Helium-induced cardioprotection: in sickness and in health, for better or for worse?
Oei, Gezina

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"If we knew what it was we were doing, it would not be called research; would it?"

— Albert Einstein

Helium-induced cardioprotection: In sickness and in health, for better or for worse?
Helium-induced cardioprotection: in sickness and in health, for better or for worse?

Gezina T.M.L. Oei
COLOFON

Helium-induced cardioprotection: in sickness and in health, for better or for worse?
PhD thesis, University of Amsterdam, the Netherlands.

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Helium-induced cardioprotection: in sickness and in health, for better or for worse?

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PROMOTIECOMMISSIE

Promotores: prof. dr. M.W. Hollmann  
prof. dr. B. Preckel

Co-promotor: dr. N.C. Hauck-Weber

Overige Leden: prof. dr. M.J. Schultz  
prof. dr. W.S. Schlack  
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dr. C. Boer

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CHAPTER

General introduction and outline of this thesis

Gezina Oei and Benedikt Preckel
GENERAL INTRODUCTION

According to the World Health Organization (WHO; www.who.org) an estimated 17.3 million people died in 2008 from cardiovascular disease - about 30% of all deaths – and this number will increase to an estimated 23.3 million people per year in 2030. Of these deaths, an estimated 7.3 million were attributed to coronary heart disease. Coronary heart disease is a disease of the blood vessels supplying the heart. The severity and extension of this disease is highly influenced by co-existing risk factors such as hypertension, obesity, hypercholesterolemia, hyperglycemia, insulin resistance, atherosclerosis and heart failure, but also by sex and age of the patient.

In coronary artery disease, vascular injury, lipid deposition, thrombus formation and inflammation eventually lead to a partial or total obstruction of a coronary artery, resulting in ischemia of the downstream myocardial tissue. As myocardial ischemia is reflected by an imbalance between oxygen supply and demand, reperfusion of threatened tissue is pivotal. Reperfusion is done by fibrinolytics or percutaneous coronary intervention (PCI), but restoration of blood flow to compromised tissue is also performed by coronary artery bypass graft surgery (CABG).

Time course of reperfusion and windows of protection

In general, the time to intervention (reperfusion) after ischemia is of vital importance to the amount of myocardium that can be salvaged. Already in 1977 it was shown that cellular damage after an ischemic episode develops according to a wave front pattern, and timely reperfusion is essential. However, while ischemia itself causes substantial tissue damage, reperfusion also contributes to the final size of the infarcted area, a phenomenon called: “reperfusion injury”. In an experimental study in mice undergoing regional ischemia it was shown that both duration of ischemia as well as reperfusion contributed to the final extent of infarct size. In this study it was demonstrated that extension of index ischemia resulted in larger infarct sizes; mice exposed to 120 minutes of reperfusion had an infarct size of 18% after 30 minutes of ischemia, which was increased to 69% after 60 minutes of ischemia. Simultaneously, an increase of reperfusion time after an equal index ischemia extended the infarcted area. To illustrate: after 30 minutes of ischemia, infarct size increased from 18% to 69% when reperfusion time was prolonged from 120 to 240 minutes. Similarly, an increase of reperfusion time from 120 to 240 minutes increased infarct size from 40% to 72% after 45 minutes of ischemia.

Taken together, the final extent of the infarct size thus consists out of ischemic cell damage and reperfusion injury, as shown in Figure 1. Nonetheless, the very existence
of reperfusion injury has been debated vigorously as some argued that reperfusion only hastened the initial injury after ischemia and others considered it as a contributor to de novo injury⁴,⁵.

Regardless of its definition, the amount of research conducted to attenuate or even eradicate ischemia/reperfusion injury is vast. In 1986, Murry and colleagues first presented the preconditioning phenomenon⁶. In dogs exposed to 40 minutes of coronary artery occlusion and 4 days of reperfusion it was shown that precedence of index ischemia by 4

Figure 1. In the upper graph, the extent of cell death is depicted on the y-axis and time is depicted on the x-axis. Underneath the graph an overview of the different conditioning protocols is shown for each category: ischemic and anesthetic conditioning. I-EPC= ischemic early preconditioning, I-LPC= ischemic late preconditioning, I-PostC= ischemic postconditioning, A= anesthetic.

Ischemic pre- and postconditioning

Regardless of its definition, the amount of research conducted to attenuate or even eradicate ischemia/reperfusion injury is vast. In 1986, Murry and colleagues first presented the preconditioning phenomenon⁶. In dogs exposed to 40 minutes of coronary artery occlusion and 4 days of reperfusion it was shown that precedence of index ischemia by 4
cycles of 5 min of coronary occlusion interspersed with 5 min of reperfusion significantly reduced infarct size from 29% in controls to 7% in treated animals without a difference in collateral blood flow. Moreover, they also showed that once the index ischemia was prolonged, 3 hours instead of 40 minutes, preconditioning could not exert protection anymore. Apparently, cardioprotective effects of preconditioning are abrogated or cannot be induced when the index ischemia is too long, i.e. the initial damage is too big. These findings emphasize the importance of timely reperfusion.

According to the original ‘conditioning’ experiments in animal behavior, preconditioning of the heart is based on the idea that a ‘memory’ for ischemic circumstances is created, thereby rendering increased tolerance to future prolonged ischemic episodes. Preconditioning-induced cardioprotection is therefore not to be confused with cardioprotective ‘treatments’. An example is the use of ACE-inhibitors in patients with hypertension and myocardial infarction; ACE-inhibitors have to be taken on a chronic, daily basis and lower the blood pressure but also protect the heart against ischemia/reperfusion injury. The difference between conditioning and a treatment is that discontinuation of the treatment (i.e. one stops taking the pills) results in a loss of the effects (the blood pressure rises, the protection is abrogated). In preconditioning this is typically not the case; preconditioning results in a rapid development of the protective state, and lasts for 1-2 hours (early preconditioning, EPC). Preconditioning also has a late phase (late preconditioning, LPC), in which protection does not start until 24 hours after the initial stimulus, and lasts for approximately 72 hours.

The preconditioning stimulus is thus biphasic and applied before the index ischemia. Although preconditioning proved to be a very effective method to reduce infarct size, its clinical applicability is limited as occurrence of an ischemic episode is not predictable in many clinical situations. Postconditioning seemed to solve this problem; it was protective in dogs exposed to 60 min of ischemia and 3 hours of reperfusion. In this study, application of 3 cycles of 30 seconds of reperfusion interspersed by 30 second cycles of reocclusion at the onset of reperfusion reduced infarct size from 25% in controls to 14% in treated animals. Infarct size in the postconditioning group was moreover comparable to the preconditioning group, showing the great potential of the postconditioning stimulus. This study first of all showed the great effectiveness of postconditioning, but secondly proved the very existence of reperfusion injury: when a treatment administered at reperfusion successfully lowers infarct size, some kind of damage should occur in this time window.

Nevertheless, successful application of postconditioning strongly depends on the duration of the postconditioning cycles and the time point of administration. In the first experiments that were done to investigate the concept of postconditioning, five-minute cycles of ischemia followed by reperfusion were used, comparable to the first
preconditioning protocols. To the investigator’s surprise this did not work and it took another 11 years before it was shown that short cycles of 30 seconds were effective in infarct size reduction. The first minute of reperfusion proved to be very critical as well, as delay of the first occlusion in the postconditioning protocol until one minute after onset of reperfusion abrogated cardioprotection in comparison to the application of the first stimulus at 30 seconds after the onset of reperfusion. Apparently, critical events take place during the first minute(s) of reperfusion.

Other ways of “conditioning”-induced cardioprotection

Conditioning agents are numerous and comprise a wide array of stimuli. A protective effect of a potential detrimental stimulus was first described when the phenomenon of ischemic preconditioning was discovered. Since then, other potential dangerous circumstances were used as conditioning stimuli; short episodes of those circumstances, such as heat-stress, hypothermia, lipopolysaccharide exposure and hypoxia exerted protective effects. They can therefore be categorized as “stress-induced cardioprotection”.

“Anesthetic conditioning” refers to the use of volatile anesthetics as conditioning agents, a technique investigated since the 1990s. The volatile anesthetics such as sevoflurane induce preconditioning and postconditioning. Additionally, the anesthetic noble gas xenon also induces pre- and postconditioning. The non-anesthetic noble gas helium is chemically similar to xenon as they both belong to the family of noble gases. In 2007 it was first shown that helium gas is able to induce preconditioning. The advantages of helium are numerous: (1) it is much cheaper than xenon and readily available, (2) it does not have cardiovascular effects, (3) it has beneficial physical properties such as low density and viscosity, (4) it does not induce anesthesia and (5) it can therefore be used in various clinical settings. This makes it a perfect candidate for use in the clinical setting as a conditioning agent.

The mechanisms underlying anesthetic preconditioning have been extensively investigated. Generally said, the preconditioning stimulus triggers a cascade of events, starting with alteration of multiple protein kinases, and signal transduction through G-protein coupled receptors, eventually leading to a set of end-effectors, among which are sarcolemmal and mitochondrial $K_{ATP}$ channels and the mitochondrial permeability transition pore. Eventually, all pathways and its end-effectors result in cell death or survival. For an overview of ischemic, and anesthetic conditioning protocols in relation to ischemia/reperfusion injury, also see Figure 1.
Cellular changes during reperfusion

At the onset of reperfusion, many cellular, molecular and metabolic processes take place simultaneously, which result directly from the events that take place during an ischemic episode. Ischemia results in depletion of ATP and high-energy phosphates, which cease aerobic metabolism. Consequently, the cell is bound to switch to anaerobic glycolysis. The concomitant decrease of pH will deactivate troponin C and cause Ca<sup>2+</sup> overload. Simultaneously, activation of intracellular proteases, e.g. calpain results in hypercontracture, necrosis and activation of apoptotic cascades as well as opening of the mitochondrial permeability transition pore<sup>23</sup>. Prompt return of blood flow immediately increases oxygen levels and restores substrates essential to generation of ATP. While this situation is beneficial for cell survival, the quick reestablishment of normo-pH abolishes the low-pH induced inhibition of mitochondrial permeability transition, hypercontracture and calpain activity, resulting in cell damage. The recovery of pH also leads to changes in membrane ion transportation, resulting in Ca<sup>2+</sup> overload. Furthermore, the sudden recovery of aerobic metabolism by activation of xanthine oxidase induces a reactive oxygen species (ROS) burst. ROS induce damage in proteins, DNA and cellular structures such as organelles, and evoke an inflammatory response<sup>23</sup>.

The interplay of the inflammatory response with the endothelium and the effects on the vascular bed play an important role during reperfusion. Shortly after the onset of reperfusion, the cardiac release of cytokines activates and recruits neutrophils. Neutrophils in turn produce ROS, proteases and cytokines, which damage the endothelium. The damaged endothelium now increases its production of ROS, cytokines and adhesion molecules, which further enhances endothelial dysfunction: intercellular tight junctions are compromised and lead to increased vascular permeability, facilitating leukocyte influx. Once migrated to tissue, leukocytes will produce more ROS and proteases, leading to oxidative stress and degradation of the extracellular matrix<sup>24,25</sup>.

The endothelium

The endothelium is ‘at rest’ under normal circumstances and serves dilator, anti-platelet and anti-neutrophil functions. Ischemia/reperfusion poses a stress signal for the endothelium and causes its activation. Activated or dysfunctional endothelium is characterized by a pro-thrombotic and pro-inflammatory state as it increases the adhesion of neutrophils and platelets<sup>26</sup>. Ischemia/reperfusion is also associated with a decrease in availability of endothelial mediators that are associated with vasodilation<sup>27,28</sup>. Prolonged vasoconstriction after reperfusion results in the amplification of expression of adhesion molecules and production of inflammatory cytokines, both of which account
for the recruitment of inflammatory cells. These inflammatory cells in turn produce more cytokines, further stimulating inflammation and tissue damage.

The association of endothelial dysfunction with cardiovascular disease is well known. Endothelial dysfunction in cardiovascular disease is associated with 2 hallmarks: (i) diminished nitric oxide production from vascular endothelium, resulting in prolonged vasoconstriction, (ii) the systemic character; endothelial dysfunction can be measured in different vascular beds and cardiovascular events may occur distant from the diseased endothelial bed.

**Conditioning of the diseased heart**

One of the challenges we face in the translation of experimental data of myocardial conditioning to clinical practice is the presence of co-existing disease in the target patient group. Whereas most conditioning techniques were first investigated in the laboratory in healthy animals with isolated myocardial infarction, development and extension of cardiovascular disease in humans depends on co-existing risk factors. Chronic diseases such as hypertension, obesity, hypercholesterolemia, hyperglycemia, insulin resistance, atherosclerosis and heart failure are important in the development and severity of coronary artery disease. These risk factors alter the possibilities to protect the heart by pre- and postconditioning. In addition, sex and aging might have an influence on the protective strategies.

Chronic hypertension leads to left ventricular hypertrophy and is an independent risk factor for cardiovascular heart disease. In hypertrophic myocardium cell signaling is altered, making the heart more susceptible to ischemic injury, possibly by abrogating the cardioprotective effects of conditioning. Ischemic postconditioning was indeed abrogated in spontaneously hypertensive rats (SHR). Alteration of cell signaling seems to be one underlying cause of the abrogated protection in diabetic hearts. New therapies to render the diabetic heart more susceptible to cardiac conditioning are therefore targeted on protein kinases that are altered by the presence of diabetes and are supposed to be involved in the conditioning-induced survival pathways.

Another way to protect the diseased myocardium is the administration of multiple protective stimuli, as a combination of separate protocols might induce larger protection together than they do separately. The combination of ischemic late preconditioning with early preconditioning reduced infarct size in rabbits in a stronger fashion than each stimulus alone. A stronger reduction of infarct size was also found after the combination of ischemic late preconditioning with sevoflurane-induced early preconditioning. As the threshold of conditioning seems to be higher in diseased subjects, this approach might be a solution for patients with comorbidities such as
hypertension and diabetes.

AIMS OF THIS THESIS

The aim of this thesis was to investigate new possible conditioning agents characterized by the ability to reduce infarct size. Our first aim was to investigate whether hypoxia could induce preconditioning in vivo. Due to the limited applicability of hypoxic gas mixtures in clinical practice, we thereafter focused on helium gas as a potential cardioprotective agent. From literature it was already known that the anesthetic noble gas xenon induces preconditioning and postconditioning. We hypothesized that the non-anesthetic noble gas helium can also induce cardioprotection. Therefore, the second aim of this thesis was to find out the correct duration and time point of administration of helium as a conditioning agent, and the underlying mechanisms of cardioprotection. As comorbidity is a huge compromising factor in patients, the third aim of this thesis was to search for ways to protect the diseased myocardium against ischemia/reperfusion injury by the use of helium conditioning.

OUTLINE OF THIS THESIS

Part I (chapters 1-2) is the main introduction of this thesis and comprises chapter 1 with the general introduction and chapter 2 with an introduction to the primary conditioning agent that was investigated in this manuscript: helium gas.

In Chapter 2 the main characteristics of helium gas are described. The biological effects of helium gas on different organs are explained as well as its effects on different molecular pathways.

Part II (chapter 3-7) of this thesis completely consists of animal studies and is split in 2 sections; section A (chapter 3-5) has been conducted in healthy animals and section B (chapter 6-7) in diseased animals.

In Chapter 3 we investigate whether hypoxia can induce cardioprotection. Three different oxygen concentrations are investigated and compared to anesthetic induced
preconditioning by sevoflurane. Additionally we investigate the difference between continuous and short administration of sevoflurane. This chapter basically emphasizes on the intensity and duration of a preconditioning stimulus and the correct timing.

In Chapter 4, three different helium postconditioning protocols are investigated in order to determine the correct duration of the stimulus. This study was also designed to investigate the involvement of the innate immune response in the myocardium during ischemia and early reperfusion, as it studies the influence of helium postconditioning on the magnitude of the hyper acute cytokine burst during early reperfusion.

In Chapter 5 infarct size measurement and histology is used to investigate the extent of cardiomyocyte damage after ischemia/reperfusion. By use of a PCR array of cell death pathways, it is investigated which cell death- and cell survival pathways are triggered after helium postconditioning, focusing on necrosis, apoptosis and autophagy.

Using diabetic and hypertensive rats, chapters 6 and 7 focus on the differences between ‘healthy’ and ‘diseased’ myocardium. These studies have been designed to investigate differences in cell signaling between healthy and diseased myocardium and the possibilities of protecting the diseased heart against ischemia/reperfusion injury by conditioning.

In Chapter 6 we investigate whether the diabetic Zucker rat can be protected against ischemia/reperfusion injury by helium-induced pre- and postconditioning. Moreover, we study the involvement of the pro-survival kinases of the reperfusion injury signaling kinases (RISK)-pathway by analyzing extracellular signal-regulated kinases 1/2 (ERK1/2) and Akt/Protein kinase B in helium induced preconditioning. To assess whether attenuated disruption of mitochondrial function plays a role in helium-induced conditioning, we investigate phosphorylation of glycogen synthase kinase-3beta (GSK-3beta) and mitochondrial uncoupling.

In Chapter 7 we evaluate the use of multiple helium conditioning stimuli to protect the hypertensive myocardium. Additionally we investigate the roles of GSK-3beta and protein kinase C- epsilon (PKC-epsilon) in helium-induced conditioning.

Part III (chapter 8-9) of this thesis has been conducted in healthy volunteers.

Chapter 8 focuses on possible endothelial protection by helium conditioning in a model of forearm ischemia/reperfusion. Forearm endothelial function is measured as
the vasodilatory response to acetylcholine and nitroprusside. As endothelium exposed to damage becomes activated and secretes markers in the circulation, plasma levels of cytokines, adhesion molecules and microparticles were also investigated. In Chapter 9 the safety of helium regarding the immune response is investigated. This study was designed to rule out the possibility that helium inhalation depresses the capacity of the innate immune system to respond to pathogens.
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Cellular effects of helium on different organs

Gezina Oei, Nina Weber, Markus Hollmann, Benedikt Preckel
Anesthesiology 2010 112(6): 1503-151
Helium is an odorless, tasteless, colorless monatomic element. It belongs to the family of noble gases, characterized by filled valence orbitals, meaning that a maximum amount of electrons is carried in the outer shell of the atom. This fact suggests that noble gases are ‘inert’ thus less capable of interacting with other compounds. In contrast to the noble gas xenon, helium lacks anesthetic properties\(^1\) and application of 80-90 atmospheres of helium pressure even increased the minimum alveolar concentration for volatile anesthetics. Therefore, helium is categorized as a ‘non-immobilizer’, a gas that is not able to induce anesthesia (immobilize) but that might have other behavioral effects\(^2\). Experimental research has convincingly shown that, although ‘inert’ and non-anesthetic, helium exerts cellular effects in vitro and in vivo: it reduces ischemia/reperfusion damage in cardiac and brain tissue. Organ protective properties of helium gas might also become relevant for clinical practice. This review summarizes the current knowledge on cellular effects of helium in various organs.
Physical and chemical properties of helium

Helium is the lightest noble gas (molecular weight 4 g/mol) and has the lowest melting and boiling points of all elements. It has a lower density (0.179 g/m$^3$) compared to oxygen (1.43 g/m$^3$) and nitrogen (1.25 g/m$^3$), and its absolute viscosity is 201.8 m poise (oxygen: 211.4 m poise; normal air: 188.5 m poise). As flow depends on density and viscosity of each element within a gas mixture, the physical properties of helium reduce airway resistance, thereby promoting airflow through the lungs.

