as percentages, where 0% corresponds to oxygenated perfusion and 100% as the intensity of epicardial NADH fluorescence after 2 min of ischemia, just before reperfusion with oxygen saturated Tyrode.

OFF- and ON-KINETICS. The time needed for 50% increase of fluorescence intensity ($T_{1/2\text{-increase}}$) during occlusion was measured in CON, CON hearts loaded with rotenone, LPS1 and LPS2 hearts and was used to quantify the mitochondrial response to hypoxia. An increased $T_{1/2\text{-increase}}$ is interpreted as a decreased capacity of mitochondria to consume oxygen. The $T_{1/2\text{-increase}}$ is referred to as the OFF-KINETICS. The time needed for 50% decrease of fluorescence intensity ($T_{1/2\text{-decrease}}$) during reperfusion in CON, CON hearts loaded with rotenone, LPS1 and LPS2 hearts is referred to as ON-KINETICS. It is interpreted as a measure of the rate of oxygen transport from the microcirculation to the mitochondria.

Heterogeneity of microcirculatory perfusion during ON-KINETICS. To compare the heterogeneity of NADH fluorescence recovery during the reperfusion phase we compared the frequency distribution histograms of ischemic surface areas between CON, LPS1 and LPS2 hearts. The method of acquiring the frequency distribution histograms has been described in detail elsewhere [11]. Utilizing image analysis software (Matlab version R210B), we measured the surface area of all weak microcirculatory units in images acquired exactly halfway between maximum fluorescence intensity (at the end of occlusion) and minimum fluorescence (at the start of occlusion), which have a slower return to baseline fluorescence levels. To accomplish this, fluorescence images were segmented in areas with a peak in fluorescence intensity surrounded by a border of low intensity. The surface area of the microcirculatory weak unit was defined as the area (in pixels) with an intensity of fluorescence larger than or equal to the intensity halfway between the peak intensity and

lowest intensity in that area on the border (Fig. 1). Data were corrected for size of surface area analyzed and correlated to the surface of the uranyl square. Also we did not include microcirculatory units on the borders of the selected areas because the size of these areas are not known.

Figure 1. A: NADH fluorescence image of control heart. Black rectangle is the selection of the image used for analysis of the heterogeneity pattern and is shown in B. C is the resulting image of spots in B, where only those areas of the spots are shown that have an intensity equal or larger than the intensity halfway between the lowest intensity and the peak intensity of each area.

Statistical analysis. Group means were tested for difference using paired t-tests. The criterion for significance was taken to be $p < 0.05$ for all comparisons. Descriptive data are presented as mean $\pm$ SEM (standard error of the mean).

RESULTS

Endotoxemia induced a significant hypotension in all animals. Table 1 shows the effects of endotoxemia upon hemodynamic parameters and blood gas analysis during the ventilation setup period, before the excision of the beating heart. As can be seen, endotoxemia induced acidosis and a decrease of MAP. However, there was no significant difference in MAP between the LPS1 and LPS2 groups. The death of a subject from the ‘lighter’ endotoxemia protocol (LPS1) may indicate that probably the sepsis in the LPS1 group was not less severe than in the LPS2 group. At the start of the ventilation period there was no significant difference between any of the three groups for the parameters depicted in Table 1.

	CON	LPS1	LPS2
MAP	100 ± 4.1	46 ± 2.6	61 ± 3.9
HR	266 ± 28	265 ± 6	265 ± 15
PH	7.42 ± 0.03	7.22 ± 0.03	7.28 ± 0.03

Table 1. Effects of sepsis on hemodynamic parameters and serum pH, just before the end of the ventilator period. While HR did not differ significantly between groups, MAP and pH were significantly reduced during sepsis when compared to control.

OFF-KINETICS. Upon cessation of myocardial oxygenation by halting the perfusion with oxygenated Tyrode, an increase of the surface NADH fluorescence is observed, indicating the development of tissue hypoxia. In the endotoxemic hearts groups (LPS1 and LPS2) this increase was delayed in comparison to the control hearts (CON) (Fig. 2).

Figure 2. Example of the NADH-fluorescence trace of a control heart (open circles) and an endotoxemic heart (closed squares) (LPS2). $T_{1/2-increase}$ is the elapsed time between the black arrow and the gray arrows. The black arrow indicates the moment of occlusion of coronary flow; the gray arrows indicate the moment that 50% NADH fluorescence is reached. The fat black arrow indicates the moment of reperfusion. Every marker represents a measurement per second.

The time needed for 50% increase of fluorescence intensity ($T_{1/2\text{-increase}}$) during occlusion was measured in CON, LPS1, and LPS2 hearts. To demonstrate that an increased $T_{1/2\text{-increase}}$ is indeed caused by a decreased capacity of mitochondria to consume oxygen 3 control hearts were perfused (after the posthypoxic recovery protocol) with rotenone, a known inhibitor of mitochondrial respiration by blocking complex I of the respiratory chain. Subsequently $T_{1/2\text{-increase}}$ was measured in these control hearts in which the mitochondria were inhibited with rotenone (example in Fig. 3). LPS2 was carried out to evaluate whether more severe endotoxemia would produce more increased $T_{1/2\text{-increase}}$. When comparing the mean $T_{1/2\text{-increase}}$ of the NADH fluorescence of the endotoxemic hearts to that of control hearts, there was a significantly delayed ischemia in endotoxemic hearts (Fig. 6). LPS2 was performed to evaluate whether a more severe endotoxemia could induce a more attenuated mitochondrial metabolism; however, while the mean $T_{1/2\text{-increase}}$ was greater in LPS2 than in LPS1, the difference did not achieve significance (Fig. 6).

Figure 3. Example of the NADH-fluorescence trace of a control heart (open circles) and the same heart after being loaded with rotenone (closed squares) (CON). $T_{1/2\text{-increase}}$ is the elapsed time between the black arrow and the gray arrows. The black arrow indicates the moment of occlusion of coronary flow; the gray arrows indicate the moment that 50% NADH fluorescence is reached. As can be seen, $T_{1/2\text{-increase}}$ greatly increased due to inhibition of oxygen consumption by rotenone induced inhibition of respiration. Also note that baseline fluorescence significantly increased due to rotenone loading. Fat black arrow indicates reperfusion.

ON-KINETICS. During reperfusion of control hearts (CON) with oxygen-saturated medium, NADH fluorescence swiftly decreased to a lower intensity with patchy, strong fluorescent areas lagging behind normoxic areas, as described previously [10]. In both the LPS1 and LPS2 groups, the post-ischemic reperfusion patterns did not differ significantly from those of control hearts (Fig. 4); endotoxemic hearts had post-reperfusion ischemic areas that were similar in size and frequency distribution pattern as those observed in control hearts; there was no significant difference between the frequency distribution histograms in Fig. 5 even though some endotoxemic hearts displayed ischemic areas clearly larger as those seen upon reperfusion in control hearts.

Figure 4. Left is shown the heterogeneous NADH fluorescence pattern upon reoxygenation in a control heart (CON); the right image shows the pattern of a septic heart (LPS1).

The mean relative frequency distribution of ischemic surface areas in septic hearts showed a pattern as was also seen in control hearts and was clearly different from the patterns of hearts embolized with 9.8 and 15 μm microspheres [12, 13]: there were larger high fluorescent areas present which were not present in CON hearts and septic hearts (Fig. 4).

Figure 5. Frequency distribution histogram of ischemic surface areas in control hearts (CON) and endotoxemic hearts (LPS1 and LPS2). Columns represent mean $\pm$ SEM.

Upon reperfusion after ischemia, whether in CON hearts, LPS hearts or CON hearts loaded with rotenone, NADH fluorescence decreased very quickly (when compared to the OFF-KINETICS) to baseline levels, while there was no significant difference between all the groups (Fig. 2, 3 and 6).

DISCUSSION

Septic shock is ultimately characterized by a persistent oxygen extraction deficit despite adequate systemic hemodynamic and oxygen derived variables, resulting in organ failure. Macro-hemodynamic pathophysiologic mechanisms alone cannot explain this oxygen extraction deficit, which is occurring at (intra)cellular and thus microcirculatory level [15, 16, 17]. This defect in oxygen extraction has been mainly attributed to microcirculatory alterations well described for sepsis resulting in shunting of oxygen transport of weak microcirculatory units resulting in a decreased oxygen extraction (24). In addition a reduction in the ability to utilize oxygen by the mitochondria could fuel this process. Our findings in the present study may add a piece of the puzzle that mitochondrial dysfunction could contribute to this deficit in addition to microcirculatory alterations. The main finding of the present study was that signs of mitochondrial dysfunction precede over the microcirculatory alterations in early endotoxemia. This does not preclude the presence in vivo of red blood cell flow abnormalities due to red blood cell and endothelial cell interaction since our study was conducted in a crystalloid perfused isolated model. In addition as we showed earlier, stressing the septic heart did reveal the vulnerability of the septic microcirculation by early induction of ischemia of MWU's [5]. However, that study [5] was performed after a much longer endotoxemic induction and therefore utilized a more severe sepsis model. Nevertheless our in vitro model was able to identify early mitochondrial dysfunction under non-stressed conditions.

In the present study we found that in endotoxemic hearts the increase of NADH fluorescence after occlusion of coronary flow was slower than in control hearts (Fig. 6). To test whether a decreased oxidative capacity of myocardial mitochondria indeed results in an increased $T_{1/2\text{-increase}}$ of NADH fluorescence during occlusion of coronary flow, 3 control hearts were loaded with rotenone, an inhibitor of mitochondrial electron transport. Repeating the occlusion and reperfusion indeed showed a significantly increased $T_{1/2\text{-increase}}$. We concluded therefore that the increased $T_{1/2\text{-increase}}$ probably was attributed to a decreased capacity of mitochondria to consume oxygen. Vanasco et al. described for the first time this "Complex I syndrome" in endotoxemia in a study on isolated mitochondria [17]. Further evidence of the "Complex I syndrome" was also found in a clinical study in skeletal muscle biopsies of septic patients where an inverse correlation was found between complex I activity and the severity of sepsis [15, 16]. Recently, Agarwal et al [18], showed in a study describing the cardioprotective effects of isoflurane on cardiac mitochondrial bioenergetics, the well-known fact that rotenone attenuated the magnitude and increased the rate of state 3 NADH oxidation in isolated mitochondria. This effect was also clearly shown in this study in the, intact, beating isolated perfused heart (Fig. 3) which is, as far as we know, the first description of evaluating complex I dysfunction in the intact heart.

Control hearts show consistently that the increase of fluorescence upon induction of ischemia or hypoxia, is always much slower, and homogeneous, than the decrease of fluorescence upon reoxygenation which in addition is always heterogeneous [10, 11]. We hypothesized that in the case of ischemia, the rate of increase of NADH fluorescence is related to the rate of oxygen consumption. This explanation is also supported by the study of Coremans et al. [19] showing that a decreased rate of NADH oxidation in isolated perfused kidneys is caused by impaired mitochondrial function. A study by Duboc et al. [23] using fiber NADH fluorescence measurement also supports this hypothesis by showing that rejection of transplanted hearts is accompanied by a decreased rate of NADH fluorescence decrease upon reperfusion after an episode of ischemia which was caused by a decreased capacity for mitochondrial oxygen consumption. Another argument in favor of this hypothesis is that an earlier study showed that the increase of fluorescence during hypoxia was almost twice as fast during high flow anoxia when compared to ischemia [10]. This effect was caused by the washing-out of tissue oxygen via oxygen depleted nitrogen saturated Tyrode. In other words, the increase of fluorescence is related to changes in tissue oxygen content, and tissue oxygen content can be altered by either wash-out or change in consumption. The main determinant of oxygen consumption in myocardium is myocardial work; therefore the left ventricle was cannulated in all Langendorff hearts to ascertain that cardiac work was nearly completely prevented [13], thereby eliminating as much as possible that cardiac work influenced the results. LPS2 was carried out to evaluate whether a more severe model of sepsis would produce increased impairment in mitochondrial function. We did not find a significant difference of mitochondrial function between LPS1 and LPS2 although there was a larger impairment in LPS2. Here again, as mentioned before, the level of severity in endotoxemia between the LPS1 and LPS2 groups may not have differed possibly due to the relative small size of the experimental groups.

An explanation for the differences between the rates and patterns of increase and decrease of fluorescence during ischemia and reperfusion might be that the increase upon ischemia is mainly dictated by consumption whereby oxygen diffusion in tissue levels out local differences in pO_2 , resulting in a more or less homogeneous increase, the speed of which is mainly dictated by the rate of oxygen consumption. The decrease of fluorescence upon reoxygenation on the other hand is faster and heterogeneous because it is mainly dictated by oxygen transport and diffusion; there is no oxygen consumption worth mentioning due to the very lack of oxygen. Upon reoxygenation the myocytes closest to the oxygen supply swiftly oxidize the reduced respiratory chain, while the myocytes furthest away from the supply remain reduced (the microcirculatory weak units [10]). Because transport and diffusion of oxygen are always much faster (indeed, must always be faster under physiological conditions) than its consumption, the decrease of fluorescence upon reoxygenation is much quicker than the increase upon initiation of ischemia or hypoxia. Furthermore, the decrease of fluorescence upon reoxygenation was not different between control hearts and septic hearts in this study. This would imply that sepsis induced changes in metabolism do not affect the rate of decrease upon reoxygenation. Therefore, one could also state that this rate of decrease is not dependent on metabolism. This statement is supported by the finding that in rotenone perfused control hearts (CON, $n = 3$); the $T_{1/2\text{decrease}}$ is not different between groups (Fig. 6).

Endotoxemia induced mitochondrial dysfunction would be expected to decrease $T_{1/2\text{-decrease}}$. However, we did not find this; a possible explanation is that the differences are too small to be detected with the small size of the experimental groups in this study. Also there are technical limitations in the model used here to measure differences in $T_{1/2\text{-decrease}}$ between groups. The mitochondrial redox balance very quickly shifts to NAD^+ upon reoxygenation because oxidation of NADH occurs at a very low mitochondrial- and tissue oxygen concentration. The experimental model used in this study cannot measure the response time of changes in mitochondrial NADH upon reoxygenation as this is a process occurring in milliseconds while the time-resolution of the measurements in this model is 1 second. The $T_{1/2\text{-decrease}}$ measured in this study is however closely related to rate of oxygen transport in the microcirculation including the rate of diffusion of oxygen to the mitochondria. A mild attenuation of mitochondrial functioning as caused by endotoxemia will not result in an increased $T_{1/2\text{-decrease}}$ because impaired oxygen metabolism would result in a faster decrease of NADH and thus a smaller $T_{1/2\text{-decrease}}$. When, on the other hand, the rate of mitochondrial oxygen consumption is so low that it is even lower than the rate of oxygen transport, then one might expect an increase of $T_{1/2\text{-decrease}}$ as we showed to occur in an earlier study (21) where rotenone in the perfusate caused a delayed recovery of high NADH fluorescence after ischemia in normal hearts.