Helium has a high thermal conductivity and heat loss of the body may occur when it is completely surrounded by helium. Because lower body temperature may result in reduced metabolism, embedding of a body in a helium environment could result in lower energy expenditure. In rats, extended breathing of 75% helium induced hypothermia. However, in humans, hypothermia during helium breathing –at least for short episodes- has not been reported yet.

Possible mechanisms of action underlying biological effects of helium

As anesthetic potency is nearly linearly correlated with the oil/water partition coefficient (known as the ‘Meyer-Overton rule’), it can be predicted that noble gases with lower solubility in fat and water have negligible anesthetic effects. For helium this holds true, in contrast to xenon and krypton that can produce the full state of general anesthesia. Helium’s low fat solubility can be overcome by elevation of partial pressure above the atmospheric level, delivering a sufficient amount of the gas into the central nervous system. However, in rats exposed to 84.6 ± 22.2 atmospheres of helium, tremors and convulsions have been observed, resembling activation of the central nervous system rather than depression of neural cell activity. This marked helium as a non-immobilizer; a compound producing convulsions near the estimated partial pressures needed to produce anesthesia as predicted by the Meyer-Overton rule. In addition, the counteracting antagonism of pressure itself, proven by reversal of general anesthesia in newts and mice after exposure to hydrostatic- or gas pressure, makes a depressive effect of helium at high pressures unlikely. However, only a direct comparison between high pressures of helium and high pressure per se could completely elucidate this matter. In fact, helium pressure could actually reduce duration and frequency of convulsions.

Biological effects of gases were once attributed to both indirect and direct actions on cytosolic and membrane bound (specific) proteins. The exact mechanism behind biological effects or the possible role for pressure in this matter is currently still not understood. The same holds true for helium and its effect on brain tissue or its biological
effects in general.

Helium-induced organ protection

A. Heart

Myocardial tissue can be protected against ischemia/reperfusion injury by subjecting it to one or more short ischemic episodes according to a specific protocol; early (EPC) or late preconditioning before ischemia, or postconditioning after myocardial ischemia\(^\text{12}\). In addition to ischemia, also pharmacologic compounds can trigger the signaling cascades of ischemic conditioning, thereby inducing organ-protection. Activation of adenosine, muscarinic, α-adrenergic, opioid or bradykinin receptors by ischemic preconditioning is known to trigger subsequent transduction pathways\(^\text{13}\) and therefore drugs that act directly upon these receptors contribute to organ protection.

Halogenated fluorocarbons like isoflurane, desflurane and sevoflurane, as well as the noble gas xenon are also known to exert cardioprotective effects\(^\text{14-17}\). In rabbits that underwent 3 cycles of 5 minutes (min) inhalation of 70% helium, neon or argon with 30% oxygen, interspersed with 5 min wash out of the respective noble gas, or three cycles of brief ischemia interspersed with 5 min of reperfusion, prior to the index ischemia of 30 min and 3 hours (h) of reperfusion, a reduction of infarct size was observed after ischemic- as well as after noble gas-induced preconditioning\(^\text{18}\).

Volatile anesthetics activate multiple pathways, in which reactive oxygen species, protein kinase C and various mitochondrial channels play a crucial role (for a detailed overview see Weber and Schlack\(^\text{14}\)). For an overview of currently discussed signaling kinases and targets involved in helium-induced conditioning, see Figure 1. In helium-induced EPC, the use of selective inhibitors of phosphatidylinositol-3-kinase, extracellular signal-regulated kinase and 70-kDa ribosomal protein s6 kinase abolished cardioprotection\(^\text{18}\). Blockade of glycogen synthase kinase or the apoptotic protein p53 lowers the threshold of helium EPC, as the combination of only one cycle of helium preconditioning with a glycogen synthase kinase- or apoptotic protein p53 -inhibitor provided a comparable infarct size reduction as 3 cycles of helium alone\(^\text{19}\). Not only inhibition of glycogen synthase kinase and apoptotic protein p53 pathways were shown to lower the threshold of helium EPC, also administration of morphine did; the combination of one 5 min cycle of helium administration along with morphine resulted in an equal infarct size reduction as 3 cycles of helium\(^\text{20}\). In contrast to a previous study\(^\text{19}\), one cycle of helium alone did not reduce infarct size in comparison to control. Use of a nonselective opioid receptor antagonist prevented the infarct size reduction\(^\text{20}\). Above described data imply involvement of the so-called reperfusion injury signaling kinase pathways and an opioid receptor-mediated mechanism in helium induced preconditioning.
It has been suggested that preconditioning affects cardiac mitochondrial function by regulation of extracellular signal-regulated kinase 1/2, phosphatidylinositol-3-kinase/Akt and glycogen synthase kinase-3beta. Opening of the mitochondrial permeability transition pore (mPTP) can lead to mitochondrial dysfunction. Pagel and colleagues showed in an in vivo study in rabbits, that application of a selective mPTP opener abolished helium EPC, suggesting a role for the mPTP in helium induced EPC. Opening of the mPTP during reperfusion is enhanced by normalization of acidic pH.
after restoration of blood flow\textsuperscript{23}. Thus, helium EPC may reduce infarct size by maintaining modest intracellular acidosis during early reperfusion, which keeps the mPTP closed. This is supported by data showing that helium preconditioning is not cardioprotective in rabbits that underwent transient metabolic alkalosis during reperfusion. In these experiments, myocardial protection could be restored after co-administration of cyclosporin A, an mPTP inhibitor\textsuperscript{24}. Next to the mPTP, the mitochondrial adenosine triphosphate-regulated potassium channel has been demonstrated to be involved in helium preconditioning: administration of the adenosine triphosphate-regulated potassium channel antagonist 5-hydroxydecanoate blocked helium-induced infarct size reduction\textsuperscript{24}.

In rats subjected to 25 min of regional myocardial ischemia and 2 h of reperfusion, cardiac mitochondrial function was analyzed by the rate of oxygen consumption by isolated mitochondria after administration of a complex 2 substrate (‘state 2’), adenosine-diphosphate (‘state 3’), and after complete phosphorylation of adenosine-diphosphate to adenosine-triphosphate (‘state 4’).\textsuperscript{25} The respiratory control index, calculated as state 3 /state 4, represents mitochondrial coupling between respiration and oxidative phosphorylation. The respiratory control index was reduced after helium preconditioning mainly by an increase of state 4 respiration, indicating a mild mitochondrial uncoupling after helium-induced preconditioning\textsuperscript{25}.

Involvement of the mitochondrial calcium sensitive potassium channel has also been investigated in helium induced organ protection\textsuperscript{25}. In rats, infarct size reduction after helium preconditioning and the concomitant reduction of the respiratory control index were abolished by addition of a mitochondrial calcium sensitive potassium channel blocker. Beside effects on the mitochondria, other enzymes and mediators might be affected by helium: application of a nonselective blocker of the endothelial nitric oxide synthase abolished infarct size reduction by helium and omitted nitric oxide production\textsuperscript{26}. Additionally, administration of the reactive oxygen species scavengers N-acetylcysteine or N-2 mercaptopropionyl glycine blocked cardioprotection after helium EPC in an in vivo study in rabbits, suggesting that a basal amount of reactive oxygen species is necessary for the mediation of the protective effect of helium EPC\textsuperscript{27}.

It has to be noted that although these experimental studies point out the involvement of different kinases and their targets in helium induced EPC, actual expression and activity of these kinases have not been measured yet or no changes could be demonstrated until now\textsuperscript{28}.

The previously mentioned experimental studies have been conducted in young and healthy animals. Cardioprotective effects, however, are diminished in disease states like diabetes or aged subjects\textsuperscript{29}. A well-known animal model for diabetes type II is the Zucker obese rat\textsuperscript{30}. These animals display insulin-resistance and become hyperlipemic.
and hyperinsulinemic but are normoglycemic, representing a prediabetic state of type II diabetes. In an in vivo study in Zucker obese rats, helium preconditioning did not lead to an infarct size reduction nor to mild mitochondrial uncoupling in comparison to non-diabetic controls.

In aged 22-24 months old Wistar rats, cardioprotection by helium preconditioning was abolished. These findings are in line with findings from anesthetic preconditioning: isoflurane preconditioning was attenuated in aged human atrial cardiomyocytes. In Langendorff perfused hearts of aged Fischer rats, sevoflurane preconditioning was abolished. In the same rat strain age-related changes in myocardial ischemic tolerance occur during the course of life, represented by an enhanced increase in intracellular Na⁺ content during an ischemic episode. This suggests that changes within the myocardium occur with progression of age. In fact, several changes in the senescent myocardium have been described that might influence cardioprotection. Helium EPC is most likely influenced by age, as infarct size reduction was abolished in aged rats. In addition, effects of helium on mitochondrial respiration could not be found in aged animals either.

Beside EPC, helium also induces late preconditioning: the administration of 30%, 50% or 70% helium 24 h before the sustained ischemic episode reduced infarct size from 55% in control to 40%, 34% and 37%, respectively. In contrast, 10% helium was not cardioprotective. Repetitive administration of helium on subsequent days within the time window of late preconditioning did not further enhance infarct size reduction. Helium-induced late preconditioning was abolished by a cyclooxygenase-2 inhibitor, a mechanism that was previously shown to be involved in xenon late preconditioning as well. An effect of helium on mitochondrial function could not be detected outside the early preconditioning window, suggesting that mitochondrial uncoupling is a trigger of helium-induced late preconditioning, not a mediator.

B. Brain and neuronal tissues
The discovery of sustained neuroprotective effects of xenon in various in vitro and in vivo models caused a shift towards the investigation of helium as a neuroprotectant. Xenon, a low-affinity N-methyl-D-aspartate receptor antagonist, causes a reduction of detrimental neurotransmitter presence in the brain. For helium, four studies currently describe its possible role in neuroprotection but can only speculate about an underlying mechanism.

In an in vitro mouse model of traumatic brain injury, brain slices were treated with elevated pressures of up to 2 atm of helium (75% helium, 20% oxygen, 5% carbon dioxide) on top of 1 atm of air (75% nitrogen, 20% oxygen, 5% carbon dioxide) in a specific gas chamber. Helium reduced cell damage as measured by a fluorescent technique for cell injury. Despite evidence for protection, a big drawback of this study...
design is the difficulty to distinguish between direct pharmacological effects of helium and effects of pressure per se. In addition, the absence of nitrogen in the helium-oxygen mixture may partly cause the observed differences between helium and control. However, further experiments with addition of nitrogen pressure instead of helium pressure were conducted. A significantly worse injury outcome was shown under nitrogen pressure in comparison to helium, suggesting that the protection was caused by removal of detrimental effects of nitrogen.\(^{39}\)

Detrimental effects of nitrogen and protective effects of noble gases were also investigated in neuronal cultures from mouse brain tissue.\(^{41}\) Cellular injury was provoked by oxygen and glucose deprivation, and cells were subsequently exposed to 90 minutes of nitrogen-hypoxia (95% nitrogen, 5% carbon dioxide) or noble gas-hypoxia (75% noble gas, 20% nitrogen, 5% carbon dioxide), under normobaric conditions. Injured cells in the nitrogen group showed more functional damage in comparison to injured cells in the xenon and argon group, indicating protective effects of these gases and/or detrimental effects of nitrogen. While neon and krypton had no effect at all, helium worsened the damage. Interestingly, in un-injured cells, exposure to neon or helium did not have damaging effects, while krypton negatively influenced cellular function.\(^{41}\) This suggests a difference between healthy and injured cells.

A theory that supports a detrimental role of nitrogen is the “nitrogen-washout theory.”\(^{42}\) This theory hypothesizes that nitrogen obstructs backflow of oxygen to the mitochondria at the time of reperfusion. Normally, oxygen availability is three times that of nitrogen, suggesting oxygen rather than nitrogen uptake in the case of fully functional mitochondria. This is explained by their respective solubility in water (nitrogen 1.6% and oxygen 3.0%) and the delivery rate of oxygen to tissues via hemoglobin at 1000 ml per minute. During ischemia, adenosine triphosphate-depletion in cells results in swelling and break down of the mitochondrial membrane. Since no oxygen is present and the membrane is partially disrupted, mitochondria are limited to nitrogen resorption only. After restoration of blood flow during reperfusion, the nitrogen-filled mitochondria are incapable of quick oxygen reuptake, possibly caused by a delay in the nitrogen outflow along a concentration gradient.

The question is whether any neuroprotective effect at all can be found from helium under normobaric circumstances and how this relates to the amount of nitrogen present. In rat heart, various gas mixtures with or without nitrogen (70% helium/30% oxygen, 50% helium/30% oxygen/20% nitrogen, and 30% helium/30% oxygen/40% nitrogen) were tested and all of them induced myocardial infarct size reduction.\(^{28}\) This proves a potential beneficial effect of helium itself, and not the removal of detrimental effects of nitrogen. Whether this also holds true for brain tissue has yet to be evaluated.

Effects of helium on the ischemic brain were investigated in rats subjected to 2 h of focal ischemia caused by occlusion of the middle cerebral artery and 1 h of reperfusion.
The infarct volume was assessed by 2,3,5-triphenyltetrazolium staining\(^{40}\). Three groups (helium, hyperoxia, control) inhaled the intervention gas during the entire procedure of ischemia and reperfusion. Infarct volume in the 30% oxygen/70% helium group was smaller than in the 100% oxygen group (hyperoxia) and the 100% oxygen treatment was beneficial compared to the 30% oxygen/70% nitrogen treatment (control). Neurological scores in the helium treated group were significantly better compared to control\(^{40}\). The fact that helium at atmospheric pressure levels improves neurological outcome suggests a pharmacological effect of helium itself. On the other hand, the low thermal conductivity of helium might also play a significant role: in the same rat model 75% helium after reperfusion exerted neuroprotection and improved neurological outcomes, but simultaneouly induced hypothermia\(^{5}\).

C. Lung
Barach first proposed the use of helium in a mix with oxygen as a therapeutic agent in obstructive pulmonary diseases\(^{43}\). Nowadays, helium has been implemented in clinical therapies against pulmonary diseases. However, the results from using helium in asthma and chronic obstructive pulmonary disease are inconclusive\(^{44,45}\). Studies focusing on gas behavior in the tracheobronchial system demonstrate a benefit for patients with severe acute asthma, measured as improved peak expiratory flow or forced expiratory volume in one second\(^{45}\). In intubated patients with chronic obstructive pulmonary disease, helium reduced the work of breathing and intrinsic positive end expiratory pressure compared to control\(^{46}\).

The vasodilator effects of nitric oxide on pulmonary vessels were investigated in dogs using either helium or nitrogen as a carrier gas\(^{47,48}\). This study demonstrated that nitric oxide in combination with helium led to lower mean pulmonary artery pressure and pulmonary vascular resistance compared to nitric oxide with nitrogen, probably due to enhanced diffusion velocity of nitric oxide in helium.

In asthma and chronic obstructive pulmonary disease, bronchodilators and inhalational corticosteroids are cornerstones of treatment. Theoretically, the low density of helium can lead to an improved aerosol penetration into the lungs. However, no consensus about the use of helium-driven nebulizers exists, as some studies show positive results\(^{48-51}\) while others failed to show clinical improvement of the treated patients\(^{52-54}\). Nawab and coworkers investigated the effect of helium inhalation on lung inflammation and resultant structural alterations. In a neonatal animal model of acute lung injury animals receiving helium/oxygen showed improved ventilation parameters compared to a control group receiving nitrogen/oxygen. The changes in ventilation parameters were associated with increased alveolar recruitment, lower inspired oxygen requirements and ultimately an attenuated inflammatory profile\(^{55}\). The authors postulated that this decrease is caused by a reduction in biochemical and --physical stress to the lung\(^{55}\). So
far, a direct effect of helium on the airways and parenchyma has not been shown.

D. Immune system
Recent publications showed that the anesthetic technique used in patients with malignant disease undergoing surgery can affect recurrence of malignancy. The underlying cause is possibly related to the influence of anesthetic agents on cell-mediated immunity and implicates that some agents are more harmful than others. The assumption that a smaller surgical trauma may lead to beneficial changes in the immune response stimulated the development of laparoscopic surgery. However, the notion that a disadvantage of the laparoscopic technique is increased tumor spread during surgery caused by establishment of pneumoperitoneum, is still not completely ruled out for all types of oncologic surgery. In an in vivo study in mice, intraperitoneal tumor distribution was more widespread in groups that underwent pneumoperitoneum compared to a group of animals that did not undergo insufflation of the abdomen. The authors hypothesized that cancer cells are literally blown through the cavity and therefore easily disseminate. Regarding this effect, the type of gas used for pneumoperitoneum was investigated, and interestingly it was found that tumor weight in helium pneumoperitoneum was smaller than in the carbon dioxide pneumoperitoneum. In contrast, Gutt and coworkers could not show any oncologic or immunologic differences between helium or carbon dioxide-induced pneumoperitoneum in rats. In a rat model of peritonitis, a lower incidence of bacteremia was observed using helium compared to carbon dioxide. Chemical and physical properties of the used gas might influence tumor implantation and development of malignancies. In vitro studies showed differences in cell viability between malignant cell lines that were incubated with carbon dioxide or helium, with lower cell proliferation under helium incubation compared to control or compared to control and carbon dioxide. The question which specific cellular factors affect tumor growth and dissemination has still to be answered. Incubation of cells in carbon dioxide turns the culture medium acid and it was shown that carbon dioxide pneumoperitoneum caused parietal peritoneal acidosis in pigs. In contrast, helium resulted in an alkalotic parietal peritoneal pH. Environmental pH may alter macrophage differentiation and function, which in turn affects the ratio of specific collagens in wound healing. Ex vivo it was shown that helium increased the collagen 1 to 3 ratio after laparotomy in rats, leading to improved wound healing. In healthy human volunteers helium breathing had a modest anti-inflammatory effect determined by attenuated expression of inflammatory cell surface markers on leukocytes and platelets in blood.

E. Blood vessels
An increase in immune activation may be beneficial against tumor spread; but also
Cellular effects of helium on different organs

means a higher adherence of circulating cells to vascular endothelium in a site of injury. The latter may play a role in ischemia/reperfusion damage\(^{68}\). In one study in rats, an increased leukocyte-endothelium interaction in tumor and liver vessels was observed after helium application\(^{69}\). In contrast, in healthy human volunteers using an ischemia/reperfusion model of the forearm, no effect of helium on endothelial dysfunction after ischemia was observed\(^{67}\).

Clinical considerations in the use of helium

Helium –non-expensive and easy to administer- has been used in patients with respiratory diseases since 1934\(^{43}\). No (hemodynamic) side effects have been found, making it appealing for a clinician dealing with patients with cardiovascular risk factors and/or cardiac disease: especially in the anesthetic practice, where the prevention of myocardial ischemia in the perioperative period is a daily challenge.

Organ protective properties of helium gas have been shown mainly in animal studies, and results of clinical studies are awaited. In humans, anesthetic-induced conditioning depends on the conditioning protocol used\(^{70}\). In patients, poly-pharmacy and comorbidity (such as simultaneous presence of diabetes or hypertension), but also age influence the outcome, probably by increasing a ‘threshold’ for a protective agent\(^{33,71}\). For helium-induced EPC it was shown that co-administration of pharmacological agents like morphine and glycogen synthase kinase- and apoptotic protein p53-inhibitors lowered the threshold for helium preconditioning\(^{19,20}\). Co-administration of these agents in patients who are susceptible for ‘higher conditioning thresholds’ might be a promising strategy in clinical practice to further enhance cardioprotection.