Figure 6. Histogram above: $T_{1/2\text{-increase}}$ of NADH fluorescence during ischemia (OFF-KINETICS). Histogram below: $T_{1/2\text{-decrease}}$ of NADH fluorescence during reperfusion (ON-KINETICS). Columns represent mean (black columns) $\pm$ SEM (white columns). $T_{1/2\text{-decrease}}$ is not different between groups whereas $T_{1/2\text{-increase}}$ is significantly larger in LPS and rotenone groups than in control groups.

The differences in workload between septic and control hearts also have to be taken into account. To this end we prevented nearly all work by connecting the left ventricle to the atmosphere to prevent development of left ventricular pressure [13]. A consideration when

interpreting these results is whether the total myocardial oxygen content (not concentration) in septic and control hearts was comparable at the beginning of the occlusion of coronary flow, because lower oxygen content would of course lead to a faster development of ischemia.

Earlier studies showed that in control Langendorff hearts there exist areas with decreased oxygen supply upon reoxygenation, described as MWU's [10] and these small areas were shown to originate at the capillary level. In sepsis arterioles display a decreased response to vasoconstrictors and vasodilators whereby the attenuated responsiveness to vasoconstrictor agents predominates [4, 5]. In sepsis there is also a decrease in functional vascular (capillary) density (for instance by means of occlusion due altered red blood cell deformability, endothelial cell swelling, disseminated intravascular coagulation (DIC) or plugging by neutrophils). In our present study this could be the explanation for the appearance of hemorrhagic foci on the myocardial surface in 2 septic rat hearts. An alteration of the heterogeneous NADH fluorescence pattern upon reoxygenation could therefore be expected in septic hearts if the vascular bed itself was altered. Larger areas of relatively long and persisting hypoxia would hint at disturbance of flow at the arteriolar/arterial level (as was shown earlier in experiments where arterioles and arteries were occluded with microspheres [11, 12]). Smaller areas would indicate disturbance at the level of the microcirculatory weak units (capillary level) [5, 10, 20]. However, we found in this study that upon reperfusion with oxygenated Tyrode, endotoxemic hearts displayed heterogeneity with identical ischemic areas as in control hearts. Therefore, in this study at least, we conclude that sepsis did not induce an alteration of oxygen transport pattern in the microcirculation at the arteriolar or capillary level, which could be revealed with NADH-videofluorimetry. A consideration with this conclusion is that there may well be very significant alterations of the microcirculation but that these go undetected because of their balanced effect on capillary perfusion, an effect already shown earlier [10], where plugging the capillary compartment with microspheres obviously dramatically decreased perfusion while the heterogenic pattern of ischemia was identical to the patterns resulting from reperfusion after ischemia or hypoxia. In this study however three hearts also displayed ischemic areas at the beginning of perfusion with a much larger area. These could have been caused by acidosis occurring in the septic hearts. It was shown earlier that acidosis results in larger myocardial areas with a persisting high NAD fluorescence intensity [14]. During perfusion these areas cleared indicating either recovery from acidosis and/or recovery of perfusion at local arteriolar/arterial level due to washout of blood clots.

In conclusion our study has demonstrated mitochondrial alterations to occur independent of microcirculatory effects in this model of the septic myocardium. The direct effects of endotoxemia on mitochondrial metabolism could be well studied using NADH-videofluorimetry in intact beating hearts. The pattern of oxygen transport to myocardium in the microcirculation appeared not to be altered although the kinetics of NADH accumulation dependent on Complex I were clearly attenuated. It is concluded that experimental model presented here can be of use in further studies concerning the effects of sepsis (and effects of therapeutic interventions in sepsis) on metabolism and microcirculation of the myocardium.

REFERENCES

- 1 Schorr CA, Zanotti S, Dellinger RP (2014). Severe sepsis and septic shock: management and performance improvement. *Virulence*; 5(1): 190-199
- 2 Zanotti-Cavazzoni SL, Hollenberg SM (2009). Cardiac dysfunction in severe sepsis and septic shock. *Curr Opin Crit Care*; 15(5): 392-397
- 3 Levy RJ (2007). Mitochondrial dysfunction, bioenergetic impairment, and metabolic down-regulation in sepsis. *Shock*; 28(1): 24-28
- 4 Lush CW, Kvietys PR (2000). Microvascular dysfunction in sepsis. *Microcirculation*; 7(2): 83-101
- 5 Avontuur JA, Bruining HA, Ince C (1995). Inhibition of nitric oxide synthesis causes myocardial ischemia in endotoxemic rats. *Circ Res*; 76(3): 418-425
- 6 Huet O, Dupic L, Harrois A, Duranteau J (1995). Oxidative stress and endothelial dysfunction during sepsis. *Front Biosci (Landmark Ed)*; 16: 1986-1995
- 7 Carré JE, Singer M (2008). Cellular energetic metabolism in sepsis: the need for a systems approach. *Biochim Biophys Acta*; 1777 (7-8): 763-771
- 8 Levy RJ, Deutschman CS (2007). Cytochrome c oxidase dysfunction in sepsis. *Crit Care Med*; 35(9 Suppl): S468 – S475
- 9 Sharma AC (2007). Sepsis-induced myocardial dysfunction. *Shock*; 28(3): 265-269
- 10 Ince C, Ashruf JF, Avontuur JAM et al. (1993). Heterogeneity of the hypoxic state in rat heart is determined at capillary level. *Am J Physiol*; 264: 294-301
- 11 Ashruf JF, Ince C, Bruining HA (1999). Regional ischemia in hypertrophic Langendorff-perfused rat hearts. *Am J Physiol*; 277: 1532-1539
- 12 Ashruf JF, Ince C, Bruining HA et al. (1994). Ischemic areas in hypertrophic Langendorff rat hearts visualized by NADH-videofluorimetry. *Adv Exp Med Biol*; 345: 259-262
- 13 Ashruf JF, Coremans JMCC, Bruining HA et al. (1995). Increase of cardiac work is associated with decrease of mitochondrial NADH. *Am J Physiol*; 269: 856–862
- 14 Hulsman WC, Ashruf JF, Bruining HA et al. (1993). Imminent ischemia in normal and hypertrophic Langendorff rat hearts; effects of fatty acids and superoxide dismutase monitored by NADH surface fluorescence. *Biochimica et Biophysica Acta*; 1181(3): 273-278
- 15 Brealy D, Brand M, Hargraves I et al. (2002). Association between mitochondrial dysfunction and severity and outcome of septic shock; 360: 219-223
- 16 Svistunenko DA, Davies N, Brealy D et al. (2006). Mitochondrial dysfunction in patients with severe sepsis: An EPR interrogation of individual respiratory chain components. *Biochimica et Biophysica Acta*; 1757: 262-272
- 17 Vanasco V, Magnani ND, Cimolai MC et al. (2012). Endotoxemia impairs heart mitochondrial function by decreasing electron transfer, ATP synthesis and ATP content without affecting membrane potential. *J Bioenerg Biomembr*; 44: 243-252
- 18 Agarwal B, Dash RK, Stowe DF et al. (2014). Isoflurane modulates cardiac mitochondrial bioenergetics by selectively attenuating respiratory complexes. *Biochimica et Biophysica Acta*; 1837: 354-365
- 19 Coremans JMCC, van Aken M, Naus DCWH et al. (2000). Pretransplantation assessment of renal viability with NADH-fluorimetry. *Kidney International*; 57: 671-683

- 20 Vetterlein F, Prange M, Lubrich D et al. (1995). Capillary perfusion pattern and microvascular geometry in heterogeneous hypoxic areas of hypoperfused rat myocardium. *Am J Physiol*; 268: H2183-H2194
- 21 Eerbeek O, Milstein DMJ, Ince C (2004). Microcirculatory dysfunction in Langendorff endotoxemic rat hearts. *Shock*; 21: 81 Suppl.1, Abstract: 323
- 22 Barlow CH and Chance B (1976). Ischemic areas in perfused rat hearts: measurement by NADH fluorescence photography. *Science*; 193: 909-910
- 23 Duboc D, Abastado P, Muffat-Joly M et al. (1990). Evidence of mitochondrial impairment during cardiac allograft rejection. *Transplantation*; 50(5): 751-755
- 24 Ince C and Sinaasappel M (1999). Microcirculatory oxygenation and shunting in sepsis and shock. *Crit Care Medicine*; 27: 1369-1377

9

NEW INSIGHTS INTO THE PATHOPHYSIOLOGY OF CARADIOGENIC SHOCK: THE ROLE OF THE MICROCIRCULATION

Ashruf JF^{1,2}, Ince C¹, Bruining HA³

¹Department of Translational Physiology, Academic Medical Center, University of Amsterdam, Amsterdam

²Department of Surgery, OZG Hospital, Groningen

³Department of Surgery, Erasmus University of Rotterdam, Rotterdam, the Netherlands

Published in: Curr Opin Crit Care; 19(5): 381-386, 2013

PURPOSE OF REVIEW

The ultimate goal of therapy for cardiogenic shock is to restore microcirculatory function and thereby restore the oxygen supply to sustain cellular function. Therapeutic measures mainly focus on improving pressure-derived macrocirculatory parameters. However, it is increasingly clear that to achieve significant progress in treatment, microcirculatory physiopathological mechanisms must be considered.

RECENT FINDINGS

Microcirculatory function deteriorated during cardiogenic shock and improved after treatment. Post cardiogenic shock microcirculatory disturbances, both myocardial and peripheral, were a prognostic factor for the long-term outcome. Hypothermia, whether pharmacologically or physically induced, improved post resuscitation myocardial and cerebral function, an effect associated with improved post resuscitation microcirculation. The impact of cardiogenic shock on cerebral and myocardial microcirculation could be evaluated with MRI. In severe heart failure, pharmacological interventions improved microcirculation. An assessment of the microcirculation was often performed using handheld video microscopy for direct observation of the sublingual microcirculation, which proved to be useful for evaluating the effects of interventions during cardiogenic shock. A large multicenter study on critically ill patients is now being conducted using this technique.

SUMMARY

Cardiogenic shock induces microcirculatory disorders that can be monitored and influenced in various manners, both pharmacologically and physically. In addition to global hemodynamic optimization, interventions must also ameliorate the microcirculation.

KEY POINTS

- Post resuscitation hypothermia has a beneficial effect on outcome, but in itself has a negative influence on the microcirculation.
- The inhibition of arginase could be a therapeutic strategy to rescue microcirculation in patients with SHF.
- Cardiac arrest initiates compensatory sympathetic nervous system activation, which leads to persistent microcirculatory abnormalities similar to those abnormalities found in septic shock.
- EECP could be a potential treatment for patients after cardiac arrest and the ROSC; the effects of EECP are mediated by improving cerebral microcirculation by enhancing the endothelial function.

INTRODUCTION

Cardiogenic shock is characterized by inadequate organ perfusion caused by primary cardiac dysfunction, mainly caused by damage to the heart muscle (myocardial infarction or cardiomyopathy), arrhythmia or cardiac valve disease. Three other types of shock are recognized: hypovolemic, obstructive and distributive shock [1]. Specifically in the latter type of shock, a key characteristic associated with hemodynamic derangement concerns microcirculatory malfunction with shunting [2]. All types of shock, however, cause microcirculatory abnormalities beyond obvious global hemodynamic derangement. It is increasingly clear that the microcirculation needs to be addressed, beyond the cardiac disorder, to effectively manage cardiogenic shock [3, 4, 5-7]. Of course, cardiogenic shock patients constitute a heterogeneous group of pathologies, with cardiogenic shock in relatively healthy patients (i.e. ventricular fibrillation resulting in shock) at one end of the spectrum and cardiogenic shock in patients with chronic heart failure (i.e. a large myocardial infarction), who already have significantly abnormal microcirculation because of preshock illness at the other end [8, 9]. Cardiogenic shock patients who develop a systemic inflammatory response syndrome (SIRS) have more serious impairment of the microcirculation because of SIRS-related vasodilatation and the resulting hypotension, and are more at risk of persistence of shock [10]. In addition, the physiopathology of the microcirculation differs between organs, which must be taken into account when evaluating microcirculation in therapy and research [3, 4, 11, 12].

Cardiogenic shock results in decreased cardiac output with adequate intravascular volume. Fluid therapy is one of the first interventions in the management of shock. Management of cardiogenic shock requires restoring primary cardiac function; however, restoration of systemic hemodynamics does not necessarily imply restoration of microcirculation in the various organ systems [13]. In severe cardiogenic shock, microvascular perfusion is altered, even when global circulation is restored. A potential hazard, for instance, is that excessive administration of fluid to restore global circulation can cause deterioration of microcirculatory function because of edema formation [11]. In successful electrical cardioversion in patients with atrial fibrillation, sublingual microvascular perfusion improved, whereas this improvement was not clearly related to global hemodynamic parameters [14]. An organized cardiac rhythm is important for optimal microvascular perfusion [15].

To develop treatment strategies that target microcirculation in cardiogenic shock, it is necessary to evaluate known therapeutic and diagnostic measures and their relation to the physiopathological mechanisms. In this review period, new insights related to hypothermia in cardiopulmonary resuscitation (CPR), the use of vasoactive agents, fluid therapy, the prognostic value of microcirculatory alterations post cardiac arrest, cardiac arrest and (cerebral) microcirculation, the myocardial microcirculatory effects of myocardial infarction and muscle oxygen metabolism in heart failure are assessed. In addition, an international multicenter study is being performed, which will hopefully establish a basis for future studies related to microcirculation in critically ill patients [16]. A traditional parameter to evaluate microcirculatory disorder is the measurement of serum lactate levels, which have a poor correlation with the microcirculatory disorders at the organ level [5]. Our introduction of handheld video microscopes to observe microcirculation at the bedside,

based on orthogonal polarization spectral (OPS) imaging, and later sidestream dark-field (SDF) imaging, mainly applied to observe the sublingual microcirculation, has provided great insight into the importance of this physiological compartment in perioperative medicine [3, 4, 5, 7, 17, 18]. These previous generations of handheld microscopes, however, have been criticized because of poor image quality and the inability to implement automatic analysis software, limiting the microscopes' use as research tools [19]. Recently, however, a new handheld intravital microscope has been introduced, based on incident darkfield (IDF) imaging [20]. This microscope contains a computer-controlled high-resolution imaging sensor [4] with higher image resolution, allowing the direct analysis of images for the immediate identification of microcirculatory alterations and titrating therapy. It is expected that such technological advances in microcirculatory monitoring will introduce the diagnosis and treatment of this important physiological compartment to the clinic.

HYPOTHERMIA IN CARDIOPULMONARY RESUSCITATION

Pharmacologically induced hypothermia with WIN55, 212-2 was shown to improve post resuscitation neurological and myocardial function and survival in a rat model [21]. Outcome was related to significant improvements in microcirculatory parameters, as observed by sublingual SDF imaging. A suggested advantage of WIN55, 212-2 induced hypothermia over physical hypothermia is the reduction in systemic vascular resistance and increased cardiac output following WIN55, 212-2 administration, thereby reducing myocardial oxygen consumption and afterload, and improving tissue perfusion.

Laboratory and clinical studies have demonstrated that actively reducing the blood temperature to 32-34 °C after resuscitation significantly improved the neurologically favorable survival. However, therapeutic hypothermia also has adverse effects, so limiting hypothermia's duration of use could be of benefit to the patient. A shorter duration of early hypothermia after resuscitation improved microcirculation and myocardial and neurological outcomes compared with prolonged hypothermia [22]. A possible explanation could be that prolonged hypothermia induced vasoconstriction and decreased capillary density. Another explanation might be that post resuscitation oxygen debt should be repaid as quickly as possible, which is impeded by prolonged hypothermia and concomitant microcirculatory dysfunction.

He et al. [23] attempted to clarify whether the positive effects of hypothermia in resuscitation were related to a physiological adjustment to the balance between oxygen supply and demand, which are both reduced during hypothermia, or whether hypothermia per se has deleterious effects on tissue perfusion. Sublingual microvascular flow was decreased compared with the normothermic state.

VASOACTIVE AGENTS

Factors contributing to microcirculatory dysfunction in severe heart failure (SHF) are a persistent inflammatory response, leading to increased microvascular permeability and reduced capillary density (SIRS) [10]; neurohumoral mechanisms, with the secretion of

potent vasoconstrictive agents; increased blood viscosity; altered erythrocyte rheological properties; and hypercoagulability secondary to platelet activation. Angiotensin II, the main effector of the renin-angiotensin-aldosterone system (RAAS), is an important mediator of these effects. Indeed, Salgado et al. [24] showed that angiotensin II inhibitors improved microcirculation in patients with SHF and that this finding was not related to global hemodynamic improvement. The beneficial effects of angiotensin II inhibition in SHF may be caused by microcirculatory improvement.

In SHF, there is endothelial dysfunction resulting in a reduced bioavailability of nitric oxide. Several studies showed that intravenous nitroglycerin, a nitric oxide donor, improved tissue perfusion in SHF, as observed by SDF imaging [17, 25, 26].

The reduced bioavailability of nitric oxide in SHF is further aggravated by the upregulation of arginase, a competitive enzyme for endothelial nitric oxide synthase. Topical inhibition of arginase improved sublingual microcirculation in SHF [26]. Inhibition of arginase could therefore be a therapeutic target to rescue microcirculation in SHF.

Microcirculatory blood flow was highly correlated with macrocirculatory hemodynamics, including coronary perfusion pressure in distinction with septic shock. Administration of epinephrine dramatically decreased the microcirculatory blood flow. This earlier study [27] showed that simply increasing the blood pressure by inotropic agents, without taking notice of the microcirculation, could leave the vital organs ischemic [28].

FLUID RESUSCITATION

Restoration of global hemodynamic parameters by fluid loading is one of the most important therapies in managing shock. Amelioration of the microcirculatory blood flow is the primary goal of this treatment. However, fluid loading may also alter microcirculatory oxygen delivery by hemodilution or tissue edema [13]. In this context, blood transfusion during cardiac surgery has been shown to improve red blood cell availability and oxygenation in the microcirculation [29]. The long-term impact of fluid loading on the microcirculation is minimal compared with the impact during the early phase of shock. Therefore, it is important to monitor the effect of fluid loading on the microcirculation to better balance the amount of administered fluid with amelioration of microcirculation. In SHF, the microcirculation is impaired even when global hemodynamic or laboratory signs of hypoperfusion are absent. Effective fluid loading and pharmacological treatment aimed at decreasing neurohumoral activation improve microcirculation, as evaluated by sublingual SDF imaging [30]. A similar finding was reported by Hogan et al. [31], who evaluated the microcirculation by measuring peripheral microvascular oxygen-extraction ratios on the hypothenar eminence during the emergency department treatment of SHF.

In hemorrhagic shock in pigs, fluid resuscitation guided by sublingual pCO₂ and sublingual SDF imaging significantly reduced the amount of resuscitation fluid, without compromising the outcomes of hemorrhagic shock [32]. The authors believe that this would also be the case in SHF or cardiogenic shock. Used in this way, microcirculatory monitoring could help to reduce the adverse effects of fluid overloading in shock.

PROGNOSTIC VALUE OF MICROCIRCULATORY ALTERATIONS AFTER CARDIAC ARREST

Sublingual microvascular blood flow alterations measured by OPS imaging are frequently observed in patients with SHF and are more severe in patients who do not survive [33]. This finding sustains the hypothesis that in cardiogenic shock, microvascular abnormalities result in impaired tissue oxygenation and thereby the development of multiple organ failure.

After cardiac arrest, in the early post resuscitation phase, there are significant microcirculatory abnormalities that return to normal within 48 h [34]. These abnormalities in the early phase closely resemble those abnormalities found in septic shock. However, in septic shock, they persist. This suggests that in this study, cardiogenic shock was insufficiently severe to trigger the same mechanisms of microvascular dysfunction as in sepsis. Whereas post resuscitation microvascular flow normalized, this was not the case for microvascular reactivity, as post resuscitation reactive hyperemia in the thenar muscle remained disturbed.

Patients with acute myocardial infarction (AMI) complicated by cardiogenic shock and who have impaired sublingual microcirculation at baseline or following treatment have a poor outcome [9]. Interestingly, there was a weak correlation of microcirculatory alterations with global hemodynamic parameters, as was reported earlier. Assessment of the sublingual microcirculation with SDF imaging could therefore be used as a noninvasive tool to assess the outcomes of SHF patients. Indeed, an international multicenter trial is now being performed, among others, to address this issue [16].

A later study [35] showed that early post resuscitation microcirculatory abnormalities (sublingual and peripheral) were caused by vasoconstriction because of induced systemic hypothermia, and the persistence of these alterations was associated with organ failure and death. Also, there was again no relationship between systemic hemodynamics and microcirculatory status. It was theorized that nonsurvivors of cardiac arrest suffered from ongoing sympathetic activity and the development of SIRS. A similar relationship was observed in patients with septic shock.

Another study [8] reported that impaired myocardial microcirculation (evaluated by assessing the coronary flow reserve with transthoracic Doppler echocardiography) is associated with the development of heart failure and acute coronary syndrome. This phenomenon is likely because of a reduction in the functional capacity of the myocardium caused by microcirculatory dysfunction.

A recent study [36] found that dying patients treated with extracorporeal membrane oxygenation (ECMO) for cardiogenic shock showed major functional and structural pathology in the skin microvasculature, whereas surviving patients had normal skin microcirculation. Therefore, the authors stated that finding intact skin microcirculation early after establishing ECMO is a clinically useful finding implying a good prognosis.

CARDIAC ARREST AND (CEREBRAL) MICROCIRCULATION

Because the frequency of CPR is growing, the number of patients with severe neurological deficit after the restoration of spontaneous circulation (ROSC) has steadily increased. The high mortality rate of cardiac arrest is partially caused by the complex mechanisms of brain

injury. Liu et al. [37] developed an animal model that mimics cardiac arrest and used early MRI to observe the disorders in brain microcirculation. They found that even though blood pressure returned to normal after ROSC, cerebral microcirculation did not return to physiological baseline levels. These abnormalities in the brain microcirculation were found using perfusion-weighted MRI (PWI), which conveys information about microvessels less than 100 μm , which is not obtainable by conventional angiography. Using this technique, clinicians could determine specific brain damage in cardiac arrest patients and even predict outcome.

Hu et al. [28] showed that early enhanced external counterpulsation (EECP) after the ROSC protected the brain by improving brain microcirculation. EECP could be a potential treatment for patients after cardiac arrest and the ROSC. EECP resulted in opening of the highly resistant areas of the cerebral microcirculation, thereby overcoming regional and global perfusion disturbances caused by edema and blood clots. EECP increased the left ventricular ejection fraction, carotid blood flow and endothelial shear stress, and thereby promoted nitric oxide and tissue plasminogen-activator production and decreased endothelin-1 release after the ROSC. Earlier work on the effect of mechanical circulatory support devices on the microcirculation (the Impella LP2.5 percutaneous left ventricular assist, the intra-aortic balloon pump, the HeartMate II, Centrimag and TandemHeart) [38-40] showed that the mechanical support of the failing ventricle results in improved tissue perfusion, as evaluated by SDF imaging of the sublingual microcirculation. It was also shown that improved microvascular perfusion is not necessarily correlated with an increased mean arterial pressure [40]. In contrast to EECP, treatment with norepinephrine increased carotid blood flow but had no effect on nitric oxide, tissue plasminogen activator or endothelin-1 levels [28]. The neurologic deficit in norepinephrine-treated animals was larger compared with EECP-treated animals. Treatment with vasopressors alone increased blood flow to important organs, but after ROSC the left ventricle could not tolerate the increased vascular resistance. Furthermore, α 1-adrenergic and β -adrenergic drugs also lead to greater oxygen consumption via fibrillating ventricles, which has detrimental effects on cardiac function post resuscitation. This finding explains the poor outcome of cardiac arrest with only hypertensive reperfusion.

Interestingly, whereas EECP improved cerebral microcirculation by means of a hypertensive effect, Eibers et al. [41] concluded that pulsatile perfusion did not alter human microvascular perfusion in routine cardiac surgery. However, several studies did show an improvement in microvascular flow with pulsatile perfusion, for example [42]. One explanation for this discrepancy is that these patients [41] may have been too healthy to show pulsatile perfusion-related improvement in microcirculation. In addition, measurements were performed for the sublingual microcirculation, which may not be representative of the cerebral microcirculation.

Compensatory neurohumoral mechanisms preserve the perfusion pressure and viability of vital organs, and induce early splanchnic ischemia in terminal circulatory shock. Microcirculatory perfusion pressure might be a more significant determinant of central nervous system viability than commonly recognized in clinical practice [43].

MYOCARDIAL MICROCIRCULATORY EFFECTS OF MYOCARDIAL INFARCTION

Microvascular obstruction (MVO) following AMI with reperfusion ('no-reflow' phenomenon) is associated with a poor clinical outcome. Future efforts to manage AMI should focus on maintaining the microvascular patency. Currently, cardiovascular MRI is the only modality to evaluate MVO in a clinical setting [44].

MUSCLE OXYGEN METABOLISM IN HEART FAILURE

Structural and functional (neurohumoral, inflammatory and reflex) consequences of chronic heart failure coalesce at the muscle microcirculation and abolish the rapid increase in capillary RBC flux and the RBC distribution necessary to regulate microvascular pO_2 and support rapid oxygen uptake. Strategies that enhance nitric oxide bioavailability and decrease the negative effects of inflammatory cytokines are beneficial, and increased anti-inflammatory cytokines can increase patients' exercise tolerance and quality of life [45].

CONCLUSION

Cardiogenic shock alters the microcirculation. The nature and severity of these alterations are dependent on the severity and duration of shock. Evaluation of microcirculation in the treatment of cardiogenic shock revealed several potential therapeutic targets, pharmacological and physiological, requiring further research to prove the clinical applicability. Microcirculatory alterations could be used to predict the post resuscitation outcomes: heart failure, neurological and survival. Future trials should be directed toward developing and integrating methods to evaluate microcirculation in cardiogenic shock and titrating therapy based on the normalization of microcirculatory flow, as it is becoming clear that further progress in the treatment of cardiogenic shock must have a focus on the microcirculation.

REFERENCES

- 1 Vincent JL, Ince C, Bakker J (2012). Clinical review: circulatory shock - an update: a tribute to Professor Max Harry Weil. *Crit Care*; 16: 239-243
- 2 Bonanno FG (2011). Physiopathology of shock. *J Emerg Trauma Shock*; 4: 222-232
- 3 Donati A, Tibboel D, Ince C (2013). Towards integrative physiological monitoring of the critically ill: from cardiovascular to microcirculatory and cellular function monitoring at the bedside. *Crit Care*; 17: s5-s11
- 4 Bezemer R, Bartels SA, Bakker J et al. (2012). Clinical review: clinical imaging of the sublingual microcirculation in the critically ill - where do we stand? *Crit Care*; 16: 224-232
- 5 Jung C, Fritzenwanger M, Lauten A et al. (2010). Evaluation of microcirculation in cardiogenic shock: current diagnostic and therapeutic aspects. *Dtsch Med Wochenschr*; 135: 80-83
- 6 Jung C, Lauten A, Ferrari M (2010). Microcirculation in cardiogenic shock: from scientific bystander to therapy target. *Crit Care*; 14: 193-195
- 7 Ferrari M, Jung C, Lauten A et al. (2011). Evaluation of microcirculatory disorders in shock patients. *Dtsch Med Wochenschr*; 136: 1009-1013
- 8 Nakanishi K, Fukuda S, Shimada K et al. (2012). Impaired coronary flow reserve as a marker of microvascular dysfunction to predict long-term cardiovascular outcomes, acute coronary syndrome and the development of heart failure. *Circ J*; 76: 1958-1964
- 9 Den Uil CA, Lagrand WK, van der Ent M et al. (2010). Impaired microcirculation predicts poor outcome of patients with acute myocardial infarction complicated by cardiogenic shock. *Eur Heart J*; 31: 3032-3039
- 10 Den Uil CA, Klijn E, Lagrand WK et al. (2008). The microcirculation in health and critical disease. *Prog Cardiovasc Dis*; 51: 161-170
- 11 Vincent JL, de Backer D (2012). ICU nephrology: the implications of cardiovascular alterations in the acutely ill. *Kidney Int*; 81: 1060-1066
- 12 Boerma EC, van der Voort PH, Spronk PE et al. (2007). Relationship between sublingual and intestinal microcirculatory perfusion in patients with abdominal sepsis. *Crit Care Med*; 35: 1055-1060
- 13 Harrois A, Dupic L, Duranteau J (2011). Targeting the microcirculation in resuscitation of acutely unwell patients. *Curr Opin Crit Care*; 17: 303-307
- 14 Elbers PWG, Prins WB, Plokker HWM et al. (2012). Electrical cardioversion for atrial fibrillation improves microvascular flow independent of blood pressure changes. *J Cardiothorac Vasc Anesth*; 26: 799-803
- 15 Erol-Yilmaz A, Atasever B, Mathura K et al. (2007). Cardiac resynchronization improves microcirculation. *J Card Fail*; 13:95-99
- 16 Vellinga NAR, Boerma EC, Koopmans M et al. (2012). Study design of the microcirculatory shock occurrence in acutely ill patients (microSOAP): an international multicenter observational study of sublingual microcirculatory alterations in intensive care patients. *Crit Care Res Pract*; 2012: 121752
- 17 Den Uil CA, Caliskan K, Lagrand WK et al. (2009). Dose-dependent benefit of nitroglycerin on microcirculation of patients with severe heart failure. *Intensive Care Med*; 35: 1893-1899