Helium might be used in the perioperative setting together with a protective anesthetic regimen, in order to increase organ protection. Helium is the first non-anesthetic gas inducing organ protection that can also be used safely in patients experiencing ischemic periods but not undergoing anesthesia. Additionally, helium preconditioning could play a role in organ transplantation. For a schematic overview of potential clinical applications of helium, see Figure 2. Despite promising results from experimental research, more clinical data are warranted in the specific clinical settings described above.
Figure 2. This figure shows potential clinical applications of helium in various medical fields. Current research mainly focuses on conditioning of heart and brain tissue, but in theory any organ in the body can be protected against ischemia/reperfusion injury, such as the lungs, liver, kidney and intestine. Other clinical applications of helium can be found during invasive and non-invasive ventilation in lung diseases.
CONCLUSION

Helium has been used in various clinical settings. Cellular effects of helium leading to cardio- and neuroprotection offer a novel therapeutic approach to protect patients against the detrimental effects of organ ischemia. The effects of helium in humans subjected to organ ischemia should be subject of future clinical and experimental research.
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PART

Animal studies
Animal studies
Hypoxia induced late preconditioning in the rat heart in vivo

Mark Berger, Ragnar Huhn, Gezina Oei, Andre Heinen, Andreas Winzer, Inge Bauer, Benedikt Preckel, Nina Weber, Wolfgang Schlack, Markus Hollmann
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INTRODUCTION

Ischemic heart disease and consequent heart failure are among the leading causes of morbidity and mortality in the western world\(^1\), and are of significant relevance in perioperative medicine. Perioperative myocardial infarction occurs in about 4% of patients with either an established diagnosis or risk for coronary artery disease, who undergo major non-cardiac surgery\(^3\). In patients undergoing elective vascular surgery, myocardial infarction is the most common fatal complication accounting for about 40% of postoperative fatalities within the first four years\(^5\). These data suggest that particularly in high-risk patients the choice of an anesthetic regimen that preserves myocardial function may improve the postoperative outcome.

Myocardial damage results from an insufficiency of oxygen supply to cardiac cells to meet metabolic demands. It is well established that survival of myocardial tissue subjected to ischemia can be increased by prior exposure to repeated brief episodes of sublethal ischemia induced by transient coronary artery occlusion and reperfusion, a phenomenon known as ischemic preconditioning (PC)\(^6\). Two phases of preconditioning are discriminated. An early phase (EPC), which develops within a few minutes and lasts for 2-3 hours, and a late phase (LPC), which develops more slowly (requiring 6-12 hours) but lasts for 2-3 days\(^7\). A variety of other stimuli, e.g. the volatile anesthetic sevoflurane and the noble gas xenon, are also known to offer protection against ischemia-reperfusion injury\(^8,9\). The clinical relevance of these processes has been demonstrated by Garcia and co-workers, showing that preconditioning with sevoflurane reduced the incidence of cardiac events during the first year after coronary artery bypass surgery from 17% to 3%\(^10\).

Several in vitro studies indicate that hypoxic LPC might be another powerful tool to protect against myocardial ischemia-reperfusion injury\(^11-13\). However, there are no in vivo studies yet investigating the effect and concentration-dependency of hypoxia-induced LPC on the magnitude of myocardial ischemia-reperfusion injury. Based on the foregoing issues, the goal of the present study was to investigate whether different concentrations of sustained hypoxia induce LPC in the rat heart in vivo, and how potent this effect is compared to the well established effect of EPC with sevoflurane. We also tested whether the combination of hypoxic LPC with sevoflurane EPC is more effective than each stimulus alone. The signaling pathway involved in hypoxic LPC was investigated with special emphasis on the protein kinase C (PKC) isoforms PKC-epsilon and PKC-alpha, and on the expression of the hypoxia-inducible factor (HIF)-1alpha-dependent genes heme oxygenase-1 (HO-1) and vascular endothelial growth factor (VEGF), respectively, which have previously been implicated in the genesis of hypoxic
and ischemic preconditioning.\textsuperscript{14-18}

**METHODS**

The investigation is in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Publication number 85-23, revised 1996), and was performed after approval of the Animal Ethics Committee of the University of Amsterdam, The Netherlands.

**Materials and Animals**

Hypoxic gas mixtures were purchased from Linde Gas (Linde Gas Benelux BV, Dieren, The Netherlands). The PKC-alpha antibody was purchased from Millipore (Billerica, MA, USA), and the PKC-epsilon antibody from Upstate Biotechnology (Lake Placid, NY, USA). All other chemicals were purchased from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands). Male Wistar rats (323±23 g) were obtained from Harlan (The Hague, The Netherlands), and kept with free access to standard food and water.

**Hypoxic exposure**

Rats were placed in a plexiglass chamber (Heidelberg, Germany) and randomly exposed to different concentrations of normobaric hypoxia (16%, 12%, and 8% oxygen, respectively) for 4 hours by using prepared gas mixtures with the corresponding oxygen concentrations balanced with nitrogen. Normoxic control experiments were conducted identically, except that room air (21% oxygen) instead of nitrogen was introduced into the chamber. The fresh gas flow was kept at 3 liter/minute to ensure that no accumulation of carbon dioxide occurs, which in turn might affect ventilation and thus oxygenation. The oxygen concentration within the chamber was controlled continuously (Datex Capnomac Ultima, Division of Instrumentarium Corp., Helsinki, Finland). After 4 hours of hypoxic exposure all rats were exposed to room air for 24 hours.

**Experimental protocol for infarct size determination**

Surgical preparation was performed as described previously.\textsuperscript{19,20} In brief, rats were anesthetized by intraperitoneal s-ketamine injection (150 mg/kg) followed by continuous
alpha-chloralose infusion (30 mg/kg/h), except for the groups that received sevoflurane (1 MAC) or propofol (11 mg/kg/h) continuously. After tracheal intubation, the lungs were ventilated with 30% oxygen and 70% nitrogen and a positive end-expiratory pressure of 2-3 cm H$_2$O. The right jugular vein was cannulated for saline and drug infusion, and the left carotid artery was cannulated for measurement of aortic pressure. A lateral left sided thoracotomy followed by pericardiotomy was performed and a ligature (5-0 Prolene) was passed below a major branch of the left coronary artery for a later occlusion of 25 minutes during the experiments. Aortic pressure and electrocardiographic signals were digitized using an analogue to digital converter (PowerLab/8SP, ADInstruments Pty Ltd, Castle Hill, Australia) and were continuously recorded on a personal computer using Chart for Windows v5.0 (ADInstruments).

After 120 minutes of reperfusion, the heart was excised and mounted on a modified Langendorff apparatus for infarct staining. After staining with 0.2% Evans blue, the heart was cut into transverse slices, which were then stained with 0.75% triphenyltetrazoliumchloride solution. The area of risk and the infarcted area were determined by planimetry using SigmaScan Pro computer software (SPSS Science Software, Chicago, IL).

**Study design**

The experimental protocol is shown in Figure 1. Part one of the study (all n=7-8): The purpose of this part was to investigate the effect of hypoxic LPC at different oxygen concentrations. Therefore rats were treated according to the following protocol (Fig. 1A): Twenty-four hours before the ischemia-reperfusion sequence started, rats were exposed for 4 hours to 21% oxygen (Con), 16% oxygen (LPC16), 12% oxygen (LPC12) or 8% oxygen (LPC8), respectively. To further investigate whether the effect of hypoxic LPC can be blocked by a specific PKC inhibitor, control rats and rats preconditioned with 16% oxygen received calphostin C (0.1 mg/kg) intravenously 35 minutes before myocardial ischemia was induced.

Part two of the study (all n=7-8): To compare the effect of hypoxic LPC with other preconditioning models, 3 groups of rats were subjected to the following protocol (Fig. 1B): the first group was exposed to EPC with sevoflurane (S-PC) with an end-tidal concentration of 1 minimum alveolar concentration (MAC) for 3x5 minutes interspersed with 2x5 minutes and one final 10 minutes washout period. The second group was exposed to continuous administration of 1 MAC sevoflurane (S), and the third group to continuous administration of propofol (P) in a concentration of 11 mg/kg/h. The latter two groups did not receive an anesthesia with alpha-chloralose.

Part three of the study (all n=9): The purpose of this part was to investigate whether
Figure 1. Experimental protocol. Control = normoxic control, LPC16, -12, -8 = late preconditioning with 16%, 12% and 8% oxygen, S-PC = sevoflurane early preconditioning, Sevoflurane = sevoflurane continuously, Propofol = propofol continuously. A) study design to test the cardioprotective effect of hypoxic late preconditioning at different oxygen concentrations and the functional involvement of PKC, B) study design to test the cardioprotective effect of early preconditioning with sevoflurane and continuous administration of sevoflurane and propofol, respectively, and C) study design to test the cardioprotective effect of hypoxic late preconditioning combined with the groups from part B).
the combination of hypoxic LPC (4 hours at 16% oxygen) with either EPC with sevoflurane, or with continuous administration of sevoflurane or propofol, respectively, is more cardioprotective than each stimulus alone. Therefore, the protocols from part one and two of the study were combined, resulting in the following design (Fig. 1C): The first group of rats was exposed to hypoxic LPC combined with sevoflurane early preconditioning (LPC16+S-PC). The second group was exposed to hypoxic LPC combined with continuous administration of 1 MAC sevoflurane (LPC16+S), and the third group was subjected to hypoxic LPC combined with continuous administration of 11 mg/kg/h propofol (LPC16+P).

**Western blot analysis**

For Western Blot analysis of myocardial PKC-alpha and PKCepsilon additional experiments were performed. Six and 28 hours after the hypoxic exposure (16% oxygen for 4 hours) was initiated, hearts were excised (LPC16 6h and LPC16 28h, respectively, all n=12). Hearts from control animals (Con) were excised after exposure to 21% oxygen. Before excision of the heart rats underwent a sham operation in order to follow the same surgical protocol as in the infarct size experiments.

After protein determination by the Lowry method equal amounts of the cytosolic protein fraction were mixed with loading buffer containing Tris-hydrochloric acid, glycerol and bromphenol blue. Samples were vortexed and boiled at 95°C before being subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Samples were loaded on a sodium dodecyl sulfate electrophoresis gel. The proteins were separated by electrophoresis and then transferred to a polyvinylidene membrane by tank blotting. The respective gels were stained with coomassie brilliant blue solution in order to guarantee equal loading of protein. Unspecific binding of the antibody was blocked by incubation with 5% fat dry milk powder solution in Tris buffered saline for 2 hours. Subsequently, the membrane was incubated over night at 4 °C with the respective first antibody at indicated concentrations. After washing in fresh, cold Tris buffered saline, the blot was subjected to the appropriate Alexa fluor 688 or Alexa fluor 788 infrared conjugated secondary antibody for 2 hours at room temperature. Immunoreactive bands on the membrane were visualised by the two channel laser system of the Odyssey system. The blots were quantified using the Odyssey IR Imager® (LI-COR Biotechnology, Bad Homburg, Germany).

**Semi-quantitative reverse transcriptase polymerase chain reaction assay**

HO-1 and VEGF messenger ribonucleic acid (mRNA) expression were measured by
Hypoxia induced late preconditioning in the rat heart in vivo

Reverse transcriptase polymerase chain reaction assay. Total RNA was isolated with Trizol according to the manufacturer’s instructions (Invitrogen, Darmstadt, Germany). Two micrograms total RNA were reverse transcribed (Promega, Mannheim, Germany). To quantify VEGF, HO-1 and glyceraldehyde 3-phosphate dehydrogenase mRNAs, gene-specific primers were used. Details about the primer sequences and reverse transcriptase polymerase chain reaction conditions are provided in the Supplemental Digital Content 1. Amplified products were resolved by electrophoresis on 2% agarose gels and stained with ethidium bromide. Gels were photographed under ultraviolet transillumination with a digital camera, and the images were transferred to a computer for densitometric analysis (Gelscan Software, BioSciTec, Frankfurt, Germany). Final results were expressed as the ratio of HO-1- and VEGF-mRNA to glyceraldehyde 3-phosphate dehydrogenase-mRNA for each sample.

Statistical Analysis

Normal distribution of the data was tested using the Kolmogorov-Smirnov test. Infarct sizes were analyzed by a one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test. Western blot and reverse transcriptase polymerase chain reaction data were analyzed by a Kruskal-Wallis one-way ANOVA followed by Student-Newman-Keuls post-hoc test. Data that were obtained at multiple time-points throughout the experiment, such as heart rate and blood pressure, were analyzed using one-way repeated measures ANOVA, followed by Holm-Sidak post-hoc test. Data are expressed as mean ± SD. Changes within and between groups were considered statistically significant if P<0.05. Statistics were performed using SPSS Science Software, version 17.0.
RESULTS

Infarct size measurement

As summarized in Table 1, hypoxic LPC significantly reduced infarct size at all tested oxygen concentrations (all P<0.001 vs. normoxic controls). The cardioprotective effect was similar between 16%, 12% and 8% oxygen, respectively (P=0.84, Table 1). The PKC inhibitor calphostin C completely abolished the preconditioning effect of hypoxic LPC at 16% oxygen (p<0.001), but had no effect on infarct size when it was administered to normoxic controls (p=0.30, Table 1 and Figure 2).

Early preconditioning with intermittent administration of sevoflurane (S-PC) reduced infarct size (P<0.01 vs. normoxic controls) to about the same magnitude as hypoxic LPC (P=0.87, Table 1). In contrast, continuous administration of sevoflurane (S) and propofol (P) were not cardioprotective (P=0.77 and P=0.96, respectively, vs. normoxic controls, Table 1). As shown in Table 1, the combination of LPC16 with sevoflurane early preconditioning (LPC16+S-PC) was not superior to the cardioprotective effect of each substance alone (P<0.05 vs. normoxic control, P=0.87 vs. LPC16, P=0.85 vs. S-PC). The cardioprotective effect of LPC16 was not affected by the two non-protective interventions with continuous administration of sevoflurane or propofol. In other words, hypoxic LPC was still able to reduce infarct size when it was combined with the continuous administration of sevoflurane (LPC16+S) or propofol (LPC16+P), respectively (P<0.01 vs. control).

![Figure 2. Infarct size measurement. Histogram shows the infarct size (percent of area at risk) of controls, hypoxic late preconditioning with 16% oxygen (LPC16), hypoxic late preconditioning with 16% oxygen combined with Calphostin C (LPC+Calphostin C) and Calphostin C alone. Data are presented as mean ± SD, *p < 0.05 vs. control group.](image-url)
Hemodynamic variables

Hemodynamic variables are summarized in Table 2. In all groups heart rate was decreased at the end of the experiment compared to the baseline values (P<0.05). Only when rats were exposed to continuous administration of propofol (P) the decrease in heart rate just failed to reach significance (P=0.05). The decrease in heart rate was paralleled by a decrease in mean aortic pressure (P<0.05). However, continuous administration of sevoflurane, both alone or in combination with LPC16, prevented the decrease in mean blood pressure.
### Table 2. Hemodynamic variables. Data are Mean ± SD. Con = control group; LPC16, -12, -8 = late preconditioning with 16%, 12% and 8% oxygen; Calphostin C = specific inhibitor of protein kinase C; S-PC = sevoflurane preconditioning; S = sevoflurane continuously; P = propofol continuously. *p<0.05 vs. Con, #p<0.05 vs. LPC16, $p<0.05 vs. LPC16+S-PC, §p<0.05 vs. baseline

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<th>Baseline</th>
<th>Washout 3</th>
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<td>Con</td>
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<td>360 ± 31</td>
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<tr>
<td>LPC16+S-PC</td>
<td>414 ± 32</td>
<td>368 ± 24&lt;sup&gt;i&lt;/sup&gt;</td>
<td>377 ± 24&lt;sup&gt;i&lt;/sup&gt;</td>
<td>343 ± 15&lt;sup&gt;i&lt;/sup&gt;</td>
<td>299 ± 21&lt;sup&gt;i&lt;/sup&gt;</td>
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<tr>
<td>LPC16+S</td>
<td>359 ± 24&lt;sup&gt;*&lt;/sup&gt;</td>
<td>348 ± 27&lt;sup&gt;s&lt;/sup&gt;</td>
<td>351 ± 36&lt;sup&gt;s&lt;/sup&gt;</td>
<td>349 ± 15</td>
<td>331 ± 35&lt;sup&gt;i&lt;/sup&gt;</td>
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<tr>
<td>LPC16+P</td>
<td>428 ± 24</td>
<td>418 ± 35</td>
<td>392 ± 57</td>
<td>398 ± 51</td>
<td>360 ± 27&lt;sup&gt;i&lt;/sup&gt;</td>
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| **Mean aortic pressure (mmHg)** |          |           |                 |                 |                     |
| Con              | 140 ± 16 | 135 ± 25  | 111 ± 32        | 89 ± 26<sup>s</sup> | 80 ± 29<sup>i</sup> |
| LPC16            | 130 ± 19 | 127 ± 20  | 114 ± 20        | 95 ± 23<sup>s</sup> | 89 ± 21<sup*i</sup> |
| LPC12            | 140 ± 9  | 128 ± 11  | 117 ± 17<sup>i</sup> | 103 ± 19<sup>i</sup> | 101 ± 28<sup>i</sup> |
| LPC8             | 124 ± 19 | 121 ± 23  | 108 ± 27        | 88 ± 21<sup>s</sup> | 72 ± 17<sup>i</sup> |
| LPC16+Calphostin C | 138 ± 18 | 136 ± 19  | 113 ± 24<sup>i</sup> | 100 ± 18<sup>s</sup> | 78 ± 9<sup>s</sup> |
| Calphostin C     | 143 ± 5  | 132<sup>i</sup> ± 5 | 128 ± 7<sup>s</sup> | 88 ± 14<sup>s</sup> | 72 ± 6<sup>i</sup> |
| S-PC             | 119 ± 18 | 86 ± 17<sup>s</sup> | 70 ± 12<sup>s</sup> | 65 ± 7<sup>s</sup> | 65 ± 10<sup>s</sup> |
| S                | 100 ± 21<sup>*</sup> | 116 ± 25  | 113 ± 25        | 105 ± 15        | 96 ± 9              |
| P                | 144 ± 13 | 134 ± 20  | 136 ± 10        | 116 ± 17<sup>s</sup> | 107 ± 27<sup>i</sup> |
| LPC16+S-PC       | 126 ± 10 | 107 ± 10<sup>s</sup> | 99 ± 15<sup>s</sup> | 74 ± 7<sup>s</sup> | 62 ± 4<sup>s</sup> |
| LPC16+S          | 108 ± 19 | 126 ± 13<sup>s</sup> | 125 ± 12<sup>s</sup> | 113 ± 11        | 103 ± 28            |
| LPC16+P          | 131 ± 25 | 121 ± 27  | 100 ± 41<sup>i</sup> | 101 ± 23<sup>s</sup> | 116 ± 18<sup>s</sup> |

Table 2. Hemodynamic variables. Data are Mean ± SD. Con = control group; LPC16, -12, -8 = late preconditioning with 16%, 12% and 8% oxygen; Calphostin C = specific inhibitor of protein kinase C; S-PC = sevoflurane preconditioning; S = sevoflurane continuously; P = propofol continuously. *p<0.05 vs. Con, #p<0.05 vs. LPC16, $p<0.05 vs. LPC16+S-PC, §p<0.05 vs. baseline
Expression of PKC-alpha and PKC-epsilon

Figure 3 shows that hypoxic preconditioning with 16% oxygen was associated with an increased myocardial expression of PKC-epsilon at 6 hours after the hypoxic stimulus was initiated (n=12; P=0.01 vs. control). At 28 hours the PKCepsilon expression remained elevated and was of about the same magnitude as after 6 hours (n=12; P=0.91 vs. 6 hours, P=0.01 vs. control). In contrast, at the same time points the myocardial expression of PKC-alpha was not affected by hypoxic preconditioning with 16% oxygen (PKC-alpha/tubulin ratio for control: 0.49±0.08; for LPC16 at 6 hours: 0.47±0.05, for LPC16 at 28 hours: 0.50±0.08; P=0.81; not shown).