- 18 Den Uil CA, Lagrand WK, ValksDA et al. (2009). Management of cardiogenic shock: focus on tissue perfusion. *Curr Probl Cardiol*; 34 :330-349
- 19 Mik EG, Johannes T, Freis M (2009). Clinical microvascular monitoring: a bright future without a future? *Crit Care Med*; 37: 2980-2981
- 20 Sherman H, Klausner S, Cook WA (1971). Incident dark-field illumination: a new method for microcirculatory study. *Angiology*; 22: 295-303
- 21 Weng Y, Sun S, Park J et al. (2012). Cannabinoid 1 (CB1) receptor mediates WIN55, 212-2 induced hypothermia and improved survival in a rat post cardiac arrest model. *Resuscitation*; 83: 1145-1151
- 22 Ye S, Weng Y, Sun S et al. (2012). Comparison of the durations of mild therapeutic hypothermia on outcome after cardiopulmonary resuscitation in the rat. *Circulation*; 125: 123-129
- 23 He X, Su F, Taccone FS et al. (2012). Cardiovascular and microvascular responses to mild hypothermia in an ovine model. *Resuscitation*; 83: 760-766
- 24 Salgado DR, Favory R, Rocco JR et al. (2013). Microcirculatory effects of angiotensin II inhibitors in patients with severe heart failure. *Clin Hemorheol Microcirc*; 54: 87-98
- 25 Den Uil CA, Lagrand WK, Spronk PE et al. (2009). Low-dose nitroglycerin improves microcirculation in hospitalized patients with acute heart failure. *Eur J Heart Fail*; 11: 386-390
- 26 Quitter F, Figulla HR, Ferrari M et al. (2013). Increased arginase levels in heart failure represent a therapeutic target to rescue microvascular perfusion. *Clin Hemorheol Microcirc*; 54: 75-85
- 27 Fries M, Weil MH, Chang YT et al. (2006). Microcirculation during cardiac arrest and resuscitation. *Crit Care Med*; 34: S454-S457
- 28 Hu C-L, Liu RL, Liao X-X et al. (2013). Early enhanced external counter pulsation improves neurological recovery after the return of spontaneous circulation in a mongrel dog cardiac arrest model. *Crit Care Med*; 41: e62-e73
- 29 Yuruk K, Almac E, Bezemer R et al. (2011). Blood transfusions recruit the microcirculation during cardiac surgery. *Transfusion*; 51: 961-967
- 30 Lauten A, Ferrari M, Goebel B et al. (2011). Microvascular tissue perfusion is impaired in acutely decompensated heart failure and improves following standard treatment. *Eur J Heart Fail*; 13: 711-717
- 31 Hogan CJ, Ward KR, Kontos MC et al. (2012). Peripheral tissue oxygenation improves during ED treatment of acute heart failure. *Am J Emerg Med*; 30: 196-202
- 32 Xu J, Ma L, Sun S et al. (2013). Fluid resuscitation guided by sublingual partial pressure of carbon dioxide during hemorrhagic shock in a porcine model. *Shock*; 39: 361-365
- 33 De Backer D, Creteur J, Dubois M-J et al. (2004). Microvascular alterations in patients with acute severe heart failure and cardiogenic shock. *Am Heart J*; 147: 91-99
- 34 Donadello K, Favory R, Salgado-Ribeiro D et al. (2011). Sublingual and muscular microcirculatory alterations after cardiac arrest: a pilot study. *Resuscitation*; 82: 690-695
- 35 Van Genderen ME, Lima A, Akkerhuis M et al. (2012). Persistent peripheral and microcirculatory perfusion alterations after out-of-hospital cardiac arrest are associated with poor survival. *Crit Care Med*; 40: 2287-2294

- 36 Wester T, Awan ZA, Kvernebo TS et al. (2014). Skin microvascular morphology and hemodynamics during treatment with veno-arterial extra-corporeal membrane oxygenation. *Clin Hemorheol Microcirc*; 56(2): 119-131
- 37 Liu R, Xin Li X, Hu C et al. (2012). The changes of brain water diffusion and blood flow on diffusion-weighted and perfusion-weighted imaging in a canine model of cardiac arrest. *Resuscitation*; 83: 645-651
- 38 Lam K, Sjaauw KD, Henriques JPS et al. (2009). Improved microcirculation in patients with an acute ST-elevation myocardial infarction treated with the Impella LP2.5 percutaneous left ventricular assist device. *Clin Res Cardiol*; 98: 311-318
- 39 Den Uil CA, Maat AP, Lagrand WK et al. (2009). Mechanical circulatory support devices improve tissue perfusion in patients with end-stage heart failure or cardiogenic shock. *J Heart Lung Transplant*; 28: 901 -911
- 40 Den Uil CA, Lagrand WK, van der Ent M et al. (2009). The effects of intra-aortic balloon pump support on macrocirculation and tissue microcirculation in patients with cardiogenic shock. *Cardiology*; 114: 42-46
- 41 Elbers PWG, Wijnbenga J, Solinger F et al. (2011). Direct observation of the human microcirculation during cardiopulmonary bypass: effects of pulsatile perfusion. *J Cardiothorac Vasc Anesth*; 25: 250-255
- 42 Koning NJ, Vonk AB, van Barneveld LJ et al. (2012). Pulsatile flow during cardiopulmonary bypass preserves postoperative microcirculatory perfusion irrespective of systemic hemodynamics. *J Appl Physiol*; 112: 1727-1734
- 43 Rady MY, Verheijde JL (2013). No-touch time in donors after cardiac death (nonheart-beating organ donation). *Curr Opin Organ Transplant*; 18: 140-147
- 44 White SK, Hausenloy DJ, Moon JC (2012). Imaging the myocardial microcirculation postmyocardial infarction. *Curr Heart Fail Rep*; 9: 282-292
- 45 Poole DC, Hirai DM, Copp SW, Musch TI (2012). Muscle oxygen transport and utilization in heart failure: implications for exercise (in)tolerance. *Am J Physiol*; 302: H1050-H1063

SUMMARY AND CONCLUSIONS

MYOCARDIAL MICROCIRCULATION AND MITOCHONDRIAL ENERGETICS IN THE ISOLATED RAT HEART

This dissertation focuses on the transport and metabolism of oxygen in the microcirculation. Although there is a correlation between oxygen dynamics in the macrocirculation and the microcirculation, knowledge of macrocirculatory oxygen dynamics falls short in explaining (patho-)physiological mechanisms of oxygen dynamics in the microcirculation. The isolated perfused rat heart was chosen as our model for studying the microcirculation because of its highly dynamic oxygen metabolism and transport which can be easily influenced in reproducible fashion, and also because its oxygen dynamics (intra- and extracellular) are relatively easy to monitor using various methods in absolute and relative quantities. NADH-videofluorimetry reveals ischemic myocardial areas as high-fluorescent areas. Measurement of intracellular autofluorescence from reduced pyridine nucleotide (NADH; 360-nm excitation, 460-nm emission) allows evaluation of mitochondrial energy state: NADH fluoresces when excited with ultraviolet light and NAD^+ does not. Myocardial NADH fluorescence distribution can be compared with oxygen pressure distribution in the vascular bed using Pd-porphyrin phosphorescence of intravascular Pd-porphyrin, because the phosphorescent properties of Pd-porphyrin are pO_2 -dependent.

Chapter 2. Upon re-oxygenation of the myocardium, after an episode of hypoxia, small myocardial areas reoxygenate slower than others. These areas are identical to those seen after embolization of capillaries; embolization of arteries and arterioles cause much larger ischemic areas. Ischemic areas caused by capillary embolization are identical to ischemic areas produced by increased metabolism in Langendorff perfused hearts. All these areas correlate with intravascular hypoxia as measured by Pd-porphyrin phosphorescence. Therefore, it was concluded that the myocardium possesses microcirculatory weak spots originating at the capillary level.

Chapter 3. Myocardial hypertrophy decreases muscle mass-to-vascularization ratio and thereby changes myocardial perfusion. This altered perfusion was observed during reoxygenation after an episode of hypoxia: in normal hearts small hypoxic areas reoxygenate slower than others, however in hypertrophic hearts these areas are much larger and were shown to be of similar size as the ischemic areas resulting from arterial/arteriolar embolization. Therefore it was concluded that hypertrophic hearts, in this model, contain areas with suboptimal perfusion at the arterial/arteriolar level.

Chapter 4. Normal hearts, when perfused with oxygenated medium with a pH of 7.0, develop ischemic areas of similar sizes as observed in hypertrophic hearts. This suggests the presence of acidosis induced suboptimal perfusion originating at the arterial/arteriolar level in normal hearts. Acidosis-induced ischemia is alleviated by addition of SOD (super oxide dismutase, a scavenger of free oxygen radicals) and fluorocarbons (increasing oxygen transport) to the perfusion medium. Earlier work also showed fatty acid protection from acidosis-initiated loss of myocardial flow. It is also known that normal Langendorff perfused rat hearts are borderline aerobic, as concluded from the continuous release of lactate in the effluent. These findings led us to hypothesize that the biochemical basis for the development of ischemic areas in Langendorff-perfused hypertrophic rat hearts might be the decompensation of myocardial perfusion due to local hypoperfusion at the arterial/arteriolar level exaggerated by the development of acidosis and oxygen free radicals. It was found that fatty acids added into the perfusate alleviated ischemia, probably by preventing local tissue acidosis. Similarly, the addition of oxygen free radical scavengers also alleviated ischemia. Fluorocarbons in the perfusate also had the same effect, most likely by increasing oxygen transport to ischemic tissues and thereby increasing aerobic metabolism and diminishing lactic acid formation (acidosis).

Chapter 5. For normal cardiac function, mitochondrial ATP synthesis must follow changes in ATP hydrolysis (cardiac work). Various control sites (e.g. cytosolic adenine nucleotides, intracellular calcium, and NADH/NAD⁺ + redox ratio) have been proposed as regulators of ATP synthesis. NADH/NAD⁺ redox state is mainly influenced by oxygenation level of the myocardium; however, other factors also influence this ratio. Substrate, for example, has a marked influence. We also noted that changes in workload in previous experiments altered the NADH/NAD⁺ redox state. To determine the relation between the NADH/NAD⁺ redox state and cardiac work, we measured this ratio during changes in workload while also taking into account factors influencing NADH fluorescence measurements: photo bleaching was avoided by means of intermittent UV-illumination, substrate dependency and depletion was avoided by using pyruvate as a substrate, oximetric effects were accounted for by diffuse reflectance spectroscopy, and ischemia was prevented by vasodilation thereby increasing myocardial flow and oxygenation. NADH/NAD⁺ redox ratio clearly decreases during increases in cardiac work. However, other studies reported an increase of this ratio. This may be the result of the development of relative ischemia during increases of work as is shown in chapter 2. In our experiments ischemia was avoided by the use of vasodilators and NADH-videofluorimetry to detect ischemia. We also noted that work related changes in NADH/NAD⁺ redox states were more instantaneous in pyruvate perfused hearts than in glucose perfused hearts, which we attributed to an increased capacity of mitochondrial oxidative phosphorylation due to the large increase of mitochondrial NADH in pyruvate perfused hearts.

Chapters 6 and 7. Upon illumination of tissue with ultraviolet light NADH (not NAD⁺) fluoresces in the blue color spectrum. In cardiac tissues blue fluorescence emitted from the organ surface is mainly derived from mitochondrial NADH. In this way NADH fluorescence provides direct information about the mitochondrial redox ratio. The phosphorescent properties of Pd-porphyrin compounds can be used as an indicator for oxygen concentration. The time constant of the quenching of phosphorescence is directly related to oxygen concentration. When complexes of Pd-porphyrin and bovine serum

albumin are injected into the blood, the oxygen probe is confined to the circulation and in vivo measurements of the microcirculatory O_2 concentration can be made. Images of the distribution of O_2 in the vasculature of intact organs can be obtained through continuous registration of the phosphorescence intensity of the Pd-porphyrin dye. It is expected that the combination of NADH fluorescence (oxygen in tissue) and Pd-porphyrin phosphorescence measurements (oxygen in microcirculation) in vivo can provide additional information about factors effecting mitochondrial respiration in a continuous and non-invasive way. Using NADH-videofluorimetry simultaneously with Pd-porphyrin phosphorimetry we were able to correlate intracellular hypoxia with intravascular hypoxia, which is important for gaining insight between local oxygen supply and demand in cells, tissues, and organs in vivo.

Chapter 8. Sepsis has profound (micro-)circulatory effects and also deteriorates mitochondrial function. As was shown before in hypertrophic hearts (Chap. 3, 4, and 5), microcirculatory effects could also be examined by epicardial NADH fluorescence: hypertrophic hearts displayed larger persistent ischemic areas upon reperfusion when compared to normal control hearts. However, in septic hearts (i.e. early stages of sepsis) we did not find a significant difference in fluorescence patterns between septic hearts and control hearts. This led us to conclude that in this model the microcirculation was not affected by endotoxemia, or put more accurately, with our method of evaluating the microcirculation we failed to find evidence of an altered microcirculation. We did find, however, that in this model of (early) sepsis, there was a significant deterioration of mitochondrial function as witnessed by a decreased capacity to consume oxygen.