Expression of HO-1 and VEGF

Hypoxic preconditioning with 16% oxygen transiently upregulated the myocardial expression of the HIF-1alpha-dependent genes HO-1 and VEGF (Fig. 4 and Fig. 5). Six hours after the hypoxic stimulus was initiated the expression of HO-1 and VEGF was increased by about 60% and 28%, respectively (each n=6; both P<0.05). However, at 28 hours the expression of both HO-1 and VEGF had returned to baseline values again (each n=6; P=0.50 and P=0.75 vs. control; Fig. 4 and Fig. 5).
Figure 4. Heme oxygenase (HO)-1 mRNA expression in rat myocardium. Representative reverse transcriptase polymerase chain reaction analysis experiments of a time course (Control, 6 hours, 28 hours after the hypoxic stimulus was initiated). Summarized data presenting ratio of HO-1 to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are shown. Data are presented as mean ± SD, *p < 0.05 vs. Control.

Figure 5. Vascular endothelial growth factor (VEGF) mRNA expression in rat myocardium. Representative reverse transcriptase polymerase chain reaction analysis experiments of a time course (Control, 6 hours, 28 hours after the hypoxic stimulus was initiated). Two major bands of VEGF mRNA represent polymerase chain reaction analysis fragments amplified from VEGF188 and VEGF164 mRNAs. Summarized data presenting ratio of VEGF to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are shown. Data are presented as mean ± SD, *p < 0.05 vs. Control.
DISCUSSION

It is well established that ischemic preconditioning represents a powerful and reproducible tool of cardioprotection against myocardial ischemia\textsuperscript{23}, but yet it has not been translated into routine clinical use. One of the reasons that limits the clinical applicability of ischemic preconditioning of the heart is that an invasive procedure is required to induce ischemia. Although this might be possible in some situations, e.g. by intermittent aortic cross clamping during coronary artery bypass surgery\textsuperscript{24}, it is not applicable in many others, e.g. during non-thoracic surgery. Much effort has therefore been focused on identifying preconditioning stimuli that are non-invasive, easy to administer and thus clinically more relevant.

Among various other stimuli (e.g. nitric oxide and volatile anesthetics\textsuperscript{23,25}), hypoxia may represent a potent preconditioning tool. The attraction of using hypoxia as preconditioning stimulus is that it very much resembles the stress that ultimately leads to myocardial infarction (i.e. cellular hypoxia/anoxia). An advantage of hypoxic preconditioning compared to ischemic preconditioning is that its systemic administration does not only exert a local effect but rather affects multiple organ systems which thus may also be protected against ischemic insults as it has been shown for the brain, liver, kidney, and other organ systems\textsuperscript{26-28}.

As indicated by several in vitro studies, intermittent exposure to 10% oxygen for 4 hours induced late cardioprotection in the isolated rat heart\textsuperscript{11,13}. Moreover, continuous hypoxic exposure to 10% oxygen for 4 hours followed by 24 hours of reoxygenation reduced infarct size in the isolated mouse heart, whereas shorter periods (30 minutes and 2 hours, respectively) remained uneffective\textsuperscript{12}. In contrast, in one study 10% oxygen for 4 hours had no cardioprotective effect\textsuperscript{11}. However, although different protocols of hypoxia-induced myocardial preconditioning have been proven to be effective in vitro its protective effect remains to be established in vivo.

**Hypoxia induces late preconditioning in vivo**

In the present study we demonstrate that continuous exposure to 4 hours hypoxia followed by 24 hours of normoxic reoxygenation induces LPC in the rat heart in vivo and reduces infarct size by nearly 40% compared to non-preconditioned myocardium. Interestingly, the extent of cardioprotection induced by 16% oxygen was comparable to the cardioprotection induced by 12% or 8% oxygen. However, because the infarct size slightly increased with the decrease in oxygen concentration (i.e. infarct size related to area at risk at 16% oxygen = 36%, at 12% oxygen = 38%, and at 8% = 39%), the results
do not exclude the possibility of as less pronounced cardioprotection with decreased oxygen concentrations. Indeed, a previous study by Béguin and co-workers\textsuperscript{11} showed that in a rat model intermittent hypoxia with 10% oxygen for 4 hours significantly reduced infarct size whereas the same protocol with 5% oxygen significantly increased infarct size. This observation suggests that to severe hypoxia renders the myocardium more sensitive to ischemic injury. The reason for this was not explored but is conceivable that a very low oxygen tension compromises oxygen supply to the tissue too much and itself induces cell damage with detrimental consequences. However, our results do not exclude the possibility that doses higher than 16% but lower than 21% oxygen also induce LPC.

**Potency of hypoxia-induced late preconditioning**

We next investigated, for the first time, how potent the observed cardioprotective effect of hypoxic LPC is compared to the well established effect of EPC with sevoflurane. In these experiments EPC with sevoflurane reduced infarct size by about 38%, which is in line with previous findings\textsuperscript{29}. This finding indicates that the cardioprotective effect elicited by sevoflurane EPC and hypoxic LPC is comparable. In contrast, when sevoflurane was administered continuously without a washout period before myocardial ischemia was induced, no reduction in infarct size was observed. This is in accordance with a previous clinical study from Bein and co-workers showing that continuous administration of 1 MAC sevoflurane from induction to start of cardiopulmonary bypass did not result in any protection compared with the control group\textsuperscript{30}. However, when the administration of sevoflurane before cardiopulmonary bypass was interrupted for 10 minutes, an improved myocardial performance and decreased postoperative troponin T values were observed. These data, in conjunction with our findings, suggest that interrupted administration of the anesthetic may be an essential feature for the occurrence of clinically relevant cardioprotective effects. This hypothesis is supported by our observation that continuous administration of propofol also exerted no cardioprotective effect.

These findings are of special interest for the clinical scenario when myocardial ischemia occurs by chance and cannot be timed, as it is possible, for example, in coronary artery bypass surgery. In the situation where a washout phase cannot be planned myocardial ischemia likely occurs during the administration of the anesthetics, i. e. when sevoflurane and propofol were not cardioprotective in our study. In these situations the clinical applicability of EPC is clearly limited. Thus, as pointed out elsewhere, LPC is likely to be of greater clinical relevance than EPC because it lasts 30-40 times longer and can easily be initiated before the expected ischemic event may occur\textsuperscript{23}. Because our results show that LPC with hypoxia was as cardioprotective as
EPC with sevoflurane, this finding underlines the potential relevance of hypoxia as LPC stimulus. This might especially be true in conjunction with previous data indicating that late but not early preconditioning protects against reversible postischemic contractile dysfunction (myocardial “stunning”).

**Hypoxic late preconditioning cannot be enhanced by modified anesthetic techniques**

A study by Müllenheim et al. showed that combining ischemic LPC with ischemic EPC led to enhanced cardioprotection compared to each stimulus alone. The same group showed that cardioprotection by ischemic LPC could be increased by sevoflurane-induced EPC. Therefore, we investigated whether the cardioprotective effect of hypoxic LPC could be enhanced by EPC with sevoflurane or by continuous administration of the two clinically used anesthetics sevoflurane and propofol. Interestingly, to the best of our knowledge, there is no study yet investigating the cardioprotective effect of LPC combined with continuous administration of sevoflurane or propofol. Our results demonstrate that none of the tested combinations was superior to the cardioprotection elicited by hypoxic LPC alone. However, the results also demonstrate that hypoxic LPC was not impaired by continuous administration of sevoflurane or propofol, highlighting the applicability and potential relevance of hypoxic LPC in the clinical setting.

**Signalling cascade of hypoxic late preconditioning**

Myocardial hypoxia triggers a wide range of profound cellular responses, including regulation of gene expression. In several studies protein kinase C (PKC), an ubiquitous intracellular mediator, has been shown to play a pivotal role in the signaling pathway of LPC, elicited by a variety of agents. Notably, the isoform PKC-epsilon appears to be responsible for the development of delayed cardioprotection, but in some studies PKC-alpha has also been implicated in provoking ischemic preconditioning of the heart.

Our results show that the PKC inhibitor calphostin C completely blocked the cardioprotective effect of hypoxic LPC, supporting the close association between PKC activation and the infarct size limitation conferred by hypoxic LPC. Although calphostin C is not isoform selective it has previously been shown to inhibit the novel isoforms of PKC, including PKC-epsilon, more efficiently than the conventional ones. Moreover, a previous study characterizing the expression of PKC isoforms in rat ventricular myocytes found that the dominant isoform was PKC-epsilon. Indeed, we found that 6 hours after the preconditioning stimulus was initiated the expression of PKC-epsilon was increased and that this increase remained stable until myocardial ischemia was
induced (i.e. after 28 hours). In contrast, the expression of PKC-alpha was not affected by hypoxic LPC. These observations indicate that PKC-epsilon, but not PKC-alpha, is the dominant isoform involved in the cardioprotective effect of hypoxic LPC in the rat heart in vivo. This is in line with a series of previous studies showing that translocation and activation of PKC-epsilon, but not of the PKC isoforms alpha, beta, gamma, delta, zeta, iota, lambda and mu is necessary for ischemic LPC in vivo. The reasons for the controversy between the studies regarding the involvement of PKC-alpha in mediating LPC are unclear but may be related to differences in species (canine, rabbit, humans and rat) and type of preconditioning (ischemic and hypoxic). However, we cannot exclude that other PKC isoforms that have not been studied in this report may contribute to cardioprotection elicited by hypoxic LPC in the rat.

Previous studies suggest that PKC plays a pivotal role in the upregulation of the HIF-1alpha-dependent genes HO-1 and VEGF, both of which have previously been implicated in mediating hypoxic LPC. HO-1 degrades heme and generates carbon monoxide and bilirubin, which may have antioxidant and/or anti-inflammatory effects. Indeed, mice overexpressing cardiac-specific HO-1 show a significant reduction in infarct size following ischemia-reperfusion. VEGF regulates local oxygen supply, and may be important in ischemia-reperfusion because it mediates neovascularization during myocardial ischemia. Our finding of the transient upregulation of HO-1 and VEGF mRNA provides further evidence for the involvement of these two effector genes in hypoxic LPC. Along with the hypothesis that PKC is involved in HO-1 and VEGF expression, it appeared conceivable that inhibition of PKC prevents the HO-1 and VEGF-mediated reduction in infarct size. Indeed, the observation that calphostin C completely abolished the cardioprotective effect of hypoxic LPC is consistent with this hypothesis. Moreover, our calphostin C results support the assumption that in the rat myocardium PKC lies upstream in the signaling pathway of HO-1 and VEGF expression, as proposed by others.

**Limitations of the study**

A limitation of the study is that we did not determine the oxygen threshold below which hypoxia starts to exert its cardioprotective effects. We cannot exclude that oxygen concentration higher than 16% and lower than 21% are also cardioprotective.

Another limitation of our study is that pharmacological inhibitors like calphostin C may affect other enzymes to a certain extent. Their specificity strongly depends on the concentration used. For example, calphostin C inhibits PKC with a half maximal inhibitory concentration of 50 nM, and other kinases like protein kinase A (half maximal inhibitory concentration >50 μM), p60v-src (half maximal inhibitory concentration...
>50 μM) and protein kinase G (half maximal inhibitory concentration >25 μM) are inhibited only at higher concentrations.

In summary, our results show that 1) hypoxic LPC results in profound protection against myocardial ischemia-reperfusion injury in the rat heart in vivo, as evidenced by a significant decrease in infarct size, 2) mild hypoxia (16% oxygen) induces cardioprotection of about the same magnitude as moderate (12% oxygen) and severe (8% oxygen) hypoxia, 3) the cardioprotective effect of hypoxia-induced LPC is as powerful as the cardioprotective effect of EPC with sevoflurane, and 4) PKC-epsilon is the dominant isoform involved in the cardioprotective effect of hypoxic LPC in the rat heart in vivo. These findings may provide a conceptual framework for developing novel therapeutic strategies aimed at a powerful cardioprotection especially in situations where myocardial ischemia occurs by chance and early preconditioning strategies are not applicable.
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Hypoxia induced late preconditioning in the rat heart in vivo


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Hypoxia induced late preconditioning in the rat heart in vivo


Prolonged helium postconditioning protocols during early reperfusion do not induce cardioprotection in the rat heart in vivo: role of inflammatory cytokines?

Gezina Oei, Hamid Aslami, Raphaela Kerindongo, Renske Steenstra, Charlotte Beurskens, Anita Tuip-de Boer, Markus Hollmann, Benedikt Preckel, Nina Weber
Manuscript submitted
INTRODUCTION

Early reperfusion is the cornerstone in the treatment of myocardial infarction, as quick restoration of blood flow to compromised areas will reduce infarct size, hence reducing the development of heart failure, myocardial stunning and arrhythmias. The flipside of the coin is reperfusion injury; cellular dysfunction caused by hyper acute return of blood flow and oxygen to tissue, primarily caused by oxidative stress and an inflammatory burst. The search for techniques to further reduce ischemia/reperfusion (I/R) injury has led to the discovery of pre- and postconditioning; techniques reducing infarct size by applying repetitive, short cycles of ischemia before or after the ischemic insult. ‘Conditioning’ can also be achieved by application of volatile anesthetics (sevoflurane, isoflurane) or noble gases (xenon, helium) according to specific protocols. In rats, inhalation of 15 minutes of 70% helium before or immediately after 25 minutes of myocardial ischemia reduced infarct size after 120 minutes of reperfusion.

A mechanism for helium-induced cardioprotection has yet not been elucidated, although several potential pathways have been suggested, such as signaling kinases of the RISK-pathway and the mitochondrial permeability transition pore. The role of the innate immune system in I/R injury has been under debate for years, as an excessive inflammatory burst is detrimental but at the same time a general inhibition of the innate immune system is associated with adverse outcome after myocardial infarction. During early reperfusion, several processes occur such as leukocyte activation and recruitment, cytokine and reactive oxygen species burst and concomitant endothelial dysfunction.

The exact spatial and temporal function of the different components of the innate immune response are unknown, but new insights propose the new ‘danger model’ as a concept for the initiation of the immune burst during early reperfusion. This model suggests that immunity can be triggered by release of damage-associated molecular patterns (DAMPs) from cells in danger or stress, and that tissue itself decides how the immune response is orchestrated. The myocardium and myocardial endothelial cells then release cytokines and complement, which cause a toll like receptor-mediated influx of inflammatory cells and upregulation of cytokine expression and cytokine release. Influx of neutrophils in the myocardium takes place due to changed endothelial barrier function under circumstances of stress such as exposure to high levels of reactive oxygen species. The migration, adhesion and diapedesis of neutrophils as a result of expression of adhesion molecules are subsequent events.

Exposure of human umbilical vein endothelial cells to xenon and inhalational anesthetics -which are known to be cytoprotective- lead to a reduction in protein levels and expression of adhesion molecules, suggesting an immunomodulatory effect at the
endothelial level. Administration of reactive oxygen species-scavengers in mice resulted in a smaller endothelial dysfunction, accumulation of neutrophils, expression of IL-1β and smaller infarct size. Cytokine-induced neutrophil chemoattractant (CINC-3) is a potent chemotactic factor for neutrophils and expressed in myocardium in a chronic rat model of I/R. Interleukin 1-beta (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor-alpha (TNF-alpha) are proinflammatory cytokines widely studied in myocardial ischemia/reperfusion models and are therefore assumed to play a role in the stimulation of the innate immune response during cardiac I/R.

Currently it is unknown whether the duration of helium postconditioning protocols influences the final infarct size after myocardial regional I/R, neither do we know if helium postconditioning affects the cytokine burst during early reperfusion. Therefore, in this study we exposed rats undergoing regional I/R to various lengths of helium postconditioning protocols and measured infarct size and release of biomarkers into the circulation. We hypothesized that helium-induced cardioprotection is accompanied by a reduction in the hyperacute cytokine burst during reperfusion, we therefore analyzed CINC-3, IL-6, IL-1β and TNF-alpha at protein level and at mRNA levels in myocardium exposed to I/R (‘area at risk’, AAR) and myocardium not downstream from the occluded coronary artery (‘area not at risk’, NAAR).
MATERIALS AND METHODS

Rat model of regional myocardial I/R

Helium70%/oxygen30% was purchased from Linde Gas (Linde Gas Benelux BV, Dieren, The Netherlands). Male adult Wistar rats (Charles River, Wilmington, MA, USA) were acclimatized for 7 days under conditions of 12-h light and dark cycles and had free access to food and water. Animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and all experiments were approved by the institute’s animal ethics committee (DAA102650 and 102279).

Study design

For investigation of the effect of various helium postconditioning protocols on cellular damage within the myocardium we divided animals in 5 groups (Figure 1A). Surgery of the animals was performed as previously described and comprised reversible ligation of a great branch of the left descending coronary artery. All animals were connected to blood pressure and heart frequency monitoring by cannulation of the carotid artery and had a 15 minutes baseline period for stabilization after surgical preparation. Sham operated animals (Sham) underwent surgery, but did not undergo ischemia and reperfusion. The control group (CON) underwent 25 minutes of ischemia and 120 minutes of reperfusion. The helium intervention groups also underwent 25 minutes of ischemia and 120 minutes of reperfusion, but additionally inhaled 15, 30 or 60 minutes of 70% helium/30% oxygen at the onset of reperfusion (He15, He30, He60). To make sure adequate helium concentrations were present at the onset of reperfusion, inhalation of helium was started in the last minute of the ischemic episode. Cellular damage was investigated by infarct size staining of myocardial slices and measurement of serum levels of lactate dehydrogenase (LDH) and troponine-T (TnT), both commonly used methods to assess cellular damage.

The effect of helium inhalation on mRNA and protein levels of proinflammatory markers TNF-alpha, IL-1beta, IL-6 and chemokine CINC-3 was investigated in two different time frames. As transcription of mRNA precedes translation to proteins, rats underwent short reperfusion for investigation of cytokine expression. We hypothesized that if helium postconditioning exerts its effects during early reperfusion, a direct and immediate effect on transcription of inflammatory cytokines should be found during this critical phase. In order to measure differences in mRNA levels rats should not undergo
the entire reperfusion period, but reperfusion time should be limited to the duration of helium postconditioning. Rats were assigned to 7 groups (Figure 1B). Three groups underwent 25 minutes of ischemia and 5, 15 or 30 minutes of reperfusion respectively (I/R5, I/R15 and I/R30). The helium intervention groups underwent 25 minutes of ischemia and 5, 15 or 30 minutes of reperfusion with simultaneous inhalation of helium during the entire reperfusion episode. After completion of the experimental protocol—the duration varied in each group—hearts were excised and kept at -80°C until further processing for RT-PCR experiments. In order to analyze the effect of helium postconditioning on mRNA levels of inflammatory cytokines, we compared each I/R group with its correspondent helium interventional group.

For the measurement of protein levels of cytokines in myocardial tissue, hearts were divided in groups as depicted in Figure 1A. Protein levels of cytokines during I/R not only rise due to an upregulation of transcription, but also rise due to increased release from inflammatory cells and migration of inflammatory cells towards the myocardium. We hypothesized that if helium postconditioning affects the innate immune system, an effect on protein levels of inflammatory cytokines should be found shortly after but not during the application of the protective stimulus. Therefore, these groups underwent 120 minutes of reperfusion after which hearts were excised and kept at -80°C until further processing for ELISA measurements.