Chapter 9. This review describes recent clinical and experimental studies, which show that the ultimate goal of therapy for cardiogenic shock should be restoration of microcirculatory oxygen transport to sustain cellular function. Nowadays therapeutic measures mainly focus on improving pressure-derived macrocirculatory parameters. However, to achieve significant progress in treatment, microcirculatory physiopathological mechanisms must be considered. Microcirculatory function deteriorated during cardiogenic shock and improved after treatment. Post-cardiogenic shock microcirculatory disturbances, both myocardial and peripheral, were a prognostic factor for long-term outcome predictions. Hypothermia, whether pharmacologically or physically induced, improved post-resuscitation myocardial and cerebral function, an effect associated with improved post-resuscitated microcirculation. The impact of cardiogenic shock on cerebral and myocardial microcirculation could be evaluated with MRI. In severe heart failure, pharmacological interventions improved microcirculation. An assessment of the microcirculation was often performed using handheld video-microscopy for direct observation of the sublingual microcirculation, which proved to be useful for evaluating the effects of interventions during cardiogenic shock. A large multicenter study on critically ill patients is now being conducted using this technique. Cardiogenic shock induces microcirculatory disorders that can be monitored and influenced pharmacologically and physically. In addition to global hemodynamic optimization, interventions must also ameliorate the microcirculation.

CONCLUSIONS

This thesis describes how the anatomy of the myocardial microcirculation determines the distribution pattern of oxygen to tissue and mitochondria, as evaluated with NADH- and Pd-porphyrin-videofluori-/phosphorimetry. In normal hearts this pattern reveals so-called weak microcirculatory units, originating at the capillary level, which are the first areas to become ischemic during episodes of increased oxygen consumption or decreased oxygen transport. In hypertrophic Langendorff perfused hearts, compromised or weak areas are much larger indicating a disturbance at the arteriolar/arterial level.

Furthermore, cardiac work was associated with a decrease in mitochondrial NADH, which is in contrast with the view that increased mitochondrial respiration is driven by an increase in mitochondrial NADH. An explanation for the observation in some studies that increased work is associated with increased mitochondrial NADH is that increased work in the isolated perfused heart leads to the development of local ischemia. This is because the Langendorff perfused heart is already borderline aerobic and increased work can therefore lead to local ischemia.

NADH- and Pd-porphyrin-videofluori-/phosphorimetry can also be used to measure the mitochondrial redox state and microcirculatory oxygen content in vivo, but more research is required to compensate for factors potentially introducing bias into the measurements in vivo (e.g. quenching of the fluorescence signal by hemoglobin).

Sepsis deteriorates mitochondrial function, an effect that can be examined by epicardial NADH-videofluorimetry, making this a promising instrument to study the complex effect of sepsis on the (micro-)circulation and mitochondrial function. It is also clear that further advances in the understanding and treatment of shock, regardless of its etiologic origin and its effects on whole organs, must include a component on the (patho-)physiology of the microcirculation since in this functional anatomical compartment the most relevant mechanisms occur that cannot be understood by further advances in macrocirculatory knowledge.

SAMENVATTING EN CONCLUSIES

Dit proefschrift behandelt het transport en metabolisme van zuurstof op het niveau van de microcirculatie. De kennis van zuurstoftransport en -metabolisme op macrocirculatoir niveau is ontoereikend om zuurstofmetabolisme en -transport op microcirculatoir niveau te beschrijven. Juist de kennis op microcirculatoir niveau is van essentieel belang om fysiologische en pathofysiologische processen in zuurstofmetabolisme en -transport te verklaren. De microcirculatie van het geïsoleerde geperfundeerde Langendorff rattenhart is gekozen als studiemodel omdat dit zich kenmerkt door een hoog zuurstofmetabolisme en -transport waarbij dit metabolisme en transport relatief eenvoudig - en reproduceerbaar - beïnvloed kan worden. Het zuurstoftransport en -metabolisme kunnen betrouwbaar gemeten worden, gebruikmakend van NADH-videofluorimetrie en Pd-porphyrine-fosforimetrie. Met NADH-videofluorimetrie kan ischemisch myocard geïdentificeerd worden vanwege de hoge fluorescentie intensiteit: tijdens ischemie verschuift de mitochondriale NADH/NAD^+ verhouding naar NADH doordat het door een gebrek aan zuurstof niet kan oxideren naar NAD^+ . Door intramitochondriaal NADH te exciteren met (ultraviolet) UV-licht (360 nm) zal het fluoresceren met zichtbaar licht (460 nm). Hierdoor zal ischemisch myocard een hogere fluorescentie-intensiteit vertonen. Gebruikmakend van Pd-porphyrine-fosforimetrie kan de zuurstofspanning in de microcirculatie worden beoordeeld omdat de fosforescentie intensiteit afhankelijk is van de zuurstof concentratie. Door NADH-videofluorimetrie en Pd-porphyrine-fosforimetrie te combineren, kan de intracellulaire zuurstofbeschikbaarheid worden gecorreleerd aan de microcirculatoire zuurstofspanning.

Hoofdstuk 2. Tijdens myocardiale reoxygenatie, na een ischemische episode, zullen kleine welomschreven myocardiale gebieden een langzamere reoxygenatie vertonen dan andere. Deze gebieden zijn identiek aan die welke ontstaan tijdens embolisatie van capillairen terwijl embolisatie van arteriolen en arteriën significant grotere ischemische gebieden doet ontstaan. De ischemische gebieden die ontstaan na capillaire embolisatie zijn identiek aan ischemische gebieden die ontstaan na significante toename van metabolisme in Langendorff harten. Ook correleren deze gebieden met microcirculatoire componenten (capillairen, arteriolen en arteriën) die een lage zuurstofspanning hebben (gemeten met Pd-porphyrine fosforimetrie). De conclusie is dan ook dat er “zwakke” myocardiale gebieden zijn die hun oorsprong vinden op capillair niveau.

Hoofdstuk 3. Tijdens myocardiale hypertrofie zal de spiermassa/vascularisatie ratio stijgen wat resulteert in verslechtering van myocardiale perfusie. Deze veranderde perfusie uit zich in een verandering van het reoxygenatie patroon van myocard wat meetbaar is met NADH-videofluorimetrie tijdens reoxygenatie na ischemie: het blijkt dat in hypertrofe harten gebieden met een vertraagde reoxygenatie groter zijn dan in normale harten. Deze gebieden hebben een omvang die vergelijkbaar is met de omvang die ontstaat na embolisatie van arteriën en arteriolen in normale harten. De conclusie is dan ook dat in hypertrofe harten er een verstoring optreedt van de microcirculatie op arterieel en arteriolair niveau.

Hoofdstuk 4. Perfusie van normale harten met zuur medium (pH 7.0) induceert ischemische gebieden met eenzelfde omvang als die in hypertrofe harten tijdens reperfusie. Hieruit kan geconcludeerd worden dat in normale harten acidose een verstoring oplevert

van de microcirculatie op arterieel en arteriolair niveau. Deze verstoring in normale harten, kan worden tegengegaan door SOD (superoxide dismutase, een “scavenger” van zuurstofradicalen) of fluorocarbonen (welke de zuurstoftransportcapaciteit van perfusaat verhogen) aan het perfusiemedium toe te voegen. Eerder onderzoek liet zien dat vetzuren bescherming bieden tegen acidose-geïnduceerde myocardiale flow reductie.

Bekend is ook dat Langendorff harten op de grens van aeroob metabolisme balanceren, gezien de continue productie van lactaat in het effluent. Deze overwegingen leidden tot hypothese dat het ontstaan van ischemische gebieden in hypertrofe Langendorff harten het gevolg is van lokale myocardiale hypoperfusie op arterieel/arteriolair niveau waarbij de hypoperfusie wordt versterkt door lokale acidose en vorming van zuurstof radicalen. Deze hypothese wordt ondersteund door de bevinding dat SOD, fluorocarbonen en vetzuren toegevoegd aan het perfusaat, een afname van de ischemische gebieden in hypertrofe Langendorff harten laat zien. Verklaringen hiervoor zijn dat SOD zuurstofradicalen wegvangt wat vasoconstrictie tegengaait, dat fluorocarbonen ischemie tegengaan door toename van zuurstoftransport en dat vrije vetzuren lokale acidose tegengaan door remming van glycolyse en zo ook melkzuurvorming.

Hoofdstuk 5. Een normale cardiale functie veronderstelt koppeling van mitochondriaal ATP verbruik (“cardiale arbeid”) aan mitochondriale ATP synthese. Er zijn meerdere regulatiepunten in deze complexe metabole keten bijvoorbeeld de [cytosolische adenine nucleotiden], intracellulaire $[Ca^{2+}]$ of de mitochondriale NADH/NAD⁺ redox balans.

De mitochondriale NADH/NAD⁺ redox balans wordt zeer sterk beïnvloed door de myocardiale oxygenatie maar ook andere factoren hebben een significante invloed: metabole substraten voor de citroenzuurcyclus of de glycolyse, de mate van cardiale arbeid (sterk gerelateerd aan de zuurstofconsumptie) en “photobleaching” (UV geïnduceerde daling van het fluorescentiesignaal zonder dat [NADH] veranderd). Om de relatie tussen cardiale arbeid en de mitochondriale NADH/NAD⁺ redox balans te bepalen, werden de veranderingen in de myocardiale NADH fluorescentie gemeten tijdens veranderingen in arbeid en zuurstofconsumptie. Factoren die de NADH fluorescentie beïnvloedden, werden ondervangen: pyruvaat werd gebruikt als substraat voor mitochondriaal metabolisme om substraat-afhankelijkheid van de citroenzuurcyclus tegen te gaan, photobleaching werd voorkomen door intermitterend met UV-licht te belichten en ischemie werd voorkomen door vasodilatoren toe te voegen aan het perfusaat. Tijdens een toename van arbeid nam de mitochondriale NADH/NAD⁺ redox balans duidelijk af. Deze afname was veel sneller met pyruvaat als substraat dan met glucose als substraat, mogelijk doordat pyruvaat resulteert in een toegenomen mitochondriale oxidatieve fosforylering capaciteit. Waarschijnlijk is het zo dat eerdere onderzoeken die een toename van de mitochondriale NADH/NAD⁺ redox balans lieten zien, verstoord werden door het ontstaan van ischemie tijdens hoge cardiale arbeid (en dus ischemie-geïnduceerde [NADH] stijging).

Hoofdstukken 6 en 7. Door parenchym te belichten met UV-licht zal NADH (en niet NAD⁺) fluoresceren met blauw licht. In myocard zal dit blauwe licht vooral afkomstig zijn van mitochondriaal NADH en is dus direct gerelateerd aan de mitochondriale redox balans.

De halfwaardetijd van de uitdoving van de fosforescentie van Pd-porphyrines zijn zuurstofconcentratie afhankelijk. Door Pd-porphyrine te koppelen aan albumine in de bloedbaan, is de halfwaardetijd van de fosforescentie direct gerelateerd aan microcirculatoire intravasculaire zuurstofconcentratie. Ook is het mogelijk om de verdeling

van zuurstof in de microcirculatie continu in beeld te brengen door continue registratie van fosforescentiebeelden. Door simultaan NADH-videofluorimetrie beelden te registreren is het mogelijk de microcirculatoire zuurstofspanning simultaan en continu te correleren aan de mitochondriale zuurstofspanning. Gebruikmakend van deze technieken konden wij, voor het eerst, direct intracellulaire hypoxie correleren aan intravasculaire hypoxie op cellulair niveau, in intacte organen in vitro en in vivo.

Hoofdstuk 8. Sepsis leidt tot ingrijpende veranderingen in de (micro)circulatie maar heeft ook een negatieve invloed op orgaanfunctie en meer specifiek mitochondriale functie. In hoofdstuk 3, 4 en 5 werd aangetoond dat veranderingen in de microcirculatie geëvalueerd konden worden met myocardiale NADH fluorescentie. Hypertrofe harten vertoonden grotere, relatief langdurig persisterend, ischemische gebieden tijdens reperfusie dan normale harten. In septische Langendorff harten, in de vroege fase van shock, vonden wij echter geen significant verschil ten opzichte van controle harten. In septische harten werden wel grotere gebieden gezien als in controle harten doch statistische analyse liet geen significant verschil zien. Daarom concludeerden wij dat het hier gebruikte model van sepsis geen invloed had op de verdeling van zuurstof via de microcirculatie terwijl de mitochondriale functie wel negatief werd beïnvloed: met behulp van NADH-videofluorimetrie werd waargenomen dat de mitochondriale zuurstofconsumptie duidelijk verslechterd was ten opzichte van normale harten. Eerder onderzoek had al aangetoond dat sepsis mitochondriale functie verslechterd doch deze onderzoeken werden vrijwel altijd verricht gebruikmakend van geïsoleerde mitochondria. Wij konden dit effect waarnemen in intacte, geïsoleerd geperfundeerde rattenharten.

Hoofdstuk 9. Dit overzichtsartikel beschrijft recente klinische en experimentele onderzoeken waarin de nadruk wordt gelegd op het belang van herstel van de microcirculatie in de behandeling van cardiogene shock. Therapeutische strategieën en interventies zijn nog steeds vooral gericht op herstel van macrocirculatoire parameters. Het wordt langzamerhand duidelijk dat de behandeling van (cardiogene) shock zich meer moet richten op herstel van microcirculatoire parameters. Zo werd aangetoond dat persisterende microcirculatoire afwijkingen na behandeling van shock gecorreleerd zijn aan een slechtere prognose. Hypothermie toegepast in de behandeling van shock verbetert de prognose door verbetering van de microcirculatie. Ook kon bij ernstig hartfalen de prognose verbeterd worden door de microcirculatie te ondersteunen via farmacologische interventies. Verder is aangetoond dat effecten van shock op microcirculatoire netwerken op verschillende anatomische locaties in het lichaam gecorreleerd zijn; hierdoor is het mogelijk de sublinguale microcirculatie – eenvoudig toegankelijk – te gebruiken voor het evalueren (middels een “handheld video microscope” welke de microcirculatie filmt) van therapeutische interventies tijdens de behandeling van shock. Op dit moment wordt deze techniek geëvalueerd in een multicenter internationaal onderzoek. Samenvattend kunnen we stellen dat vooruitgang in de behandeling van shock slechts mogelijk zal zijn indien deze er ook op gericht is de microcirculatie te verbeteren.

CONCLUSIES

Dit proefschrift beschrijft dat de anatomie van de microcirculatie van invloed is op het distributiepatroon van zuurstof. Hiervoor is vooral gebruik gemaakt van NADH- en Pd-porphyrine-video-/fosforimetrie. Dit distributiepatroon brengt in normale Langendorff-geperfundeerde harten de zogenaamde “weak microcirculatory units” aan het licht, die ook zichtbaar worden tijdens reperfusie na een episode van hypoxie of ischemie en tijdens periodes van toegenomen zuurstofconsumptie. Identieke distributiepatronen worden gezien na embolisatie van capillairen in normale harten. In hypertrofe Langendorff harten zijn de “weak microcirculatory units” groter, duidend op een verstoring van de microcirculatie op arteriëel/arterieel niveau.