In order to investigate the involvement of the innate immune system in helium postconditioning we distinguished two types of myocardial (ventricular) tissue. After excision of the whole heart, we first separated the ventricular from the atrial tissue and removed valves and remains of great vessels. As the (opened) ligature was left in situ, we could clearly point out the area of the ventricle downstream of the ligature and named this the ‘area at risk’ (AAR). In all groups except the Sham group, the distinct color of the AAR in comparison to the surrounding tissue further enabled separate excision. This part of the ventricle was cut from the rest of the ventricle on ice and comprised approximately one quarter to one fifth of total ventricular tissue. At the opposite side of the ventricle, not downstream of the ligature, we excised a second part of the ventricular tissue and named this the ‘area not at risk’ (NAAR). For a schematic overview of this process, also see Figure 2.

Sample size analysis for each part of the study was based on results obtained from an earlier study in which the primary endpoint was infarct size. The expected difference in mean was 18%, the standard deviation 12%, the power was 80% and the type I error 0.05, resulting in a required number of rats per group being 8. Due to a suspected drop out of 20% due to surgical error, fatal arrhythmias or technological problems with staining, enzyme linked immunosorbent assay (ELISA) or polymerase chain reaction (PCR), we performed a total of 158 experiments, of which 22 dropped out.
### Figure 1 Experimental protocols.

**A.** Experiments for determination of infarct size, measurements of biomarkers, hemodynamics and protein levels of inflammatory cytokines. 
- **Sham** = sham group, did not undergo ischemia/reperfusion; **CON** = control group; underwent 25 minutes of ischemia and 120 minutes of reperfusion; **He15, He30 and He60** groups all underwent 25 minutes of ischemia and 120 minutes of reperfusion and additionally received 15, 30 or 60 minutes of helium during reperfusion.

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**B.** Experiments for measurements of mRNA levels of inflammatory cytokines. Animals underwent 5, 15 or 30 minutes of reperfusion without (I/R5, I/R15, I/R30) or with helium (He5, He15, He30).

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*Figure 1 Experimental protocols. A. Experiments for determination of infarct size, measurements of biomarkers, hemodynamics and protein levels of inflammatory cytokines. Sham = sham group, did not undergo ischemia/reperfusion; CON = control group; underwent 25 minutes of ischemia and 120 minutes of reperfusion; He15, He30 and He60 groups all underwent 25 minutes of ischemia and 120 minutes of reperfusion and additionally received 15, 30 or 60 minutes of helium during reperfusion. B. Experiments for measurements of mRNA levels of inflammatory cytokines. Animals underwent 5, 15 or 30 minutes of reperfusion without (I/R5, I/R15, I/R30) or with helium (He5, He15, He30).*
Prolonged helium postconditioning protocols and inflammatory cytokines

Infarct size determination and measurement of LDH and TnT

At the end of the experiments (Figure 1A), the heart was excised under deep anesthesia and treated for further analysis as described previously. The area at risk and the infarcted area were determined by planimetry using SigmaScan Pro 5™ software (SPSS Science Software, Chicago, IL, USA). For measurement of injury markers LDH and TnT arterial blood sampling through the carotid cannula was done at baseline, after 24 minutes of ischemia, after application of the conditioning stimulus and after 120 minutes of reperfusion. After sampling, blood was centrifuged at 3100 RPM, 20°C for 10 min. Serum was kept at -80°C until measurement of LDH and TnT by ELISA in our clinical laboratory.

Homogenization and fractionation

After separation of the AAR from the NAAR on ice, tissue was subsequently snap frozen in liquid nitrogen for storage at -80°C until further analysis. Hearts were weighted and accordingly diluted with normal saline 1 mg of heart tissue was multiplied by 4, resulting in the amount of saline in ml to be added and Greenberger Lysis buffer (105
mM NaCl, 15 mM Tris, 1 mM MgCl₂.H₂O, 1 mM CaCl₂, 1% Triton and destilled water) with protease inhibitor mix (pepstation A, Leupeptin, Aprotinin) (1:5) upon homogenization. After homogenization, samples were left on ice for 20 minutes and subsequently centrifuged for 10 minutes at 3600 RPM at 4°C. Supernatants were stored at -80 °C for ELISA analysis. For qRT-PCR analysis, homogenates were stored with Tripure isolation reagent (1:9) at -80 °C.

**Enzyme-linked immunosorbent assay (ELISA)**

For measurement of protein levels of inflammatory cytokines, ELISA kits for TNF-alpha, IL-1beta, IL-6 and CINC-3 were purchased at R&D Systems (Abingdon, United Kingdom) and performed according to manufacturer’s instructions.

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

All qRT-PCR materials were purchased at Roche (the Netherlands), unless otherwise stated. Hundred µl of chloroform was added to heart homogenate in Tripure isolation reagent. Samples were then centrifuged for 15 minutes at 12000 RPM at 4 °C. The supernatant was added to 250 µl of 2-propanol and left untreated at room temperature for 10 minutes. After centrifugation for 10 minutes at 12000 RPM the supernatants were discarded and the pellet washed with 500 µl of 75% ethanol. This mix was again centrifuged for 5 minutes at 12000 RPM. The pellet was dissolved in RNase-free water and heated for 10 minutes at 60 °C, after which all samples were kept at -80 °C.

RNA concentration was measured on the Nanodrop 2000 (Thermo Scientific, the Netherlands). Complementary deoxyribonucleic acid (cDNA) synthesis was conducted with one microgram RNA using the Transcriptor First Strand cDNA Synthesis kit. One microliter cDNA was used in a total volume of 10 µl PCR mix per reaction. Each mixture contained 10µM of primer pairs and 2x LightCycler® 480 SYBR Green I Master. Real-time qPCR amplification was carried out using the LightCycler® 480 instrument under the following conditions: pre-incubation at 95 °C for 10 minutes, followed by 45 cycles of 95 °C for 10 seconds, 60 °C for 10 seconds and 72 °C for 15 seconds. The fluorescence measured per cycle of each sample was acquired. Primer sequences of genes for qRT-PCR were: TNF-alpha (forward CTG GGA CAG TGA CCT GGA CT; reverse GCA CCT CAG GGA AGA GTC TG), IL-1beta (forward GCC CAT CCT CTG TGA CTC AT; reverse AGG CCA CAG GTA TTT TGT CG), IL-6 (forward AGT TGC CTT GGG ACT GA; TCC ACG ATT TCC CAG AGA AC), CINC-3 (forward GTG CTA AGG CAT TGT GGT GTG T; reverse GCA ACA TCT TAT CAG TCC ATG GTT) and GAPDH (forward TGC CCC CAT GTT TGT
GAT G; reverse GCT GAC AAT CTT GAG GGA GTT GT), which were purchased at Invitrogen (the Netherlands)

Raw data was exported as text file format and subsequently converted into an Excel sheet by using the program LC480Converter. The converted data was imported to the LinRegPCR program and baseline correction was carried out, thus measuring fluorescence before amplification-specific fluorescence can be determined, consisting out of fluorescence from cDNA, primers and unbound SYBR Green. Hereafter, individual PCR efficiency of each sample was obtained from the slope of the regression line fitted to a subset of baseline- corrected data points in the log linear phase. Finally, the relative quantities of each target gene were calculated by normalization to the housekeeping gene GAPDH.

**Statistical analysis**

Statistical analysis of infarct size experiments, biomarkers and inflammatory protein levels was performed in GraphPad Prism (GraphPad Software, La Jolla, CA, USA) using one way ANOVA with a Dunnet post-hoc test comparing the control group against all other groups. Hemodynamics were tested using one way ANOVA with Tukey’s posthoc test. For inflammatory cytokine expression I/R groups were tested against the helium groups using students T-tests, testing the I/R5 against the He5 group, the I/R15 against the He15 group, and the I/R30 against the He30 group. P<0.05 was considered significant.
RESULTS

Hemodynamics

Mean aortic pressure and heart rate slowly decreased during the experimental protocol. Exposure of rats to various helium postconditioning protocols did not affect hemodynamics. For an overview of hemodynamics also see Table 1.

Infarct size and biomarkers LDH and TnT

Fifteen minutes of helium reduce infarct size as percentage of the area at risk from 43% in control animals to 21%, whereas prolonged helium inhalation for 30 or 60 minutes during early reperfusion did not, also see Figure 3A. No significant differences in area at risk as percentage of total ventricular tissue were found between groups, means +/- S.E.M. were 23% +/- 3% (Sham), 20% +/- 2% (CON), 28% +/- 2% (He15), 26% +/- 5% (He30) and 22% +/- 2% (He60) (data not shown). Analysis of biomarkers within each experimental group shows that LDH- and TnT-release in serum increases when reperfusion time increases (data not shown). Figure 3B shows LDH and TnT-levels in the Sham, CON, He15, He30 and He60 groups after 120 minutes of reperfusion.

Protein levels and expression of CINC-3, IL-6, IL-1beta, TNF-alpha

In myocardial tissue exposed to I/R (AAR), CINC-3 protein levels were significantly higher in the He30 and He60 group in comparison to the CON group. IL-1beta levels were significantly higher in the He60 group in comparison to the CON group. All cytokines were lower in the sham group in comparison to the CON group. For protein levels also see Figure 4.

Expression of cytokines in the AAR myocardium groups was not significantly different between I/R and He groups. In the NAAR myocardium, CINC-3 expression in the He30 group was significantly higher than in the I/R30 group, also see Figure 5.
<table>
<thead>
<tr>
<th></th>
<th>Baseline 15 min</th>
<th>Ischemia 24 min</th>
<th>Reperfusion 15 min</th>
<th>Reperfusion 30 min</th>
<th>Reperfusion 60 min</th>
<th>Reperfusion 120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean AP (mmHg)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>124 +/- 25</td>
<td>112 +/- 21</td>
<td>95 +/- 13</td>
<td>92 +/- 23</td>
<td>100 +/- 17</td>
<td>83 +/- 27</td>
</tr>
<tr>
<td>CON</td>
<td>117 +/- 23</td>
<td>94 +/- 21</td>
<td>96 +/- 17</td>
<td>87 +/- 13</td>
<td>90 +/- 21</td>
<td>78 +/- 22</td>
</tr>
<tr>
<td>He15</td>
<td>109 +/- 19</td>
<td>97 +/- 32</td>
<td>84 +/- 21</td>
<td>73 +/- 11</td>
<td>87 +/- 27</td>
<td>79 +/- 28</td>
</tr>
<tr>
<td>He30</td>
<td>99 +/- 23</td>
<td>78 +/- 25</td>
<td>87 +/- 24</td>
<td>82 +/- 18</td>
<td>77 +/- 19</td>
<td>62 +/- 19</td>
</tr>
<tr>
<td>He60</td>
<td>102 +/- 18</td>
<td>76 +/- 32</td>
<td>71 +/- 14</td>
<td>68 +/- 17</td>
<td>72 +/- 31</td>
<td>48 +/- 11</td>
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<td><strong>Mean HR (beats/min)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>374 +/- 39</td>
<td>387 +/- 42</td>
<td>336 +/- 51</td>
<td>332 +/- 43</td>
<td>333 +/- 47</td>
<td>312 +/- 38</td>
</tr>
<tr>
<td>CON</td>
<td>347 +/- 32</td>
<td>361 +/- 29</td>
<td>351 +/- 33</td>
<td>332 +/- 27</td>
<td>325 +/- 33</td>
<td>291 +/- 30</td>
</tr>
<tr>
<td>He15</td>
<td>356 +/- 57</td>
<td>381 +/- 33</td>
<td>379 +/- 23</td>
<td>369 +/- 39</td>
<td>361 +/- 43</td>
<td>329 +/- 50</td>
</tr>
<tr>
<td>He30</td>
<td>347 +/- 61</td>
<td>355 +/- 41</td>
<td>354 +/- 34</td>
<td>354 +/- 20</td>
<td>327 +/- 21</td>
<td>317 +/- 37</td>
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<td>He60</td>
<td>357 +/- 50</td>
<td>363 +/- 25</td>
<td>365 +/- 32</td>
<td>355 +/- 33</td>
<td>332 +/- 32</td>
<td>294 +/- 41</td>
</tr>
</tbody>
</table>

Table 1 Hemodynamics (mean arterial pressure and heart rate) sampled after 15 minutes of baseline, 24 minutes of ischemia, 15, 30, 60 and 120 minutes of reperfusion. Data are shown as mean +/- SD. At each time point there were no significant differences between different experimental groups. Mean AP = mean arterial pressure in mmHg, mean HR= mean heart rate in beats/minute.
Figure 3 Infarct size and biomarkers after 120 minutes of reperfusion. Data are shown as mean +/- S.E.M. * = p<0.05 vs CON. Amount of experiments in each group is shown below individual bars. A. Infarct sizes as percentage of the area at risk. Underneath the graph, pictures of representative slices of myocardium are shown for each group. B. Troponin T and LDH levels in the circulation after 120 minutes of reperfusion.
Figure 4 Protein levels of CINC-3, IL-6, IL-1beta, TNF-alpha. All data are shown as mean +/- S.E.M. Amount of experiments in each group is shown below individual bars. * P<0.05 significant in comparison to CON.
Figure 5. CINC-3, IL-6, IL-1beta and TNF-alpha expression. All data are shown as mean +/- S.E.M. Amount of experiments in each group is shown below individual bars. * P<0.05 I/R30 in comparison to He30.
DISCUSSION

In rat hearts we applied regional I/R and determined myocardial infarction by 2,3,5-triphenyltetrazolium chloride (TTC) staining. We investigated the release of the injury markers LDH and TnT into the circulation. In this study we show that a short episode of helium postconditioning (15 minutes) reduces infarct size, whereas prolonged helium inhalation (30 and 60 minutes) during reperfusion abrogates this protection. The main results suggest that prolonged exposure to 70% helium gas is not protective but might even induce cellular damage. This is in line with the general consensus on ischemic postconditioning; a postconditioning stimulus after ischemia should comprise short stimuli. Deleterious effects of prolonged helium exposure have been described in vitro in human tubular kidney HK2 cells exposed to three hours of oxygen glucose deprivation, 3 hours of incubation with 75% helium 24 hours before the cytotoxic stimulus resulted in reduced cell viability in comparison to oxygen glucose deprivation alone.

The results in this study do not only show that prolonged helium inhalation in cytotoxic circumstances abrogate the protective effect of short lasting helium inhalation, but also suggest a relation with an increased immune activity. The relationship between cell damage and immune activation was also investigated in animal models of laparoscopic abdominal surgery for abdominal malignancy. Helium pneumoperitoneum resulted in reduced tumor growth and spread in comparison to a CO₂ insufflated abdomen. It was hypothesized that these cytotoxic effects on tumor cells were caused by increased immune activity. Increased immune activity was indeed found after helium pneumoperitoneum in comparison to a CO₂ insufflated abdomen in rats with abdominally implanted hepatoma cells. In the helium group, a smaller tumor volume and a higher rate of leukocyte-endothelium interaction in the liver was found. This is in line with results from the present study, in which 60 minutes of helium inhalation increased protein levels of CINC-3 and IL-1beta in AAR tissue.

Taken together, in vitro and in vivo data suggest that helium can protect the heart when applied for short episodes, in contrast, prolonged inhalation can abrogate the cardioprotective effect of short lasting helium and increase the local sterile immune response. We hypothesized that a short episode of helium inhalation during early reperfusion leads to a reduction of the hyperacute reactive oxygen species- and neutrophil-induced cytokine burst. However in the present study the cardioprotective effects of helium were not accompanied by a reduction of inflammatory cytokines.

The increase of inflammatory cytokine proteins is known to rise sharply within the first 12 hours after ischemia and returns back to normal within 48 hours. Peak levels of
mRNA occur after 12 h of reperfusion, being 50-fold in the myocardium at risk and 15-fold in the myocardium not at risk. In the current study, protein levels were measured after 2 hours of reperfusion and mRNA levels after reperfusion times varying from 5 to 30 minutes. The time points of analysis could have been too early to detect the final magnitude of the cytokine burst, which could explain the results of the mRNA data. However, as helium-induced postconditioning acts within a very short time window during reperfusion (only protective within the first 15 min), significant cellular changes underlying these infarct size-sparing effects should have been found within this time frame.

Interestingly, some differences were found between AAR en NAAR myocardium. In AAR but not in NAAR myocardium, an increase in CINC-3 and IL-1beta level was found after 60 minutes of helium. This suggests that the combination of a severe damaging stimulus to the cardiomyocyte with prolonged helium inhalation not only leads to more cellular damage, but also increased protein levels of inflammatory cytokines. Possibly, only the stressed myocardium releases all of its endogenous cytokines, whereas the NAAR myocardium is not so much triggered to do this.

In the present study, the release of biomarkers and the infarct sizes in the different experimental groups showed a same trend. However, the LDH and TnT levels in blood in the intervention groups did not differ significantly from the control group. First of all, this could have been caused by the short reperfusion times that were used. In a comparable study in rats, TnT correlated with infarct size after 180 minutes of reperfusion. The serum levels of injury markers such as LDH and TnT are dependent on the release by the cell and its cytoskeletal structure, and therefore it is possible that our 2 hours of reperfusion were too short. In general, TnT is released from myocytes at a slower rate than LDH. Secondly, the use of TnT as a marker of true cellular injury during ischemia/reperfusion might not be correct as TnT is also released during heavy exercise.

In summary, the present study shows that 15 minutes of helium inhalation during early reperfusion protects the myocardium and reduces infarct size whereas a prolonged inhalation of helium (30 or 60 minutes) during early reperfusion is not protective. Helium-induced cardioprotection is not accompanied by a reduction of the hyperacute burst of inflammatory cytokines, but prolonged helium inhalation might contribute to the pro-inflammatory response.
Prolonged helium postconditioning protocols and inflammatory cytokines

REFERENCES


25. Li L, Hessel M, van der Valk L, Bax M, van der Linden I, and van der Laarse A. Partial and delayed release of troponin-I compared with the release of lactate dehydrogenase from necrotic cardiomyocytes. Pflugers Arch. 2004 448(2): 146-52

Prolonged helium postconditioning protocols and inflammatory cytokines
Reduction of cardiac cell death after helium postconditioning in rats: transcriptional analysis of cell death and survival pathways
INTRODUCTION

Coronary artery disease and subsequent myocardial infarction is a common cause of death\(^1\). In the acute phase of myocardial infarction, occlusion of a cardiac vessel by a thrombus or stationary embolus leads to myocardial hypoxia, which is followed by cessation of aerobic respiration and ATP production in the affected cardiomyocytes. The rapid energy depletion gradually suppresses metabolic activity and leads to the induction of cell death pathways and eventually the demise of cardiomyocytes. Reperfusion and reoxygenation of the infarcted tissue, as a result of e.g., pharmacological dissolution or dislodgement of the clot, ameliorates the extent of hypoxia-induced cell death, but in turn inflicts lethal reperfusion injury\(^2\). The type of cell death that is manifested depends on how fast reoxygenation occurs as a result of reperfusion\(^2\) and may proceed via necrosis, apoptosis, or autophagy. Cell survival is mainly mediated by activation of anti-apoptotic proteins and stimulation of pro-survival autophagy\(^3-5\).