Cardiale arbeid veroorzaakt een daling van de mitochondriale [NADH]. Dit staat haaks op de veronderstelling dat mitochondriale respiratie toeneemt door een toename van [NADH]. Deze veronderstelling zou verklaard kunnen worden doordat toename van arbeid kan leiden tot lokale ischemie (vooral in Langendorff geperfundeerde harten die balanceren op de grens van aeroob metabolisme) en dus een ischemie-geïnduceerde stijging van de [NADH], indien men er niet voor zorgt dat tijdens toename van arbeid ook zuurstof transport toeneemt.

NADH- en Pd-porphyrine-video-/fosforimetrie kunnen gebruikt worden voor evaluatie van de mitochondriale redox balans en de microcirculatorische zuurstofspanning. Wel is meer onderzoek nodig om te compenseren voor storende factoren bij deze metingen.

In de vroege fase van septische shock is al een negatief effect op de mitochondriale functie zichtbaar, nog voordat de microcirculatie negatief beïnvloed wordt. Dit is aangetoond in intacte organen in plaats van in geïsoleerde mitochondria, met behulp van NADH-videofluorimetrie. Verdere verbetering in de behandeling van (en in het begrip van de pathofysiologie van -) sepsis is te verwachten door een beter begrip van de fysiologie en pathofysiologie van de microcirculatie.

REFERENCES

- Agarwal B, Dash RK, Stowe DF et al. (2014)
Isoflurane modulates cardiac mitochondrial bioenergetics by selectively attenuating respiratory complexes
Biochimica et Biophysica Acta; 1837: 354-365
- Allen GA Laine SJ (1991)
Left ventricular myocardial edema. Lymph flow, interstitial fibrosis, and cardiac function
Circ Res; 68: 1713-1721
- Amende LM, Blanchette-Mackie JJ, Chernick SS et al. (1985)
Effect of pH on visualization of fatty acids as myelin figures in mouse adipose tissue by freeze-fracture electron microscopy
Biochim Biophys Acta; 837: 94-102
- Anderson PG, Bishop SP, Digerness SB (1987)
Transmural progression of morphologic changes during ischemic contracture and reperfusion in the normal and hypertrophied rat heart
Am J Pathol; 129(1): 152-167
- Ashruf JF, Ince C, Bruining HA (1993)
Increase of heart beat rate causes decrease of mitochondrial $[NADH]/[NAD^+]$
Pfluegers Arch; 422: R2
- Ashruf JF, Avontuur JAM, Van Bavel E et al. (1990)
Local ischemia created by microspheres in the rat heart induces heterogeneous epicardial NADH fluorescence
Pfluegers Arch; 416: S2
- Ashruf JF, Coremans JM, Bruining HA et al. (1996)
Mitochondrial NADH in the Langendorff rat heart decreases in response to increases in work: increase of cardiac work is associated with decrease of mitochondrial NADH
Adv Exp Med Biol; 388: 275-282
- Ashruf JF, Coremans JMCC, Bruining HA et al. (1995)
Increase of cardiac work is associated with decrease of mitochondrial NADH
Am J Physiol; 269: H856-H862
- Ashruf JF, Ince C, Bruining HA (1999)
Regional ischemia in hypertrophic Langendorff-perfused rat hearts
Am J Physiol; 277: H1532-H1539
- Ashruf JF, Ince C, Bruining HA (2013)
New insights into the pathophysiology of cardiogenic shock: the role of the microcirculation
Curr Opin Crit Care; 19(5): 381-386

- Ashruf JF, Ince C, Bruining HA et al. (1994)
Ischemic areas in hypertrophic Langendorff rat hearts visualized by NADH-videofluorimetry
Adv Exp Med Biol; 345: 259-262
- Avontuur JAM, Bruining HA, Ince C (1996)
Inhibition of nitric oxide synthesis causes myocardial ischemia in endotoxemic rats
Circ Res; 76: 418-425
- Balaban RS (1990)
Regulation of oxidative phosphorylation in the mammalian cell
Am J Physiol; 258: C377-C389
- Balaban RS, Heineman FW (1989)
Interaction of oxidative phosphorylation and work in the heart, in vivo
News Physiol Sci; 4: 215-218
- Balaban RS, Kantor HL, Katz LA et al. (1986)
Relation between work and phosphate metabolites in the in vivo paced mammalian heart
Science; 232: 1121-1123
- Barlow CH, Chance B (1976)
Ischemic areas in perfused rat hearts: measurement by NADH fluorescence photography
Science; 193: 909-910
- Barlow CH, Harden WR, Harken AH et al. (1979)
Fluorescence mapping of mitochondrial redox changes in heart and brain
Crit Care Med; 7: 402-406
- Barlow CH, Harken AH, Chance B (1977)
Evaluation of cardiac ischemia by NADH fluorescence photography
Ann Surg 1977; 186: 737-740
- Bassingthwaight JB, Yipintsoi T, Harvey RB (1974)
Microvasculature of the dog left ventricular myocardium
Microvasc Res; 7: 229-249
- Bezemer R, Bartels SA, Bakker J et al. (2012)
Clinical review: clinical imaging of the sublingual microcirculation in the critically ill - where do we stand?
Crit Care; 16: 224-232
- Björnsson OG, Kobayashi K, Williamson JR (1987)
Inducers of adenylate cyclase reverse the effect of leukotriene D4 in isolated working guinea pig heart
Am J Physiol; 252: H1235-H1242
- Boerma EC, van der Voort PH, Spronk PE et al. (2007)
Relationship between sublingual and intestinal microcirculatory perfusion in patients with abdominal sepsis
Crit Care Med; 35: 1055-1060

- Bonanno FG (2011)
 Physiopathology of shock
J Emerg Trauma Shock; 4: 222-232
- Brealy D, Brand M, Hargraves I et al. (2002)
 Association between mitochondrial dysfunction and severity and outcome of septic shock
Lancet; 360: 219-223
- Bruining HA, Pierik GJ, Ince C et al. (1992)
 Optical spectroscopic imaging for non-invasive evaluation of tissue oxygenation
Chirurgie; 118(5): 317-322; discussion 323
- Bussemaker JB, Lindeman J (1972)
 Comparison of methods to determine viability of small intestine
Ann Surg; 176: 97-101
- Carré JE, Singer M (2008)
 Cellular energetic metabolism in sepsis: the need for a systems approach
Biochim Biophys Acta; 1777 (7-8): 763-771
- Chance B (1976)
 Pyridine nucleotide as an indicator of the oxygen requirements for energy-linked functions of mitochondria
Circ Res; 38 (Suppl I): 31-38
- Chance B, Cohen P, Jöbsis F et al. (1962)
 Intracellular oxidation-reduction states in vivo
Science; 137: 499-508
- Chance B, Cohen P, Jobsis F et al. (1962)
 Localized fluorimetry of oxidation-reduction states of intracellular pyridine nucleotide in brain and kidney cortex of the anesthetized rat
Science; 136: 325
- Chance B, Williams CM (1956)
 The respiratory chain and oxidative phosphorylation
Adv Enzymol; 17: 65-134
- Chance B, Williamson JR, Jamieson D et al. (1965)
 Properties and kinetics of reduced pyridine nucleotide fluorescence of the isolated and in vivo rat heart
Biochem Z; 341: 357-377
- Chapman JB (1972)
 Fluorimetric studies of oxidative metabolism in isolated papillary muscle of the rabbit
J Gen Physiol. 59: 135-154
- Chemnitz JM, Burger W, Bing RJ. (1985)
 Crystalloid and perfluorochemical perfusates in an isolated working rabbit heart preparation
Am J Physiol; 249: H285-H292

- Cimini CM, Weiss HR (1990)
Isoproterenol and myocardial O₂ supply/consumption in hypertension-induced myocardial hypertrophy
Am J Physiol; 259: H346- H351
- Cistola DP, Hamilton JA, Jackson D et al. (1988)
Ionization and phase behavior of fatty acids in water: application of the Gibbs phase rule
Biochemistry; 27: 1881-1888
- Coremans JMCC, Ince C, Bruining HA (1997)
(Semi-)quantitative analysis of reduced nicotinamide adenine dinucleotide fluorescence images of blood-perfused rat heart
Biophys J 72(4): 1849-1860
- Coremans JMCC, Ince C, Bruining HA (1992)
NADH-fluorimetry and diffuse reflectance spectroscopy on rat heart. In: Medical Optical Tomography: Functional Imaging and Monitoring
Washington DC: SPIE; vol. IS11, p. 589-617
- Coremans JMCC, van Aken M, Naus DCWH et al. (2000)
Pretransplantation assessment of renal viability with NADH-fluorimetry
Kidney International; 57: 671-683
- Daut J, Elzinga G.(1989)
Substrate dependence of energy metabolism in isolated guinea-pig cardiac muscle: a microcalorimetric study
J Physiol Lond; 413: 379-397
- De Backer D, Creteur J, Dubois M-J et al. (2004)
Microvascular alterations in patients with acute severe heart failure and cardiogenic shock
Am Heart J; 147: 91-99
- Dellsperger KC, Marcus ML (1990)
Effects of left ventricular hypertrophy on the coronary circulation
Am J Cardiol; 65: 1504-1510
- Den Uil CA, Caliskan K, Lagrand WK et al. (2009)
Dose-dependent benefit of nitroglycerin on microcirculation of patients with severe heart failure
Intensive Care Med; 35: 1893-1899
- Den Uil CA, Klijn E, Lagrand WK et al. (2008)
The microcirculation in health and critical disease
Prog Cardiovasc Dis; 51: 161 -170
- Den Uil CA, Lagrand WK, Spronk PE et al. (2009)
Low-dose nitroglycerin improves microcirculation in hospitalized patients with acute heart failure
Eur J Heart Fail; 11: 386-390
- Den Uil CA, Lagrand WK, ValkSDA et al. (2009)
Management of cardiogenic shock: focus on tissue perfusion
Curr Probl Cardiol; 34: 330-349

- Den Uil CA, Lagrand WK, van der Ent M et al. (2009)
The effects of intra-aortic balloon pump support on macrocirculation and tissue microcirculation in patients with cardiogenic shock
Cardiology; 114: 42-46
- Den Uil CA, Lagrand WK, van der Ent M et al.(2010)
Impaired microcirculation predicts poor outcome of patients with acute myocardial infarction complicated by cardiogenic shock
Eur Heart J; 31: 3032-3039
- Den Uil CA, Maat AP, Lagrand WK et al. (2009)
Mechanical circulatory support devices improve tissue perfusion in patients with end-stage heart failure or cardiogenic shock
J Heart Lung Transplant; 28: 901-911
- Doeller JE, Wittenberg BA (1991)
Myoglobin function and energy metabolism of isolated cardiac myocytes: effect of sodium nitrate
Am J Physiol; 261: H53-H62
- Donadello K, Favory R, Salgado-Ribeiro D et al. (2011)
Sublingual and muscular microcirculatory alterations after cardiac arrest: a pilot study
Resuscitation; 82: 690-695
- Donati A, Tibboel D, Ince C (2013)
Towards integrative physiological monitoring of the critically ill: from cardiovascular to microcirculatory and cellular function monitoring at the bedside
Crit Care; 17: s5-s11
- Duboc D, Abastado P, Muffat-Joly M et al. (1990)
Evidence of mitochondrial impairment during cardiac allograft rejection
Transplantation; 50(5): 751-755
- Dyess DC, Bruner BW, Donnel CA et al. (1991)
Intraoperative evaluation of intestinal ischemia: A comparison of methods
South Med J; 84: 966-969
- Eerbeek O, Milstein DMJ, Ince C (2004)
Microcirculatory dysfunction in Langendorff endotoxemic rat hearts
Shock; 21: 81 Suppl.1, Abstract: 323
- Ehrly AM, Fleckenstein W, Hauss J et al.
Clinical Oxygen Pressure Measurements II, Tissue oxygen pressure and transcutaneous oxygen pressure
Blackwell Ueberreuter Wissenschaft, Berlin
- Einzig S, Leonard JJ, Tripp MR et al. (1981)
Changes in regional myocardial blood flow and variable development of hypertrophy after aortic banding in puppies
Cardiovasc Res; 15:711-723

- Elbers PWG, Prins WB, Plokker HWM et al. (2012)
Electrical cardioversion for atrial fibrillation improves microvascular flow independent of blood pressure changes
J Cardiothorac Vasc Anesth; 26: 799-803
- Elbers PWG, Wijbenga J, Solinger F et al. (2011)
Direct observation of the human microcirculation during cardiopulmonary bypass: effects of pulsatile perfusion
J Cardiothorac Vasc Anesth; 25: 250-255
- Eng J, Lynch RM, Balaban RS (1989)
Nicotinamide adenine dinucleotide fluorescence, spectroscopy and imaging of isolated cardiac myocytes
Biophys J; 55: 621-630
- Epand RM, Lester DS (1990)
The role of membrane biophysical properties in the regulation of protein kinase C activity
TIPS; 11: 317-321
- Erol-Yilmaz A, Atasever B, Mathura K et al. (2007)
Cardiac resynchronization improves microcirculation
J Card Fail; 13: 95-99
- Ferrara JJ, Dyess DL, Lasecki M (1988)
Surface oximetry: a new method to evaluate intestinal perfusion
Ann Surg; 54: 10-14
- Ferrari M, Jung C, Lauten A et al. (2011)
Evaluation of microcirculatory disorders in shock patients
Dtsch Med Wochenschr; 136: 1009-1013
- Fries M, Weil MH, Chang YT et al. (2006)
Microcirculation during cardiac arrest and resuscitation
Crit Care Med; 34: S454-S457
- Geyer RP (1975)
“Bloodless” rats through the use of artificial blood substitutes
Federation Proc; 34: 1499-1505
- Gregg DE (1963)
Effect of coronary perfusion pressure or coronary flow on oxygen usage of the myocardium
Circ Res; 13: 497-500
- Hak JB, van Beek JHGM, Eijgelshoven MHJ et al. (1993)
Mitochondrial dehydrogenase activity affects adaptation of cardiac oxygen consumption to demand
Am J Physiol; 264: H448-H453
- Harrison DG, Barnes DH, Marcus ML (1985)
The effect of cardiac hypertrophy on the coronary circulation
Circulation; 71: 1135-1145