The colorless, odorless, non-anesthetic noble gas helium has been shown to reduce the extent of cell death in myocardial, neuronal and epithelial tissue subjected to ischemia/reperfusion (I/R), as reviewed in\(^6\). In the heart, helium preconditioning considerably reduced infarct size in animal models of cardiac I/R by coronary artery ligation (summarized in Figure 1). Helium postconditioning (HePOC), the clinically more relevant form of conditioning, also protects the myocardium, as has been demonstrated in several rat strains\(^7,8\). Some mechanistic insight into the damage-ameliorating effects of helium gas during I/R has been provided in the last few years\(^9\). Apoptotic pathways have been shown to be involved in helium conditioning\(^10-12\).

Given the fact that helium preconditioning and HePOC reduce the extent of cell death following I/R (Figure 1), helium may affect the transcriptional regulation of cell death and survival pathways and thereby promote pro-survival signaling. Here we used a regional cardiac I/R model in rats to determine the differential expression patterns of genes related to apoptosis, necrosis, and autophagy following ischemia, I/R, or I/R with different regimes of HePOC. Transcriptional analysis of these pathways not only allowed us to test the hypothesis that HePOC reduces the magnitude of death signaling and stimulates survival pathways, but also provided insight in the significance of each mode of cell death and cell survival in every phase of I/R under native conditions and following HePOC.
Figure 1. Summary of literature in which the infarct sizes are plotted as a percentage of area at risk after helium preconditioning. Data are plotted as means. * = p<0.05 vs. control (CON).

Infarct size reduction by early preconditioning (EPC) entails application of five-minute cycles of helium inhalation before the ischemic episode. EPC1, EPC3, and EPC5 consist of 1, 3, or 5 five-minute cycles of helium before ischemia, respectively. Late preconditioning (LPC) is based on 15 min of helium administration 24 h before the ischemic episode and also results in infarct size reduction.

A: rats, n=8 per group, 25 min of ischemia and 2 h of reperfusion; B: rabbits, n=6 per group, 30 min of ischemia and 3 h of reperfusion; C: rabbits, n=6/7 per group, 30 min of ischemia and 3 h of reperfusion; D: rabbits, n=7/8 per group, 30 min of ischemia and 3 h of reperfusion; E: rats, n=8 per group, 25 min of ischemia and 2 h of reperfusion; F: rabbits, n=10 per group, 25 min of ischemia and 2 h of reperfusion; G: rabbits, n=6/7 per group, 30 min of ischemia and 3 h of reperfusion; H: rabbits, n=8 per group, 30 min of ischemia and 3 h of reperfusion; I: rabbits, n=8 per group, 30 min of ischemia and 3 h of reperfusion; J: rats, n=8-12 per group, 25 min of ischemia and 2 h of reperfusion.
MATERIALS AND METHODS

Animal model of ischemia/reperfusion

Animal experiments were approved by the institute's animal ethics committee (DAA102650). Animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). Male Wistar rats (354-426 g) were acclimatized for 7 d under conditions of 12-h light and dark cycles and ad libitum access to food and water.

Rats were anesthetized and surgically prepared as described previously8. In short, rats were mechanically ventilated and the carotid artery was cannulated for measurement of mean arterial pressure and heart rate and for blood sampling. The left anterior descending coronary artery (LAD) was ligated with a single puncture 5-0 Prolene suture through the myocardium. The ends of the suture were threaded through propylene tubing to enable tightening and loosening of the snare for the induction of ischemia and reperfusion, respectively. The helium postconditioning groups received helium gas (Linde Gas Benelux, Dieren, The Netherlands) at the onset of reperfusion (Figure 2). To ensure that sufficient helium was present in the lungs and normal air washed out at the onset of reperfusion, helium administration was started at 24 min of ischemia.

Study design (Figure 2)

The study was divided into two test arms for the determination of (1) histological damage and (2) quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) experiments.

Histological staining and analysis

For histological processing of the hearts, the organs were mounted on a modified Langendorff setup and perfused retrograde with isotonic saline solution to wash out blood from the coronary circulation. Next, the coronary circulation was flushed with 10 mL of ice-cold fixative (96% ethanol, acetic acid, 10% buffered formalin and MilliQ water in a 50:5:10:35 volume ratio). Adequate retrograde perfusion was confirmed visually by uniform changes in size and color of the myocardium. The heart was then removed from the Langendorff setup and stored in ice-cold fixative. Fixed rat hearts were dehydrated in graded steps of ethanol and xylene and cut into half, longitudinally at the center of the ligation of the LAD (Online Resource 1). The left halve of the
A. Histological analysis

<table>
<thead>
<tr>
<th></th>
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<td>25'</td>
</tr>
<tr>
<td>I</td>
<td>15'</td>
<td>Occlusion</td>
</tr>
<tr>
<td>I/R5</td>
<td>15'</td>
<td>Occlusion</td>
</tr>
<tr>
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<tr>
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<td>15'</td>
<td>Occlusion</td>
</tr>
<tr>
<td>He30</td>
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B. qRT-PCR

<table>
<thead>
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</thead>
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<td>Sham</td>
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<td>25'</td>
</tr>
<tr>
<td>I</td>
<td>15'</td>
<td>Occlusion</td>
</tr>
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</tr>
<tr>
<td>He15</td>
<td>15'</td>
<td>Occlusion</td>
</tr>
</tbody>
</table>

Figure 2. Schematic overview of the experimental protocols. Panel A: rats were sacrificed after 25 min of ischemia and 5, 15 or 30 minutes of reperfusion for histological analysis; I = ischemia, I/R5 = ischemia 25 min/reperfusion 5 min, I/R15 and I/R30: respectively reperfusion 15 and 30 min, He5 = ischemia 25 min/reperfusion 5 min with 5 min of helium postconditioning, He15 and He30: respectively with 15 and 30 min of helium postconditioning. Panel B: rats were sacrificed after ischemia or 15 min of reperfusion; Sham = no ischemia/reperfusion, I = ischemia, I/R15 = ischemia 25 min/reperfusion 15 min, He15 = ischemia 25 min/reperfusion 15 min with 15 min of helium postconditioning.
heart was embedded in paraffin and sectioned with a microtome along the cutting plane (4-μm thick sections). The sections were deparaffinized, stained with hematoxylin and eosin (H&E), and mounted on glass slides using VectaMount (Vector Laboratories, Burlingame, CA).

A histological scoring system was developed to semi-quantitatively analyze the extent of myocardial damage in I/R-subjected rat hearts and to determine the extent of cardiomyocyte protection by HePOC. The scoring parameters are listed in Table 1. H&E-stained sections were viewed under a light microscope (Confocal Microscope SP-8X, Leica). The area at risk was delineated on the basis of the pathological demarcation zone at low power view (10x magnification), whereby the puncture wound from the suture was used as an anchor point. The area at risk always extended from the periphery of the puncture wound to the apical end. Semi quantitative scoring of histopathological parameters of myocardial damage (contraction band necrosis, interstitial edema, granulocyte adherence/extravasation, extravasation of erythrocytes) was performed at higher power magnification (400x) in the full thickness of the myocardium.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Score</th>
<th>Quantitative value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Contraction bands/coagulation necrosis</td>
<td>0</td>
<td>Absent</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1-10% of smooth muscle cells</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>11-50% of smooth muscle cells</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>51-100% of smooth muscle cells</td>
</tr>
<tr>
<td>2. Interstitial edema</td>
<td>0</td>
<td>Absent</td>
</tr>
<tr>
<td></td>
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<td>Present</td>
</tr>
<tr>
<td>3. Granulocyte infiltration</td>
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</tr>
<tr>
<td></td>
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<td>Present</td>
</tr>
<tr>
<td>4. Platelet aggregates/thrombi</td>
<td>0</td>
<td>Absent</td>
</tr>
<tr>
<td></td>
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<td>Present</td>
</tr>
<tr>
<td>5. Extravasation of red blood cells</td>
<td>0</td>
<td>Absent</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Present</td>
</tr>
</tbody>
</table>

Table 1. Histological scoring parameters as used to semi-quantitively score myocardial damage in the different experimental groups.
endocardial segment and an epicardial segment of myocardium were scored separately. All parameters were scored in 4 fields of view (FOVs) per segment in the area at risk (x400).

**qRT-PCR**

At the end of the experiment (Figure 2B) the heart was excised under deep anesthesia. The area at risk was cut from the rest of the myocardium and sliced in two pieces on ice, which was snap frozen in liquid nitrogen and stored at -80 °C until further analysis. All analyses were performed in myocardial tissue at risk.

RNA was extracted from heart tissue using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Venlo, The Netherlands) according to manufacturer's instructions. All materials and reagents used in the procedure were from Qiagen, unless stated otherwise. In brief, ±30 mg of heart tissue was added to 300 μL of RLT lysis buffer containing 1% (v/v) alpha-mercaptoethanol and homogenized with a MagNA Lyser using MagNA Lyser Green Beads (both from Roche Applied Sciences, Penzberg, Germany). The lysate was collected and 600 μL of nuclease-free water containing 1.67% (v/v) proteinase K was added to digest residual fibrous tissue (6 min at 55°C). Total RNA was extracted using spin columns, including on-column DNAse I treatment, and eluted in 30 μL of nuclease-free water. Next, eluate RNA concentrations were determined by UV-vis absorbance spectroscopy (NanoDrop, Thermo Scientific, Rockford IL). RNA yields typically ranged from 0.3 to 3 μg/μL (data not shown). To ensure RNA purity, samples with an A260/A280 ratio of ≤1.99 were excluded from further analysis.

Next, 1 μg of RNA was reverse transcribed to cDNA using the RT2 First Strand Kit according to manufacturer's instructions. For optimal temperature control, cDNA synthesis was performed on a PTC-200 thermal cycler (MJ Research, Waltham, MA). The reaction conditions are included in Online Resource 2. cDNA was subsequently mixed with RT2 SYBR Green qPCR Mastermix and nuclease-free water according to the manufacturer's instructions, loaded (25 μL/well) onto the Rat Cell Death PathwayFinder PCR Array (SABiosciences catalogue number #PARN-212A), and run on a LightCycler 480 (Roche Applied Sciences). The run parameters are specified in Online Resource 2.

Melting curve analysis was performed at the end of the PCR run. Amplicons that showed amplification of non-specific products were excluded from analysis. The data was further processed according to Ruijter and colleagues. Each amplicon was corrected for baseline fluorescence and a common fluorescence threshold (Nq) for all arrays was set in the upper half of the log-linear phase of the amplification plot. Amplicons that did not reach Nq before cycle 40 were considered undetectable. Next, the individual PCR efficiencies were calculated and amplicons with an efficiency <1.80 or >2.00 were
excluded from further analysis. The individual efficiencies were subsequently used to calculate the starting concentration (N0) per amplicon. All samples were normalized to the mean N0 of the housekeeping genes that showed the most stable expression over all arrays (i.e., Ldha and Rplp1). Two comparisons were made. First, the differences between experimental groups (I, I/R15 and He15) and the sham group was calculated according to

\[
\frac{\left( \frac{N_0_{\text{GOI}}}{N_0_{\text{Housekeeping}}} \right)_{\text{I/R}}}{\left( \frac{N_0_{\text{GOI}}}{N_0_{\text{Housekeeping}}} \right)_{\text{sham}}}
\]

and expressed as fold difference versus the sham group. The GOI (gene of interest) within one group is thus first normalized against the housekeeping gene, afterwards a comparison between each experimental group and the sham group was made. Heat maps of these expression profiles were generated using Mayday Microarray Data Analysis software. A total of 84 genes involved in cell death and survival pathways were investigated and divided in 4 categories: necrosis, pro-apoptosis, anti-apoptosis and autophagy. For a description of each gene see Online Resource 3. Due to multiple roles for some genes, the total amount of genes in each category was 27 (necrosis), 23 (pro-apoptosis), 14 (anti-apoptosis) and 33 (autophagy). After exclusion of genes with insufficient n-numbers in either group due to an aberrant melting curve, efficiency, or undetectable levels of mRNA, a total of 20 (necrosis), 21 (pro-apoptosis), 14 (anti-apoptosis) and 29 (autophagy) genes were included in the heatmaps.

Secondly, a comparison between the I/R and He15 group was made. In order to do so, the means of the He15 group were divided by the means of the I/R group and presented as fold increase. Gene selection was performed according to the criteria as described above. A total of 20 (necrosis), 20 (pro-apoptosis), 14 (anti-apoptosis) and 30 genes (autophagy) were included in the analysis.

**Statistical analysis**

Statistical analysis was performed in GraphPad Prism (GraphPad Software, La Jolla, CA). Baseline hemodynamics were analyzed using one way ANOVA with a Tukey post hoc test for multiple comparisons. Differences in mRNA expression levels between the I/R and He15 group were tested using a Mann-Whitney test. A P-value of ≤0.05, indicated with an asterisk in the figures, was considered significant.
RESULTS

Hemodynamic parameters

Aortic pressure and heart rate (mean ± S.D.) during the experiments are shown in Table 2. Baseline hemodynamics did not vary between groups.

Histological damage profiles

Histological analysis was performed to assess cell damage at a microscopic level (n=4-6 per group). The morphology of cardiomyocytes clearly differed among the experimental groups. Hearts in the sham group contained normal unaltered cardiomyocytes; myofibrils were ordered in a structured manner and exhibited similar morphology. The absence of cardiomyocyte damage, inflammatory cell infiltrates, red blood cell extravasation, thrombosis, and edema resulted in a mean total histology scores of 0. Representative micrographs of sham-operated animals are shown in Figure 3B and Online Resource 4A.

After 25 min of ischemia, hypercontraction of myofibers was observed in some slices (Online Resource 4B), without loss of the native structure and morphology of cardiomyocytes similar to the sham operated group. In the 5-min reperfusion group, waviness of myofibers and contraction bands could be observed and were accompanied by other signs of tissue damage (Online Resource 4C). At 15 or 30 min of reperfusion the cardiac tissue exhibited clear signs of damage, that entailed necrosis, interstitial edema, granulocyte infiltration, platelet aggregates/thrombi, and extravasation of red blood cells (Figure 3C). This was observed in all histological specimens (Online Resources 4D and 4E) and was reflected in the total histology score (Figure 3A).

Fifteen min of HePOC reduced the extent of cell damage, which was reflected in the trend of an overall lower total histology score in this group compared to all other intervention groups (Figure 3A). Interestingly, 15 minutes of HePOC resulted in less cell damage compared to 30 min of helium, indicating that prolonged helium inhalation is not beneficial for cardiac I/R. The morphological features of cardiomyocytes that had been exposed to 30 min of HePOC were similar to those of cardiomyocytes in the I/R15, I/R30 and He5 group (Online Resource 4D, 4E and 4H). A representative micrograph of myocardium exposed to I/R15 and He15 is shown in Figure 3C and 3D, respectively. The extent of necrosis, edema, extravasation of red blood cells, granulocyte infiltration, and platelet aggregation is much lower after He15 (Figure 3D) compared to I/R15 (Figure 3C). This is also reflected in the total histology score (Figure 3A).
Table 2. Hemodynamics (mean arterial pressure and heart rate) sampled after 15 min of baseline, 24 min of ischemia, and 15 min of reperfusion. Data are shown as mean +/- SD. At baseline, there were no significant differences between different experimental groups. Mean AP = mean arterial pressure in mmHg, mean HR= mean heart rate in beats/minute.

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|                   |          |              |                |                 |                 |
| **Mean heart rate (BPM)** |        |              |                |                 |                 |
| Sham              | 372 +/- 20 | 373 +/- 20  | 363 +/- 24     | 352 +/- 32      |                 |
| I                 | 341 +/- 40 | 367 +/- 32  |                |                 |                 |
| I/R5              | 338 +/- 29 | 354 +/- 29  | 330 +/- 18     |                 |                 |
| I/R15             | 329 +/- 60 | 340 +/- 50  | 326 +/- 46     | 306 +/- 44      | 320 +/- 39      |
| I/R30             | 329 +/- 62 | 359 +/- 39  | 342 +/- 38     | 346 +/- 41      |                 |
| He5               | 363 +/- 36 | 366 +/- 16  | 344 +/- 35     |                 |                 |
| He15              | 338 +/- 35 | 365 +/- 36  | 350 +/- 37     | 324 +/- 34      |                 |
| He30              | 343 +/- 57 | 366 +/- 36  | 347 +/- 47     | 359 +/- 40      | 328 +/- 51      |
Figure 3. Panel A. Total histology score plotted per group. Panels B-D: Representative histological sections of hearts in the sham, I/R15, and He15 group. Slices are 400 times enlarged. E indicates tissue edema, P indicates presence of platelets and thrombi, RBC indicates extravasation of red blood cells, GRA indicates extravasation of granulocytes and stars indicate contraction band or coagulation necrosis.
mRNA expression profiles in hearts subjected to ischemia, ischemia/reperfusion and HePOC

Exposure of cardiomyocytes to I, I/R15 and He15 caused differential gene expression in all cell death pathways compared to cardiomyocytes in the sham-operated group (Figure 4). Many genes of the cell death pathways in the I and I/R15 groups are regulated in a similar manner, i.e. the expression patterns of genes involved in the execution of necrosis, apoptosis and autophagy are affected similarly under both conditions of ischemia and I/R. To specify this: most genes are similarly up- or downregulated after I and I/R15. This can be seen from the actual amount of genes that are up- or downregulated in each group of genes. Fifteen out of 20 genes involved in necrosis pathways were downregulated after I and 14/20 after I/R15. Simultaneously, genes involved in pro-apoptosis were upregulated (I 11/21; I/R15 12/21) and anti-apoptotic genes were downregulated (10/14 in both groups). I and I/R15 also downregulated most genes involved in autophagy; 21/29 and 23/29, respectively.

Addition of 15 min of helium postconditioning changed the expression profiles of whole sets of genes. This can be easily seen when one compares the last column to the third column. Helium particularly upregulated genes involved in anti-apoptotic pathways (10/14) and autophagy (16/29). In contrast, I and I/R15 downregulated the majority of anti-apoptotic genes and genes involved in autophagy. This suggests that the protective effect of 15 min of helium postconditioning is linked to expression of genes involved in autophagy and anti-apoptotic signaling.

Figure 4 first of all shows that gene expression patterns that are visible during reperfusion already emerge during ischemia. Additionally it shows that exposure to a short 15-minute episode of HePOC is powerful enough to exert changes on expression patterns of genes involved in cell death pathways, which occurs already during early reperfusion. Taken together, the data suggests that effects of helium postconditioning are immediate and undo some of the detrimental changes in gene expression that have been initiated during ischemia and are extended during reperfusion.

Effects of HePOC on cell death, cell survival, and autophagy

Beside the general changes in gene expression profiles during ischemia, reperfusion and POC in comparison to sham animals, a direct comparison between the I/R15 and He15 group has been made. In Figure 5, the fold increase and decrease of each gene within one of four categories of cell damage/survival after He15 in comparison to I/R15 is depicted. This figure shows which genes are upregulated and downregulated (most strong upregulation to downregulation from left to right in the figure) after HePOC
and could therefore play a key role in helium’s underlying cardioprotective mechanism.

In line with results shown in Figure 4, in comparison to I/R15, He15 particularly upregulates genes involved in autophagy and anti-apoptosis: 27/30 genes involved in autophagy were upregulated, and 12/14 anti-apoptotic genes. This underlines the general finding that the cardioprotective mechanism of HePOC is related to an increase in expression of genes employed in autophagy and against apoptosis.