- Harrois A, Dupic L, Duranteau J (2011)
Targeting the microcirculation in resuscitation of acutely unwell patients
Curr Opin Crit Care; 17: 303-307
- He X, Su F, Taccone FS et al. (2012)
Cardiovascular and microvascular responses to mild hypothermia in an ovine model
Resuscitation; 83: 760-766
- Heineman, F. W., and R. S. Balaban (1993)
Effects of afterload and heart rate on NAD(P)H redox state in the isolated rabbit heart
Am J Physiol 264: H433-H440
- Hobbbahn J, Gonzen P, Hansen E et al. (1990)
Myocardial surface oxygen tension is an indicator of transmural tissue oxygenation of the in vivo beating pig heart.
In: Clinical Oxygen Pressure Measurements II; 315-326. Editors: Ehrly AM et al. Blackwell Ueberreuter Wissenschaft, Berlin
- Hogan CJ, Ward KR, Kontos MC et al. (2012)
Peripheral tissue oxygenation improves during ED treatment of acute heart failure
Am J Emerg Med; 30: 196-202
- Hu C-L, Liu RL, Liao X-X et al. (2013)
Early enhanced external counter pulsation improves neurological recovery after the return of spontaneous circulation in a mongrel dog cardiac arrest model
Crit Care Med; 41: e62-e73
- Huet O, Dupic L, Harrois A, Duranteau J (1995)
Oxidative stress and endothelial dysfunction during sepsis
Front Biosci (Landmark Ed); 16: 1986-1995
- Hulsmann W C, Ashruf JF, Bruining HA et al. (1993)
Imminent ischemia in normal and hypertrophic Langendorff rat hearts; effects of fatty acids and superoxide dismutase monitored by NADH surface fluorescence
Biochim Biophys Acta 1181: 273-278
- Hulsmann WC (1976)
Coronary vasodilation by fatty acids
Basic Res Cardiol; 71: 179-191
- Hulsmann WC, de Wit LEA (1990)
Acidosis, cardiac stunning and its prevention by oxygen
Cell Biol Int Rep; 14: 311-315
- Hulsmann WC, De Wit LEA, Schneydenberg C et al. (1990)
Loss of cardiac contractility and severe morphologic changes by acutely lowering the pH of the perfusion medium: protection by fatty acids
Biochim Biophys Acta; 1033: 214-218

- Hulsmann WC, De Wit LEA, Stam H et al. (1990)
Hormonal control of cardiac lipolysis by glyco(geno)lysis
Biochim Biophys Acta; 1055: 189-192
- Hulsmann WC, Dubelaar ML (1987)
Early damage of vascular endothelium during cardiac ischemia
Cardiovasc Res; 21: 674-677
- Hulsmann WC, Dubelaar ML (1992)
Carnitine requirement of vascular endothelial and smooth muscle cells in imminent ischemia
Mol Cell Biochemistry; 116: 125-129
- Ince C, Sinaasappel M (1999)
Microcirculatory oxygenation and shunting in sepsis and shock
Crit Care Medicine; 27: 1369-1377
- Ince C, Ashruf JF, Avontuur JAM et al. (1993)
Heterogeneity of the hypoxic state in rat heart is determined at capillary level
Am J Physiol; 264: H294-H301
- Ince C, Ashruf JF, Sanderse EA et al. (1992)
In vivo NADH- and Pd-porphyrin- videofluori-/phosphorimetry
Adv Exp Med Biol; 317: 267-275
- Ince C, Bruining HA (1991)
Optical spectroscopy for the measurement of tissue hypoxia
In: Update in Intensive Care and Emergency Medicine: Update 1991 (Vincent JL, ed.); 161-171, Springer-Verlag, New York
- Ince C, Coremans JMCC, Bruining HA (1992)
In vivo NADH fluorescence
Adv Exp Med Biol; 317: 277-296
- Ince C, Sinaasappel M (1999)
Microcirculatory oxygenation and shunting in sepsis and shock
Crit Care Med 27(7): 1369-1377
- Ince C, van der Sluijs JP, Sinaasappel M et al. (1994)
Intestinal ischemia during hypoxia and experimental sepsis as observed by NADH-videofluorimetry and quenching of Pd-porphyrin phosphorescence
Adv Exp Med Biol 36: 105-110
- Ince C, Vink H, Wieringa PA et al. (1990)
Heterogeneous NADH fluorescence during post-anoxic reactive hyperemia in saline perfused rat heart
Adv Exp Med Biol; 277: 477-482
- Jöbsis FF (1977)
Noninvasive, infrared monitoring of cerebral and myocardial oxygen sufficiency and circulatory parameters
Science; 198: 1264-1267

- Jung C, Fritzenwanger M, Lauten A et al. (2010)
Evaluation of microcirculation in cardiogenic shock: current diagnostic and therapeutic aspects
Dtsch Med Wochenschr; 135: 80-83
- Jung C, Lauten A, Ferrari M (2010)
Microcirculation in cardiogenic shock: from scientific bystander to therapy target
Crit Care; 14: 193-195
- Kang Y, Mallet HRT, Bungler R. (1992)
Coronary autoregulation and purine release in normoxic heart at various cytoplasmic phosphorylation potentials: disparate effects of adenosine
Pfluegers Arch; 421: 188-199
- Katz AM (1990).
Cardiomyopathy of overload. A major determinant of prognosis in congestive heart failure.
N Engl J Med; 323: 100-110
- Katz LA, Koretsky AP, Balaban RS (1987)
Respiratory control in the glucose perfused heart. A ^{31}P NMR and NADH fluorescence study
FEBS Lett; 221: 270-276
- Kirber MT, Ordway RW, Clapp LH et al. (1992)
Both membrane stretch and fatty acids directly activate large conductance $\text{Ca}(2+)\text{-activated K}^+$ channels in vascular smooth muscle cells
FEBS Lett; 297: 24-28
- Kobayashi K, Neely JR. (1979)
Control of maximum rates of glycolysis in rat cardiac muscle
Circ Res; 44: 166-175
- Koning NJ, Vonk AB, van Barneveld LJ et al. (2012)
Pulsatile flow during cardiopulmonary bypass preserves postoperative microcirculatory perfusion irrespective of systemic hemodynamics
J Appl Physiol; 112: 1727-1734
- Koretsky AP, Balaban RS. (1987)
Changes in pyridine nucleotide levels alter oxygen consumption and extramitochondrial phosphates in isolated mitochondria: a ^{31}P NMR and fluorescence study
Biochim Biophys Acta; 893: 398-408
- Kwan CY, Daniel EE (1989)
In: Calcium in essential hypertension (Aoli K, Frohlich ED, eds.), pp 201-230, Academic Press Japan
- Lam K, Sjaauw KD, Henriques JPS et al. (2009)
Improved microcirculation in patients with an acute ST-elevation myocardial infarction treated with the Impella LP2.5 percutaneous left ventricular assist device
Clin Res Cardiol; 98: 311-318

- Lange K, Boyd IJ (1942)
The use of fluorescein to determine the adequacy of the circulation
Med Clin North Am; 26: 943-952
- Lauten A, Ferrari M, Goebel B et al. (2011)
Microvascular tissue perfusion is impaired in acutely decompensated heart failure and improves following standard treatment
Eur J Heart Fail; 13: 711-717
- Levy RJ (2007)
Mitochondrial dysfunction, bioenergetic impairment, and metabolic down-regulation in sepsis
Shock; 28(1): 24-28
- Levy RJ, Deutschman CS (2007)
Cytochrome c oxidase dysfunction in sepsis
Crit Care Med; 35(9 Suppl): S468 – S475
- Liu R, Xin Li X, Hu C et al. (2012)
The changes of brain water diffusion and blood flow on diffusion-weighted and perfusion-weighted imaging in a canine model of cardiac arrest
Resuscitation; 83: 645-651
- Locher R, Weisser B, Mengden L et al. (1992)
Lysolecithin actions on vascular smooth muscle cells
Biochim Biophys Res Commun; 183: 156-162
- Lush CW, Kvietys PR (2000)
Microvascular dysfunction in sepsis
Microcirculation; 7(2): 83-101
- Malik AB, Shapiro JM, Yanics J et al. (1974)
A simplified method for producing rapid ventricular hypertrophy in rats
Cardiovasc Res; 8: 801-805
- Marcus ML, Koyanagi S, Harrison DG et al. (1983)
Abnormalities in the coronary circulation that occur as a consequence of cardiac hypertrophy
Am J Med; 75, Suppl I: I62-I66
- Marcus ML, Mueller TM, Gascho JA et al. (1979)
Effects of cardiac hypertrophy secondary to hypertension on the coronary circulation
Am J Cardiol; 44: 1023-1028
- Mik EG, Ince C, Eerbeek O et al. (2009)
Mitochondrial oxygen tension within the heart
J Mol Cell Cardiol 46(6): 943-51
- Mik EG, Johannes T, Freis M (2009)
Clinical microvascular monitoring: a bright future without a future?
Crit Care Med; 37: 2980-2981

- Mik EG, Johannes T, Zuurbier C et al. (2008)
In vivo mitochondrial oxygen tension measured by a delayed fluorescence lifetime technique
Biophys J:95(8): 3977-3990
- Mik EG, Stap J, Sinaasappel M et al. (2006)
Mitochondrial PO₂ measured by delayed fluorescence of endogenous protoporphyrin IX
Nature Methods. 3(11): 939-945
- Millikan GA (1943)
Oximeter, an instalment for measuring continuously the oxygen saturation of arterial blood in man
Rev Sei Instrum; 13: 434
- Moncada S, Palmer RMJ, Higgs EA (1991)
Nitric oxide: physiology, pathophysiology, and pharmacology
Pharmacol Rev; 43: 109-142
- Nakanishi K, Fukuda S, Shimada K et al. (2012)
Impaired coronary flow reserve as a marker of microvascular dysfunction to predict long-term cardiovascular outcomes, acute coronary syndrome and the development of heart failure
Circ J; 76: 1958-1964
- Nishiki K, Erecinska M, Wilson DF (1978)
Energy relationships between cytosolic metabolism and mitochondrial respiration in rat heart
Am J Physiol; 234: C73-C81
- Nuutinen EM (1984)
Subcellular origin of the surface fluorescence of reduced nicotinamide nucleotide in the isolated perfused rat heart
Basic Res Cardiol; 79: 49-58
- Pierik EGJM, Ince C, Avontuur JAM et al. (1991)
The application of NADH fluorescence to identify noninvasively tissue hypoxia in vivo
Eur Surg Res 1991; 23: 12-13
- Ploem JS (1990)
In: Standardization in Immunofluorescence, edited by E. J. Holborow. Oxford, UK: Blackwell, 1970, p 137-153
- Poole DC, Hirai DM, Copp SW, Musch TI (2012)
Muscle oxygen transport and utilization in heart failure: implications for exercise (in)tolerance
Am J Physiol; 302: H1050-H1063
- Quitter F, Figulla HR, Ferrari M et al. (2013)
Increased arginase levels in heart failure represent a therapeutic target to rescue microvascular perfusion
Clin Hemorheol Microcirc; 54: 75-85
- Rady MY, Verheijde JL (2013)
No-touch time in donors after cardiac death (nonheart-beating organ donation)
Curr Opin Organ Transplant; 18: 140-147

- Rakusan K, Flanagan MF, Geva T et al. (1992)
Morphometry of human coronary capillaries during normal growth and the effect of age in left ventricular pressure-overload hypertrophy
Circulation; 86: 38-46
- Rakusan K, Moravec J, Hatt PY (1980)
Regional capillary supply in the normal and hypertrophied rat heart
Microvasc Res; 20: 319-326
- Rakusan K, Wicker P (1990)
Morphometry of the small arteries and arterioles in the rat heart: effects of chronic hypertension and exercise
Cardiovasc Res; 24: 278-284
- Renault G, Raynal E, Sinet M et al. (1984)
In situ double-beam NADH laser fluorimetry: choice of a reference wavelength
Am J Physiol; 246: 491-499
- Roberts JT, Wearn JT (1941)
Quantitative changes in the capillary-muscle relationship in human hearts during normal growth and hypertrophy
Am J Heart; 21: 617-633
- Rumsey WL, Schlosser C, Nuutinen EM et al. (1990)
Cellular energetics and the oxygen dependence of respiration in cardiac myocytes isolated from adult rat
J Biol Chem; 265: 15392-15399
- Rumsey WL, Vanderkooi JM, Wilson DF (1988)
Imaging of phosphorescence: a novel method for measuring oxygen distribution in perfused tissue
Science; 241: 1649-1651
- Salgado DR, Favory R, Rocco JR et al. (2013)
Microcirculatory effects of angiotensin II inhibitors in patients with severe heart failure
Clin Hemorheol Microcirc; 54: 87-98
- Schaffer SW, Safer B, Ford C et al. (1978)
Respiratory acidosis and its reversibility in perfused rat heart: regulation of citric acid cycle activity
Am J Physiol; 234: H40-H51
- Scholz PM, Grover GJ, Mackenzie JW et al. (1990)
Regional oxygen supply and consumption balance in experimental left ventricular hypertrophy
Basic Res Cardiol; 85: 575-584
- Schorr CA, Zanotti S, Dellinger RP (2014)
Severe sepsis and septic shock: management and performance improvement
Virulence; 5(1): 190-199

- Seleye H, Bajusz E, Grasso S et al. (1960)
Simple techniques for the surgical occlusion of coronary vessels in the rat
Angiology; 11: 398-407
- Sharma AC (2007)
Sepsis-induced myocardial dysfunction
Shock; 28(3): 265-269
- Sherman H, Klausner S, Cook WA (1971)
Incident dark-field illumination: a new method for microcirculatory study
Angiology; 22: 295-303
- Steenbergen CG, Deleeuw C, Barlow B. et al. (1977)
Heterogeneity of the hypoxic state in perfused rat heart
Circ Res 4: 606-615
- Svistunenko DA, Davies N, Brealy D et al. (2006)
Mitochondrial dysfunction in patients with severe sepsis: An EPR interrogation of individual respiratory chain components
Biochimica et Biophysica Acta; 1757: 262 – 272
- Tamura M, Oshino MN, Chance B et al. (1978)
Optical measurements of intracellular oxygen concentration of rat heart in vitro
Arch Biochem Biophys; 191: 8-22
- Territo PR, French SA, Dunleavy MC et al. (2001)
Calcium activation of heart mitochondrial oxidative phosphorylation: rapid kinetics of mVO_2 , NADH, AND light scattering
J Biol Chem 276(4): 2586-2599
- Udenfriend S (1962)
In: *Fluorescence Assay in Biology and Medicine*. New York: Academic; vol. 1: 104
- van der Laan L, Coremans A, Ince C et al. (1998)
NADH-videofluorimetry to monitor the energy state of skeletal muscle in vivo
J Surg Res 74(2): 155-160
- Van Genderen ME, Lima A, Akkerhuis M et al. (2012)
Persistent peripheral and microcirculatory perfusion alterations after out-of-hospital cardiac arrest are associated with poor survival
Crit Care Med; 40: 2287-2294
- Vanasco V, Magnani ND, Cimolai MC et al. (2012)
Endotoxemia impairs heart mitochondrial function by decreasing electron transfer, ATP synthesis and ATP content without affecting membrane potential
J Bioenerg Biomembr; 44: 243–252