Apart from general trends, individual genes were found to be significantly upregulated after He15 in comparison to I/R15. Within the necrosis group of genes, Olr1583, Symp2, and...
Cybb, Txnl4b, and Dpysl4 were all significantly upregulated after He15 in comparison to I/R15 only. A short description of each gene is given in Online Resource 3 and the relation of these genes to HePOC will be addressed in the discussion.
Figure 5. Regulation of genes after 15 min of helium postconditioning shown as fold increase or decrease in comparison to I/R15 per category. * p<0.05
DISCUSSION

In this study helium-induced postconditioning was investigated by microscopic assessment of cell damage and analysis of its transcriptional effects on cell death and survival pathways (necrosis, apoptosis and autophagy). We showed that signs of cell damage in H&E-stained histological slices were reduced after 15 min of helium. Additionally we showed that in comparison to I/R15 only, He15 upregulated genes in all categories; necrosis, pro- and anti-apoptosis and autophagy. However, He15 predominantly upregulated genes involved in autophagy and inhibition of apoptosis. Taken together, these data suggested that the HePOC-induced reduction of I/R-induced cell damage, is mediated by an instantaneous upregulation of genes employed in autophagy and the inhibition of apoptosis during early reperfusion. We therefore suggest that the upregulation of these genes at least counterbalance or even overrule the upregulation of the pro-death genes, resulting in cardioprotection after HePOC.

In earlier studies from our laboratory, infarct size reduction (analyzed by triphenyl tetrazolium chloride (TTC) - Evans blue staining) after HePOC was found in several rat strains. Fifteen min of 70% helium during early reperfusion reduced infarct size as percentage of area at risk from 47 ± 2% (mean ± S.E.M.) in control to 30 ± 2% in the HePOC group (Online Resource 5), while the area at risk as percentage of total ventricular tissue was similar in both groups; 21 ± 2% in CON and 22 ± 2% (data not shown). This is in line with the results of the current study, in which histological analysis showed less cellular damage after He15 in comparison to I/R15. He15 slices exhibited less contraction band necrosis, edema, extravasation of RBC and granulocytes and less platelets/thrombi than I/R15 slices. Interestingly, histological slices of 30 min of helium showed excessive cellular damage in comparison to 15 min of helium. Again, this is in line with results from infarct size experiments analyzed by TTC-staining, in which 30 min of helium during early reperfusion abrogated the protection that was seen after 15 min of HePOC.

From enzymatic and histologic assessment of cell damage after HePOC we therefore drew the conclusion that (1) helium reduces cardiomyocyte damage which results in smaller infarct size and (2) it does so only after 15 min of HePOC. This led to the investigation of transcriptional profiles of genes employed in cell death and survival pathways after 15 min of HePOC. Although mRNA expression profiles after 25 min of ischemia were also analyzed in this study, we focused on reperfusion because that seems to be the time window in which HePOC exerts its effects. Histological analysis showed a reduction of cell damage after HePOC at 15 min of reperfusion. From TTC-staining we know that the protective effect of 15 min of HePOC can also be found after 2 h of
reperfusion\(^7,8,21\); 1 h and 45 min after the postconditioning stimulus is discontinued. It is very likely that within this time window both necrotic and apoptotic cell death are reduced by helium postconditioning, and that pro-survival mechanisms such as autophagy contribute substantially. We aimed to investigate whether signs of cellular survival on a mRNA expression level could be found as early as the 15 min reperfusion episode.

Separate studies investigating the effects of helium conditioning on specific proteins and their concomitant posttranslational modifications in the cell death and survival pathways have been conducted\(^9\). However, cell death and survival pathways interact and various ways of cellular stress might trigger necrotic, apoptotic and autophagic pathways simultaneously, leading to activation of common downstream cell death elements or might offset each other\(^1\). We therefore used PCR arrays to investigate 4 categories of cell death/survival simultaneously. Generally it is thought that necrosis occurs quickly and centrally, whereas apoptotic cell death takes a bit longer due to the slowly orchestrated execution of the apoptotic cell death program and mainly occurs in the border zone of the area at risk\(^3\). In this study we investigated gene expression in the area at risk, to find out which type of cell death is particularly affected by HePOC. The trend of the current study suggests that orchestration of genes against apoptosis and pro-autophagy leads to the reduced cellular damage that is found in histological analyses at 15 min of reperfusion. This could make sense, as programmed cell death might play a far more important role than anticipated: in rats, chronic (7 d) ligation of a coronary artery resulted in a peak-myocyte death within the first 4.5 h after ligation in which apoptosis predominated\(^22\).

Autophagy is originally categorized as a survival mechanism in which cells consume their own proteins, lipids and organelles in order to maintain protein and organelle quality and to provide amino acids, energy and free fatty acids in case of nutrient deficiency. Cell constituents and parts of the cytoplasm are first engulfed in autophagosomes, after which fusion with lysosomes take place. Hereafter, degradation and recycling occurs. It is suggested that once this process becomes overactive, it becomes detrimental to cells and might end in autophagic cell death or in another type of cell death, such as apoptosis\(^5,23,24\). Yet, inhibition of autophagy might lead to cellular damage, for example due to an increased sensitivity to apoptotic signs, stressing the potential pro-survival role of autophagy\(^25\). In vivo, sevoflurane late preconditioning\(^26\) and ischemic POC\(^27\) increased autophagic vacuoles and reduced infarct size.

In the current study, we found a simultaneous upregulation of genes employed in pathways against apoptosis and pro-autophagy; this could relate to the infarct size reduction that was observed after HePOC\(^7,8,21\) as well as the results from histological analysis in this study. Three significantly regulated genes - Becn1 and Sqstm1 (autophagy)
and Nol3 (anti-apoptosis) - in this analysis were of particular interest. The inactivation of Beclin-1 (Becn1), a protein involved in autophagosome formation, reduced infarct sizes28 thereby suggesting a detrimental effect of autophagosome formation on infarct size. Becn1 loses its pro-autophagic function after interaction with Bcl-2, which is logic according to the hypothesis that an increased rate of autophagy in apoptotic cells probably leads to cell death29. Thus, a combined downregulation of the anti-apoptotic Bcl-2 with an upregulation of Becn1 during reperfusion facilitates cell death, presumably necrosis. In our study, I/R caused a downregulation of both Becn1 and Bcl-2 in comparison to sham, which was diminished by helium postconditioning. These findings are comparable to results from a study in rats, in which ischemic postconditioning increased protein- and mRNA-levels of Beclin-127.

Sqstm1 (Sequestosome 1) is a stress-inducible intracellular protein affecting autophagy activity. Protein levels of Sqstm1 are regulated by autophagy as they are degraded in its machinery, but Sqstm1 is also involved in the delivery of ubiquitinylated cargo to autophagosomes30. The latter is particularly important with regard to mitochondrial damage caused by I/R injury and the need to clear cells from mitochondrial debris. In mice, infarct size reduction after ischemic postconditioning was accompanied by translocation of Sqstm1 to mitochondria and presence of Parkin, an E3 ubiquitin ligase. In Parkin -/- mice the translocation of Sqstm1 to mitochondria was absent and infarct size reduction was blunted31. These findings are in line with our results, as the I/R-induced downregulation of Sqstm1 was attenuated after helium postconditioning.

Nol3, nucleolar protein 3, also known as apoptosis repressor with caspase recruitment domain (ARC), inhibits apoptosis on a level further down the apoptotic cascade as it directly binds to and inhibits caspase-8 activity. Phosphorylation of ARC by protein kinase CK2 (CK2) activates this protein, while calcineurin dephosphorylates and inactivates ARC32. Not only did ARC overexpression decrease infarct size after I/R5, anesthetic-induced preconditioning was associated with an increase of phosphorylation of ARC, a reduction in activity of calcineurin and a reduction in caspase-8 activity and cytochrome c release32. This in line with our results; I/R decreases Nol3 expression while He15 increases it.

Although Figure 5 is particularly useful to observe trends, it also shows that some genes categorized in the necrotic pathway were upregulated significantly after He15: Olr1583, Sycp2, Cybb, Txn4 and Dpysl4. A short description of the genes are given in Online Resource 3. After a quick glance at these genes, it looks as if some unexpected genes play a role in cardioprotective mechanisms. Olr1583 and Dpysl4 are not directly known to be expressed in the heart, but do play a role in HePOC. Olr1583 (Olfactory receptor 1583), member of the olfactory gene family, is predominantly found in the olfactory epithelium of the nose and is involved in the recognition of specific odorants33.
However, evidence exists that at least one specific member of the olfactory receptor family also exists in the heart. Olfactory receptor 1 transcripts were detected in the developing heart, suggesting that the olfactory receptor might play a role in cardiac development. In the current study we show that Olr1583 is expressed in the heart and that this expression is downregulated by I/R, but upregulated after He15. Dpysl4 (dihydropyrimidinase-like 4) -also known as CRMP3- is expressed in the developing brain but its function is unclear. An inhibitory effect on brain development as well as a crucial role in neurite outgrowth and axonal differentiation has been described. We showed a downregulation of CRMP3 in heart tissue after I/R in comparison to sham operated animals and an attenuation of downregulation after He15.

Two other genes - Sycp2 and Txnl4b - are involved in the cell cycle and biological diversity. Sycp2 (synaptonemal complex protein 2) is part of the so-called synaptonemal complex, a meiosis-specific nuclear structure that is involved in recombination of chromosomes during the prophase, resulting in chromosomal crossover. Sycp2 therefore plays a role in genetic variation. Txnl4b (Thioredoxin-like 4B) or DIM-2 is a gene required in cell cycle progression as it transits cells from the synthesis (S) –phase to the Growth 2 (G2) phase, and it is involved in pre-mRNA splicing. Pre-mRNA leads to different types of mRNA, which in turn result in different proteins. In a way it plays a role in the establishment of the huge diversity of proteins that exists in eukaryotes. I/R downregulated both Sycp2 and Txnl4b, while He15 attenuated the downregulation of Sycp2 and even upregulated Txnl4b.

Cybb (Cytochrome b-245, beta polypeptide) -also known as Gp91-phox or Nox2- encodes for a protein called cytochrome b-245, which is a constituent of the NADPH-oxidase. The NADPH-oxidase produces superoxide and hydrogen peroxide in phagocytes that need them for killing of pathogens, in vascular smooth muscle cells, endothelial cells and cardiomyocytes. Nox2 is upregulated in infarcted areas after myocardial infarction in the rat and after hypoxia-reoxygenation in porcine coronary artery endothelial cells (PCAEC). Pharmacologic or genetic blockade of the NADPH oxidase in PCAECs reduced the hypoxia/reoxygenation induced reactive oxygen species levels. Interestingly this was accompanied by a reduction in vessel outgrowth, suggesting a role for the NADPH oxidase in angiogenesis and neovascularization.

Nox2 and NADPH oxidase upregulation might be associated with cell death due to increased levels of oxidative stress, however under certain circumstances upregulation could be pro-survival. It is not unlikely that the outcome depends on the intensity and extent of the ROS signal, the present kinases and caspases in the tissue, the stress signal that induced it and the type of tissue. Nox-induced ROS was shown to be involved in the differentiation of cardiomyocytes from embryonic stem cells and neonatal cardiomyocytes. Nox2 expression peaked at embryonic day 12, suggesting a critical role in the differentiation process.
role for Nox2 in early cardiomyogenesis. As Nox2 is also upregulated after myocardial infarction, it is not unlikely that it plays a role in differentiation of cardiac progenitor cells. In our study, I/R downregulated the Cybb/Nox2 gene while He15 upregulated it. Taken together, the relative upregulation of Cybb/Nox2, Sycp2 and Txnl4b suggest that HePOC is possibly related to organ development and cell reproduction. This idea is underlined by the finding that Olr1583 and Dpysl4 are expressed in the heart and affected by HePOC.

A limitation of this study is the lack of subsequent experiments investigating proteins corresponding with the found genes/ mRNA expression profile. Increased levels of mRNA do not necessarily translate in a rise of the correlative protein. Furthermore, posttranslational modifications often determine net function and the effect of a protein. Our study does indicate that HePOC exhibits a wide array of effects on cell death and survival. A clue as to the underlying mechanism of HePOC could be found within the complex interplay of the above-mentioned cellular processes. Future research should in any case comprise anti-apoptotic and autophagic pathways.

In conclusion, helium-induced cardioprotection by 15 min of POC appears to be associated with activation of pro-survival cell mechanisms. Helium influences the balance between pro- and anti-apoptosis, in favor of genes directed against apoptosis. Simultaneously, it stimulates genes involved in autophagy and possibly cell reproduction and tissue development. This suggests that helium exerts its protective effects through a cell surviving mechanism that comprises a whole set of pathways.
NON-STANDARD ABBREVIATIONS AND ACRONYMS

POC: postconditioning; helium postconditioning: HePOC
REFERENCES


Helium postconditioning and cell death- and survival pathways


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29. Takagi H, Matsui Y, and Sadoshima J. The role of autophagy in mediating cell survival and death during ischemia and reperfusion in the heart. Antioxid Redox Signal. 2007 10(9): 1373-81


38. Simeoni F, and Divita G. The Dim protein family: from structure to splicing. Cellular and Molecular Life Sciences. 2007 64(16): 2079-2089


Helium postconditioning and cell death- and survival pathways


Online Resource 1. Preparation of histological slices. Hearts were cut in half, through the ligation of the LAD.
Online Resource 2. cDNA synthesis. RNA was reverse transcribed to cDNA using the RT2 First Strand Kit (Qiagen, catalogue number #330401) according to manufacturer’s instructions. Before starting the procedure, the RNA concentration of all samples was diluted to 250ng/μL with nuclease-free water. Next, 1μg (4μL) of RNA was mixed with 2μL of GE-buffer and 2μL of nuclease-free water and incubated at 42°C for 5min to eliminate any residual DNA. Following incubation at 4°C (1min), 10μL of reverse transcriptase mix was added to each 10μL sample and reverse transcription was started by incubating the samples at 42°C for 15min and subsequently terminated by raising the temperature to 92°C for 5min. Last, 91μL of nuclease-free water was added to each sample.

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Helium postconditioning and cell death- and survival pathways

5

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- NF-κB  
- Arc  
- Adprt2  
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- Osi, ZIP, ZIP3  
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Online resource 4A. Representative slices of three sham animals, 200 times enlarged. The pictures clearly show normally structured cardiomyocytes without edema or signs of cell damage.
Online resource 4B. Representative slices of three animals that underwent 25 minutes of ischemia, 200 times enlarged. Arrows indicate ischemic hypercontracture, E indicates edema.
Online resource 4C. Representative slices of three animals that were exposed to 25 minutes of ischemia and 5 minutes of reperfusion, 200 times enlarged. E indicates edema, RBC indicates extravasation of red blood cells and asterisk indicates contraction band necrosis.
Online resource 4D. Representative slices of three animals that were exposed to 25 minutes of ischemia and 15 minutes of reperfusion, 200 times enlarged. E indicates edema, RBC indicates extravasation of red blood cells, GRA indicates extravasation of granulocytes and asterisk indicates contraction band necrosis.
Online resource 4E. Representative slices of three animals that were exposed to 25 minutes of ischemia and 30 minutes of reperfusion, 200 times enlarged. E indicates edema, RBC indicates extravasation of red blood cells, GRA indicates extravasation of granulocytes and stars indicate contraction band necrosis.
Online resource 4F: Representative slices of three animals that were exposed to 25 minutes of ischemia and 5 minutes of reperfusion with helium postconditioning for 5 minutes, 200 times enlarged. Arrows indicate hypercontracture, E indicates edema, RBC indicates extravasation of red blood cells, GRA indicates extravasation of granulocytes, P indicates presence of platelets and thrombi and asterisk indicates contraction band necrosis.
Online Resource 4G. Representative slices of three animals that were exposed to 25 minutes of ischemia and 15 minutes of reperfusion with helium postconditioning for 15 minutes, 200 times enlarged. GRA indicates extravasation of granulocytes, P indicates presence of platelets and thrombi, and asterisk indicates contraction band necrosis.
Online Resource 4H. Representative slices of three animals that were exposed to 25 minutes of ischemia and 30 minutes of reperfusion with helium postconditioning for 30 minutes, 200 times enlarged. E indicates edema, RBC indicates extravasation of red blood cells, GRA indicates extravasation of granulocytes, P indicates presence of platelets and thrombi and asterisk indicates contraction band necrosis.
Online Resource 5
Infarct sizes as percentage of area at risk. Data are plotted as mean ± S.E.M. * refers to statistical significance of HePOC vs CON. Below the graph, representative TTC-stained cross-sections of myocardium are shown for the control (CON) and 15-min HePOC group. Infarct sizes from 3 earlier studies\textsuperscript{4-6} were compiled and analyzed using an unpaired Student's t test.
REFERENCES


Animal studies
Helium-induced early preconditioning and postconditioning are abolished in obese Zucker rats in vivo

Ragnar Huhn, Andre Heinen, Nina Weber, Raphaela Kerindongo, Gezina Oei, Markus Hollmann, Wolfgang Schlack, Benedikt Preckel

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INTRODUCTION

Diabetes mellitus is a known risk factor for the development of ischemic heart disease and myocardial infarction. It was shown that acute myocardial infarction is consistently associated with an increased mortality in patients with type 2 diabetes. Furthermore, diabetes mellitus is associated with a loss of the protective potency of cardioprotective strategies, e.g. preconditioning, both in humans and animals. Katakam et al. demonstrated that both, ischemic and pharmacological preconditioning by the mitochondrial ATP-activated potassium (mK\textsubscript{ATP}) channel agonist diazoxide is abolished in Zucker obese rats, a widely used animal model of insulin resistance and type 2 diabetes.

Recently it was shown that exposure to the noble gas helium initiates a pronounced protection of the myocardium against ischemia reperfusion injury. Helium is easy and safe to administer, and when compared to volatile anesthetics or xenon, the absence of anesthetic effects, as well as the lack of hemodynamic side effects would make helium an optimal agent for cardioprotection. These properties might offer the possibility to use helium in various groups of patients, e.g. during the perioperative period in patients at risk for cardiac events, as well as in non-surgical patients, e.g. in patients with unstable angina or myocardial infarction. Helium is already safely used in the therapy of asthma and chronic obstructive pulmonary disease, as well as in young children with ventilation disorders.

Cardioprotective effects of helium are mediated by activation of prosurvival signaling kinases and prevention of mitochondrial permeability transition pore (mPTP) opening. Opening of the mPTP can be regulated by different mechanisms including alterations in mitochondrial bioenergetics or regulation of glycogen synthase kinase-3beta (GSK-3beta) activity. The underlying mechanism, however, by which helium confers cardioprotection via mPTP is unknown.

We aimed to investigate (1) if the noble gas helium initiates cardiac preconditioning in the Zucker obese rat in vivo, and (2) the underlying sub-cellular mechanism by which helium prevents mPTP opening, i.e. regulation of mitochondrial bioenergetics and/or inhibition of prosurvival kinase dependent pathways.
METHODS

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and were approved by the Institutional Committee for Animal Care and Use (Academic Medical Center Amsterdam, The Netherlands).

Materials

Helium was purchased from Linde Gas (Linde Gas Benelux BV, Dieren, The Netherlands). KCl was purchased from EMD Chemicals (Gibbstown, NJ, USA); all antibodies were purchased from Cell Signaling Technology Inc. (Danvers, USA) except the anti-alpha-tubulin and the anti-actin antibodies (Sigma, Saint Louis, USA). All other chemicals were purchased from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands). Rat insulin samples were measured with a Rat Insulin ELISA from Orange Medical (Orange Medical, Tilburg, The Netherlands).

Surgical preparation

Animals had free access to food and water at all times before the start of the experiments. Male Zucker lean rats (248±5 g) and male Zucker obese rats (334±5 g) were anesthetized by intraperitoneal injection of S-ketamine (150 mg/kg) and Diazepam (1.5 mg/kg).