- Vandeplasse G, Borgers M (1990)
Ultrastructure and Ca²⁺ reallocation during ischemia, the Ca²⁺ paradox and metabolic acidosis
Cell Biol Int Rep; 14: 317-334
- Vanderkooi JM, Maniara G, Green TJ et al. (1987)
An optical method for measurement of dioxygen concentration based upon quenching of phosphorescence
J Biol Chem; 262: 5476-5482
- Vellinga NAR, Boerma EC, Koopmans M et al. (2012)
Study design of the microcirculatory shock occurrence in acutely ill patients (microSOAP): an international multicenter observational study of sublingual microcirculatory alterations in intensive care patients
Crit Care Res Pract: 2012: 121752
- Vetterlein F, Prange M, Lubrich D et al. (1995)
Capillary perfusion pattern and microvascular geometry in heterogeneous hypoxic areas of hypoperfused rat myocardium
Am J Physiol; 268: H2183-H2194
- Vincent JL, de Backer D (2012)
ICU nephrology: the implications of cardiovascular alterations in the acutely ill
Kidney Int; 81: 1060-1066
- Vincent JL, Ince C, Bakker J (2012)
Clinical review: circulatory shock - an update: a tribute to Professor Max Harry Weil
Crit Care; 16: 239-243
- Weng Y, Sun S, Park J et al. (2012)
Cannabinoid 1 (CB1) receptor mediates WIN55, 212-2 induced hypothermia and improved survival in a rat postcardiac arrest model
Resuscitation; 83: 1145-1151
- Wester T, Awan ZA, Kvernebo TS et al. (2014)
Skin microvascular morphology and hemodynamics during treatment with veno-arterial extra-corporeal membrane oxygenation
Clin Hemorheol Microcirc; 56(2): 119-131
- White RL, Wittenberg BA (1993)
NADH fluorescence of isolated ventricular myocytes: effects of pacing, myoglobin and oxygen supply
Biophys J; 65: 196-204
- White SK, Hausenloy DJ, Moon JC (2012)
Imaging the myocardial microcirculation postmyocardial infarction
Curr Heart Fail Rep; 9: 282-292
- Wieringa PA, Spaan JAE, Stassen HG et al. (1982)
Heterogeneous flow distribution in a three dimensional network simulation of the myocardial microcirculation: a hypothesis
Microcirculation; 2: 195-216

- Wieringa PA, Stassen HG, Laird JD et al. (1988)
Quantification of arteriolar density and embolization by microspheres in rat myocardium
Am J Physiol; 254: H636-H650
- Williamson JR, Davis KN, Medine-Ramirez G (1982)
Quantitative analysis of heterogeneous NADH fluorescence in perfused rat hearts during hypoxia and ischaemia
J Mol Cell Cardio; 14, Suppl 3: 29-35
- Williamson JR, Safer B, Rich T et al. (1976)
Effects of acidosis on myocardial contractility and metabolism
Acta Med Scand Suppl; 587: 95-112
- Wilson DE (1992)
Oxygen dependent quenching of phosphorescence: a perspective
Adv Exp Med Biol; 317: 195-201
- Wilson DF, Pastuszko A, DiGiacomo JE et al. (1991)
Effect of hyperventilation on the oxygenation of the brain cortex of newborn piglets
J Appl Physiol; 70: 2691-2696
- Wright C, Weiss HR, Kedem J et al. (1991)
Effect of dopamine on regional myocardial function and oxygen consumption in experimental left ventricular hypertrophy
Basic Res Cardiol; 86: 449-460
- Wright CB, Hobson RW (1975)
Prediction of intestinal viability using Doppler ultrasound techniques
Am J Surg; 129: 642-645
- Wyatt JS, Delpy DT, Cope M et al. (1986)
Quantification of cerebral oxygenation and hemodynamics in sick newborn infants by near infrared spectrophotometry
Lancet; 2: 1063-1066
- Xu J, Ma L, Sun S et al. (2013)
Fluid resuscitation guided by sublingual partial pressure of carbon dioxide during hemorrhagic shock in a porcine model
Shock; 39: 361-365
- Ye S, Weng Y, Sun S et al. (2012)
Comparison of the durations of mild therapeutic hypothermia on outcome after cardiopulmonary resuscitation in the rat
Circulation; 125: 123-129
- Yuruk K, Almac E, Bezemer R et al. (2011)
Blood transfusions recruit the microcirculation during cardiac surgery
Transfusion; 51: 961-967

Zanotti-Cavazzoni SL, Hollenberg SM (2009)

Cardiac dysfunction in severe sepsis and septic shock

Curr Opin Crit Care; 15(5): 392-397

Zuurbier CJ, van Iterson M, Ince C (1999)

Functional heterogeneity of oxygen supply-consumption ratio in the heart

Cardiovasc Res 44(3): 488-497

PORTFOLIO

Geboren 26-11-1967 te Paramaribo, Suriname

Scholing, opleidingen, werkzaamheden:

Atheneum B te Paramaribo en Delft, eindexamen augustus 1986, cum laude

Propedeuse geneeskunde, Erasmus Universiteit Rotterdam, juli 1987, cum laude

Wetenschappelijk onderzoek Erasmus Dierexperimenteel Centrum, afdeling Heelkunde, onder leiding van Prof. dr. H.A. Bruining en C. Ince, 1988 – 1993

Doctoraal geneeskunde, Erasmus Universiteit Rotterdam, september 1990, cum laude

Militaire dienst, januari 1992 – maart 1993

Basisarts examen, Erasmus Universiteit Rotterdam, mei 1995, cum laude

Opleiding tot algemeen chirurg, inschrijving MSRC januari 2002

Voorzitter medische staf Delfzicht ziekenhuis te Delfzijl in 2008 en 2009 (tijdens het fusietraject met het St. Lucas ziekenhuis te Winschoten)

Wetenschappelijk onderzoek Academisch Medisch Centrum Amsterdam, afdeling Translationele Fysiologie, onder leiding van Prof. dr. C. Ince, 2011 - heden

Laparoscopische en endoscopische chirurgie, meerdere opleidingen/cursussen o.a. LIMIS, April 2009

Geregistreerd nvco chirurg-oncoloog, februari 2014

Geregistreerd nvgc gastro-intestinaal chirurg, juni 2014

Werkzaam als chirurg (algemene chirurgie, laparoscopische/endoscopische chirurgie, oncologische chirurgie en gastro-intestinale chirurgie), mei 2002 tot heden, in de Ommelander Ziekenhuis Groep, locatie Delfzijl en Winschoten

PhD training:

Cursus proefdierkunde, mei 1995

Internationale conferenties/presentaties: ISOTT (Mainz, Duitsland, 1992), ISOTT (Istanbul, Turkije, 1994), en te Beaune, Frankrijk, 1993

LIST OF PUBLICATIONS

Ashruf JF, Avontuur JAM, van Bavel E et al. (1990)

Local ischemia created by microspheres in the rat heart induces heterogeneous epicardial NADH fluorescence

Pfluegers Arch. 416, S2

Ashruf JF, Ince C, Pierik EGJM et al. (1992)

Videofluorimetrie en fosforimetrie ter beoordeling van orgaanvitaliteit

Ned Tijdschr Geneeskd. 136, nr 12: 608

Ince C, Ashruf JF, Sanderse EA et al. (1992)

In vivo NADH- and Pd-porphyrin-videofluori-/phosphorimetry

Adv Exp Med Biol. 317: 267-275

Ashruf JF, Ince C, Bruining HA (1993)

Increase of heart rate causes decrease of mitochondrial $[NADH]/[NAD^+]$

Eur J Physiol. 424: R2

Ince C, Ashruf JF, Avontuur JAM et al. (1993)

Heterogeneity of the hypoxic state in rat heart is determined at capillary level

Am J Physiol. 264: H294-H301

Hulsmann WC, Ashruf JF, Bruining HA et al. (1993)

Imminent ischemia in normal and hypertrophic langendorff rat hearts; effects of fatty acids and superoxyde dismutase monitored by NADH surface fluorescence

Biochimica et Biophysica Acta 1181: 273-278

Ashruf JF, Ince C, Bruining HA et al. (1994)

Ischemic areas in hypertrophic langendorff rat hearts visualized by NADH-videofluorimetry

Adv Exp Med Biol. 345: 259-262

Ashruf JF, Coremans JMCC, Bruining HA et al. (1995)

Increase of cardiac work is associated with decrease of mitochondrial NADH

Am J Physiol. 269: H856-862

Ashruf JF, Coremans JMCC, Bruining HA et al. (1996)

Mitochondrial NADH in the Langendorff rat heart decreases in response to increases in work: increase of cardiac work is associated with decrease of mitochondrial NADH

Adv Exp Med Biol. 388: 275-282

Ashruf JF, Ince C, Bruining HA (1999)

Regional ischemia in hypertrophic langendorff-perfused rat hearts

Am J Physiol. 277: H1532-H1539

Ashruf JF, Ince C, Bruining HA (2013)

New insights into the pathophysiology of cardiogenic shock: the role of the microcirculation

Curr Opin Crit Care 19(5): 381-386

Ashruf JF, Ince C

Myocardial microcirculation and mitochondrial energetics in the isolated rat heart

Accepted for publication in Adv Exp Med Biol. 2015

Eykyn T, Mariotti E, Orton M, Eerbeek O, Ashruf JF et al.

Modelling non-linear kinetics of hyperpolarized [1-¹³C] pyruvate in the crystalloid perfused rat heart

Submitted to NMR in Biomedicine, 2015

Ashruf JF, Ergin B, Milstein DMJ et al.

Epicardial NADH fluorescence of the isolated perfused rat heart reveals mitochondrial dysfunction in early endotoxic shock

To be submitted

DANKWOORD

Dit proefschrift is tot stand gekomen door de bijdrage van velen (over een veel langere periode dan gebruikelijk). Ik wil een ieder hiervoor van harte bedanken. Een aantal mensen wil ik in het bijzonder noemen. Prof. dr. ir. C. Ince, beste Can, we kennen elkaar al heel lang. Wat mij bijzonder raakte was de hartverwarmende manier waarop je mij weer binnenhaalde in je huidige afdeling en huis, nadat ik na vele jaren van omzwervingen weer bij je aanklopte met de vraag of ik mijn onderzoek kon afronden. Dr. O. Eerbeek, beste Otto, ik ben je zeer erkentelijk voor het weer in orde maken van de “NADH-videofluorimeter” en het mij weer op weg helpen in de praktijk van laboratorium onderzoek. Dr. ir. C. Zuurbier, beste Coert, dank voor het mogen lenen van je perfect werkende laboratorium en Langendorff opstelling. Nog steeds zit het me niet lekker dat ik, na je verzoek heel voorzichtig om te gaan met een fragiele kwetsbare miniatuur warmtewisselaar, deze nog dezelfde dag onherstelbaar beschadigde. Dr. D.M. Milstein, beste Dan, bedankt voor je kritische beoordeling van mijn schrijfsels. Als de tekst goed loopt is het te danken aan jou en als het Engels hapert, zal ik er in de toekomst nog aan moeten schaven. Ik zal proberen het woord “the” in de toekomst zoveel mogelijk te vermijden. Heel veel dank ben ik verschuldigd aan Bulent Ergin. Beste Bulent, je was zo vriendelijk om weken achtereen tot in de kleine uurtjes, wanneer de zon weer bijna opkwam, het sepsis protocol uit te voeren. Het is verbazingwekkend hoe vaardig je bent met het cannuleren van aders en slagaders met een diameter van kleiner dan 1 mm, en ook nog zonder bril! Ook dank voor je bereidheid me terzijde te staan als paranimf. Dr. R. Bezemer, beste Rik, heel veel dank voor het weer aan de gang krijgen van de videocamera en de bijbehorende software. De leden van de commissie, prof. dr. T.W.L. Scheeren, dr. E.G. Mik, prof. dr. R.J.A. Wanders, prof. dr. ir. J.A.E. Spaan en prof. dr. R.J. de Winter, ik wil jullie van harte bedanken voor de moeite die jullie genomen hebben bij het beoordelen van dit proefschrift. Ramses, mijn broer, bedank ik voor zijn bereidwilligheid paranimf te zijn (dat jij er plezier in schept om - met een levensgroot simulatiestuur met 3D-bril op, en pet op met speciale sensoren, gezeten voor een enorme Samsung TV in je woonkamer - racesimulatiegames te spelen blijft onder ons, gezien je werkzaamheden als psychiater). Verder ben ik Jurgen Avontuur, Erik Sanderse, Annemiek Coremans, Robert Pierik en prof. dr. W.C. Hulsmann zeer erkentelijk voor de intensieve, vruchtbare en gezellige samenwerking in de vroege periode van dit onderzoek. Een speciaal woord van dank gaat uit naar prof. dr. H.A. Bruining, die mij niet alleen op het pad van wetenschappelijk onderzoek zette in 1989 maar die ook een groot aandeel heeft gehad in mijn vorming tot chirurg in de rol van opleider. Ik heb u ervaren als een man van weinig woorden, tomeloze energie en een hart van goud. Lieve pa, ik denk dat jij misschien nog de grootste aanjager geweest bent in de afronding van dit proefschrift. Ik mag hopen dat ik maar de helft van jou drive, energie en werklust heb, en ik zit geramd. Lieve Svati, dank voor al je steun en ook adviezen en tips vooral in de “vroege”periode. Ik heb de kunst ook een beetje afgekeken van jou toen je met jouw proefschrift bezig was. Talin, Raphael, Raimi: ook jullie moet ik bedanken voor het geduld en begrip die jullie opbrachten voor mijn inspanningen. Wel hoop ik dat jullie, Ratar, datgene waar je aan begint iets sneller afronden dan ik. Lieve Petra, ik heb veel gehad aan je hulp bij het nalezen en verbeteren. Nu het proefschrift af is, komt er weer meer tijd voor de rest. En mijn lieve moeder, dat is iets tussen haar en mij en dat zit goed.