Surgical preparation was performed as described previously. Briefly, after tracheal intubation, the lungs were ventilated with 30% oxygen and 70% nitrogen and a positive end-expiratory pressure of 2-3 cm H_2O. During the experiments the endtidal CO_2 (etCO_2) concentration was measured in the expiratory gas (Datex Capnomac Ultima, Division of Instrumentarium Corp., Helsinki, Finland). Respiratory rate was adjusted to maintain etCO_2 between 35 - 45 mmHg. Body temperature was maintained at 38°C by the use of a heating pad. The right jugular vein was cannulated for saline and drug infusion, and the left carotid artery was cannulated for measurement of aortic pressure. Anesthesia was maintained by continuous alpha-chloralose infusion. A lateral left sided thoracotomy followed by pericardiotomy was performed and a ligature (5-0 Prolene) was passed below a major branch of the left coronary artery.

All animals were left untreated for 20 minutes before the start of the respective experimental protocol. Aortic pressure was digitized using an analogue to digital converter (PowerLab/8SP, ADInstruments Pty Ltd, Castle Hill, Australia) at a sampling rate of 500 Hz and were continuously recorded on a personal computer using Chart for...
Experimental protocol

Rats were assigned to seven groups (Figure 1): Animals for infarct size measurements underwent 25 min of coronary artery occlusion and 2 hours of reperfusion (I/R). Zucker lean control group (ZL Con, n=8): after surgical preparation rats received 30% oxygen/70% nitrogen. Zucker lean helium preconditioned group (ZL He-PC, n=8): rats received helium 70%/30% oxygen for three 5-min periods, interspersed with two 5-min wash-out periods 10 min before ischemia and reperfusion. Zucker obese control group (ZO Con, n=8): after surgical preparation rats received 30% oxygen/70% nitrogen. Zucker obese helium preconditioned group (ZO He-PC, n=8): rats received helium 70%/30% oxygen for three 5-min periods, interspersed with two 5-min wash-out periods 10 min before ischemia and reperfusion. Zucker obese helium preconditioned group (ZO He-PC (6x), n=8): rats received helium 70%/30% oxygen for six 5-min periods.

Figure 1. Experimental protocol. ZL = Zucker lean, ZO = Zucker obese, Con = Control, He-PC = helium preconditioning, He-PostC = helium postconditioning.
interspersed with five 5-min wash-out periods 10 min before ischemia and reperfusion. In two additional groups we investigated whether we could induce cardioprotection in ZO rats by helium postconditioning. In these groups ZL and ZO rats (each n=8) received helium 70%/30% oxygen for 15 min at the onset of reperfusion

Infarct size measurement

After 120 minutes of reperfusion, the heart was excised and mounted on a modified Langendorff apparatus for perfusion with ice cold normal saline via the aortic root at a perfusion pressure of 80 cm H₂O in order to wash out intravascular blood. After 2 minutes of perfusion, the coronary artery was re-occluded and the remainder of the myocardium was perfused through the aortic root with 0.2% Evans blue in normal saline for 10 minutes. Intravascular Evans blue was then washed out by perfusion for 10 minutes with normal saline. This treatment identified the area at risk as unstained. The heart was then cut into transverse slices, 2 mm thick. The slices were stained with 0.75% triphenyltetrazoliumchloride (TTC) solution for 10 minutes at 37°C, fixed in 4% formalin solution for 24 hours at room temperature. The area of risk and the infarcted area were determined by planimetry using SigmaScan Pro 5® computer software (SPSS Science Software, Chicago, IL) and corrected for dry weight of each slide.

To investigate the effects of helium preconditioning on mitochondrial respiration, additional experiments (n = 8 for each group) were conducted using the same preconditioning protocol except that the hearts were excised 5 min after the third helium administration (see Fig. 1). The effect of helium preconditioning on phosphorylation of the enzymes GSK-3beta (Ser9), Akt (Thr308 and Ser473) and extracellular regulated kinase (Erk1/2) (p42/p44) were determined in additional experiments in group 1-4 at four different time points (n = 4 for each time point in duplicate): time point 1 after the first helium administration, time point 2 after the third helium administration, time point 3 after 15 minutes of ischemia and time point 4 after 15 minutes of reperfusion (see Figure 1).

Mitochondrial isolation

Heart mitochondria were isolated by differential centrifugation as described previously. Briefly, atria were removed and ventricles were placed in isolation buffer [200 mmol/L mannitol, 50 mmol/L sucrose, 5 mmol/L KH₂PO₄, 5 mmol/L 3-(n-morpholino)propanesulfonic acid, 1 mmol/L Ethylene glycol-bis(2-aminoethylether)-N,N,N’,N’-tetraacetic acid, 0.1% bovine serum albumin, pH 7.15 adjusted with KOH], and minced into 1 mm³ pieces. The suspension was homogenized for 15 sec in 2.5 ml
isolation buffer containing 5 U/ml protease, and for another 15 sec after addition of 17 ml isolation buffer. The suspension was centrifuged at 3220g for 10 min, the supernatant was removed, and the pellet was resuspended in 25 ml isolation buffer and centrifuged at 800g for 10 min. The supernatant was centrifuged at 3220g for 10 min, and the final pellet was suspended in 0.5 ml isolation buffer and kept on ice. Protein content was determined by the Bradford method\textsuperscript{14}. All isolation procedures were conducted at 4°C.

**Mitochondrial respiration**

Oxygen consumption was measured polarographically at 37°C using a respirometric system (System S 200A, Strathkelvin Instruments, Glasgow, Scotland). Mitochondria (0.3 mg protein/ml) were suspended in respiration buffer containing 130 mmol/L KCl, 5 mmol/L K$_2$HPO$_4$, 20 mmol/L 5 mmol/L 3-(n-morpholino) propanesulfonic acid, 2.5 mmol/L Ethylene glycol-bis(2-aminoethylether)-N,N',N''-tetraacetic acid, 1 µmol/L Na$_4$P$_2$O$_7$, 0.1% bovine serum albumin, pH 7.15 adjusted with KOH. Mitochondrial respiration was initiated by administration of 10 mmol/L complex II substrate succinate (+10 µmol/L complex I blocker rotenone) after 60 sec. State 3 respiration was initiated after 120 sec by addition of 200 µmol/L ADP. Respiration rates were recorded under state 3 conditions and after complete phosphorylation of ADP to ATP (State 4). The respiratory control index (RCI, state 3/state 4) and the P/O ratio (phosphate incorporated into ATP to oxygen consumed) were calculated as parameter of mitochondrial coupling between respiration and oxidative phosphorylation, and mitochondrial efficiency, respectively. From each heart, respiration measurements were repeated in 3 mitochondrial samples and the average was taken (and counted as n = 1). Respiration rates are expressed as absolute rates in nmol O$_2$/mg/min.

**Separation of cytosolic fraction**

For cellular fractionation and subsequent Western blot assay, tissue specimens were prepared for protein analysis of GSK-3beta (Ser9), Akt (Thr308 and Ser473) and Erk1/2 (p42/p44), respectively. The excised hearts were frozen in liquid nitrogen. Subsequently, a cellular fractionation was performed as described previously\textsuperscript{15}. The frozen tissue was pulverized and dissolved in lysis buffer containing: Tris base, EGTA, NaF and Na$_3$VO$_4$ (as phosphatase inhibitors), a freshly added protease inhibitor mix (aprotinin, leupeptin and pepstatin) and DTT. The solution was vigorously homogenized on ice (Homogenisator, IKA, Staufen, Germany) and then centrifuged at 1000 g, 4°C, for 10 min. The supernatant, containing the cytosolic fraction, was centrifuged again at 16000 g, 4°C, for 15 min to clean up this fraction for further Western blot assay.
Western blot analysis

After protein concentration was determined by the Lowry method\(^6\) equal amounts of protein were prepared and loaded on a 10% SDS-PAGE gel. The proteins were separated by electrophoresis (100 V, 85 min) and then transferred to a PVDF membrane by tank blotting (100V, 1h). To prevent unspecific antibody binding the membrane was subsequently blocked with 5% skimmed milk solution in Tris buffered saline containing Tween (TBS-T) for 2 hours. Then, the membrane was incubated over night at 4°C with the respective primary antibody GSK-3beta (1:10.000), Akt(Thr308)(1:1000), or Akt(Ser473)(1:1000), Erk1/2 (p42/p44)(1:10.000). After washing in fresh, cold TBS-T, the blot was subjected to the appropriate horseradish peroxidase conjugated secondary antibody for 2 hours at room temperature. Immunoreactive bands were visualized by chemiluminescence detected on X-ray film (Amersham Hyperfilm ECL, GE Healthcare Limited, United Kingdom) using the enhanced chemiluminescence system Santa Cruz (Santa Cruz Biotechnology, Inc., Santa Cruz). The blots were quantified using a Kodak Image station\(^*\) (Eastman Kodak Comp., Rochester, NY) and the results are presented as ratio of phosphorylated protein to total protein. Equal loading of protein on the gel was additionally proved by detection of alpha-tubulin or actin, respectively, and Coomassie blue staining of the gels.

Statistical Analysis

Data are expressed as mean ± standard error of the mean (SE). Heart rate (in bpm) and mean aortic pressure (in mmHg) were measured during baseline, coronary artery occlusion, and reperfusion period. Comparisons of hemodynamics between groups or between time points in a group were performed (SPSS Science Software, version 12.0.1) using two-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Infarct sizes were analyzed by one-way ANOVA followed by Tukey's post-hoc test. Data from mitochondrial and Western blot experiments were analyzed by Student's t-test with Bonferroni's correction for multiple comparisons. Changes within and between groups were considered statistically significant if p<0.05.
RESULTS

Infarct size measurement

Helium preconditioning reduced infarct size in ZL rats from 52 ± 3 % in controls to 32 ± 2 % (p<0.05; Figure 2). In ZO control rats, infarct size was similar to ZL controls (54 ± 3 %, n.s. vs. ZL Con). In contrast to the protection seen in ZL rats, in ZO rats did helium not reduce infarct size (56 ± 3 %, n.s.; Figure 2).

Figure 2. Histogram shows the infarct sizes as percent of area at risk (AAR). ZL = Zucker lean, ZO = Zucker obese, Con = Control, He-PC = helium preconditioning, He-PostC = helium postconditioning. Data are presented as mean ± SE, *p < 0.05 vs. ZL Con.
Furthermore, an increased preconditioning stimulus by 6 cycles of helium could not protect the ZO rat heart (57 ± 4 %, n.s.; Figure 2). Helium postconditioning reduced infarct size in ZL rats (37 ± 2 %, p<0.05; Figure 2). This effect was also completely abolished in ZO rats (51 ± 3 %, n.s.; Figure 2).

Hemodynamic variables

Hemodynamic variables are summarized in Table 1. No significant differences in heart rate and aortic pressure were observed between the experimental groups during baseline, ischemia and reperfusion. At the end of the experiments, mean aortic pressure was significantly decreased compared with baseline in all groups with the exception of the ZO control group.

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<td>15 min</td>
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<td>ZL He-PostC</td>
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<td>ZO He-PC (6x)</td>
<td>407 ± 7</td>
<td>389 ± 13</td>
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<td>375 ± 9</td>
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<td>ZO He-PostC</td>
<td>400 ± 7</td>
<td>392 ± 3</td>
<td>398 ± 10</td>
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| **Mean AOP (mmHg)** |          |         |          |             |
| ZL Con            | 116 ± 6  | 105 ± 9 | 96 ± 7   | 80 ± 6'     | 81 ± 10'    | 69 ± 6'     |
| ZL He-PC          | 120 ± 7  | 103 ± 5 | 90 ± 8   | 74 ± 9'     | 62 ± 8'     | 67 ± 9'     |
| ZL He-PostC       | 110 ± 5  | 95 ± 4  | 87 ± 6'  | 70 ± 5'     | 58 ± 3'     | 60 ± 3'     |
| ZO Con            | 111 ± 14 | 106 ± 15| 100 ± 17 | 81 ± 11     | 73 ± 11     | 66 ± 10     |
| ZO He-PC          | 120 ± 4  | 113 ± 4 | 107 ± 7  | 91 ± 10     | 81 ± 11     | 60 ± 8'     |
| ZO He-PC (6x)     | 117 ± 3  | 101 ± 6 | 103 ± 4  | 86 ± 4'     | 61 ± 4'     | 62 ± 2'     |
| ZO He-PostC       | 121 ± 2  | 117 ± 3 | 106 ± 6  | 83 ± 5'     | 73 ± 6'     | 63 ± 2'     |

Table 1. Hemodynamic variables. Data are Mean ± SE. ZL = Zucker lean; ZO = Zucker obese; Con = control group; He-PC = helium preconditioning, He-PostC = helium postconditioning. *p<0.05 vs. baseline.
Weights and blood glucose levels

The body weights (g) of ZO rats were significantly higher than in ZL rats (Table 2). Blood glucose levels were not different between groups (Table 2). Insulin levels were significantly higher in ZO compared to ZL rats (Table 2).

Mitochondrial respiration

Mitochondrial respiration results are summarized in Figure 3. There was no significant difference in the RCI between ZL (n = 8) and ZO (n = 7) control rats (2.51 ± 0.03 vs. 2.52 ± 0.03, n.s.). Helium preconditioning reduced the RCI in ZL rats (2.27 ± 0.03; n = 8; p<0.05 vs. ZL Con), but had no effect on the RCI in ZO rats (2.52 ± 0.04; n = 8; n.s. vs. ZO Con). The reduction in the RCI in ZL He-PC was caused by an increase in state 4 respiration (155 ± 4 nmol O₂/mg/min vs. 139 ± 3 nmol O₂/mg/min, p<0.05); state 3 respiration was not affected by helium preconditioning in both ZL and ZO rats.

There was no difference between all groups in the efficiency of oxidative phosphorylation as demonstrated by no changes in the P/O ratio.

Regulation of GSK-3beta, Akt and Erk1/2 phosphorylation during helium preconditioning

Figure 4 shows that there were no differences at any time point in Akt and Erk1/2 phosphorylation during the experiments in ZL and ZO rats. Helium reduced GSK-3beta phosphorylation during ischemia in ZL rats compared with respective controls (0.49 ± 0.07 vs. 0.72 ± 0.07, p<0.05; Figure 4A3).
Table 2: Blood glucose levels and weights and heart dry weights. Data are Mean ± SD. ZL = Zucker lean; ZO = Zucker obese; Con = control group; He-PC = Helium preconditioning. *p<0.05 vs. ZL Con; #p<0.05 vs. ZL He-PC.

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<th>Blood sugar (mmol L⁻¹)</th>
<th>Insulin levels (nmol L⁻¹)</th>
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<th>Heart dry weight (g)</th>
<th>Area at risk (%)</th>
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<td>6.7 ± 0.1</td>
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</tbody>
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Figure 3. Summarized data for the effects of helium preconditioning on mitochondrial respiration. ZL = Zucker lean, ZO = Zucker obese, Con = Control, He-PC = helium preconditioning. RCI = respiratory control index, a parameter for the coupling between mitochondrial respiration and oxidative phosphorylation. P/O ratio = ratio between phosphate incorporated into ATP and oxygen consumed; a parameter for the efficiency of oxidative phosphorylation. Data are presented as mean ± SE, *p < 0.05 vs. ZL Con.

Figure 4. Effects of Helium-induced preconditioning on GSK-3beta (Ser9) (panel A), Akt (Thr308 and Ser473) (panel B and C) and Erk1/2 (panel D and E) phosphorylation. Summarized data presenting ratio of phosphorylated enzyme to total enzyme are shown. Time point 1 after the first helium administration (A1- E1 respectively), time point 2 after the third helium administration (A2 - E2 respectively), time point 3 after 15 min of ischemia (A3 - E3 respectively), time point 4 after 15 min of reperfusion (A4 - E4 respectively), LC = Zucker lean control, OC = Zucker obese control, LH = Zucker lean helium preconditioning, OH = Zucker obese helium preconditioning. Data are presented as mean ± SE, *p < 0.05 vs. LC.
Helium conditioning in the obese Zucker rat
DISCUSSION

The main findings of our study are that the cardioprotective effect of helium-induced preconditioning a) is abolished in the prediabetic rat heart, and b) is mediated in the non-diabetic heart rather by regulation of mitochondrial respiration, i.e. mild mitochondrial uncoupling, than by activation of pro-survival signaling kinases. We also demonstrate that helium induces postconditioning, but that this protection is abolished in the prediabetic rat.

It is well known that besides brief periods of ischemia, also pharmacological interventions can initiate cardiac preconditioning to enhance the resistance of the myocardium against ischemia and reperfusion injury. Helium is a non-anesthetic gas without significant hemodynamic side effects. These properties would make helium an ideal agent for cardioprotection in patients with cardiovascular disease not only in the perioperative setting like during cardiac surgery, but also for interventional procedures like during percutaneous coronary interventions.

There is evidence that the protective potency of both ischemic and pharmacological preconditioning is diminished in the diabetic heart. In the present study, we show that besides the rabbit heart also the rat heart can be protected by helium induced pre- and postconditioning. Furthermore, we demonstrate that the cardioprotective effect of helium is abolished in the prediabetic rat heart. Kristiansen et al. demonstrated in obese Zucker diabetic fatty and lean Goto-Kakizaki rats, two widely used rat models of type 2 diabetes, that ischemic preconditioning does not reduce infarct size. In contrast, Tsang et al. showed that preconditioning can be induced in hearts from Goto-Kakizaki rats, but the threshold that is required to achieve preconditioning is elevated in diabetic compared with non diabetic hearts. In our study, even six cycles of helium preconditioning did not result in infarct size reduction in ZO rats (Figure 2). The experimental diabetes models used in the two earlier cited studies were characterized by a significant hyperglycemia. Katakam et al. demonstrated that both, ischemic and pharmacological preconditioning induced by the mK\textsubscript{ATP} channel agonist diazoxide, are abolished in Zucker obese rats. The experiments were conducted in 10-12 weeks old rats. At this age, ZO rats are hyperinsulinemic and normoglycemic, representing a prediabetic state of type 2 diabetes. In the present study, we used 10-12 weeks old ZO rats that were also normoglycemic and hyperinsulinemic (Tab. 2), and our results are in line with the earlier findings showing that cardioprotection is abolished in the prediabetic heart (Fig. 2).

The mechanism by which helium-induced preconditioning is blocked in the prediabetic heart is unknown. Ischemic and pharmacological preconditioning failed to
Helium conditioning in the obese Zucker rat

protect the diabetic myocardium, possibly caused by dysfunctional potassium channels in the inner mitochondrial membrane. Alterations in mitochondrial function caused by potassium channel activation have been proposed to protect the myocardium by reducing mPTP opening. Helium-induced preconditioning is abrogated by administration of the mPTP opener atracyloside. It has also been suggested that preconditioning prevents mPTP opening by regulation of prosurvival signaling kinases including Erk1/2, Akt and GSK-3beta, and/or by regulation of mitochondrial bioenergetics, i.e. mild uncoupling of mitochondrial respiration. Pagel et al. demonstrated that helium preconditioning is abolished by 5-hydroxydecanoate (5-HD), a mK_ATP channel blocker. Interestingly, activation of the K_ATP channel with Diazoxide could not induce preconditioning in the Zucker obese rat heart, suggesting that the blockade of Diazoxide-induced preconditioning is related to defects at the level of the mK_ATP channel or its downstream signaling cascade.

Very recently we could demonstrate that opening of another mitochondrial potassium channel, namely the mitochondrial calcium sensitive potassium channel (mK_Ca) is involved in helium-induced preconditioning. This study, together with the study from Pagel suggest a crucial role of mitochondrial potassium channels in helium preconditioning. mK_Ca channel opening causes a slight increase in mitochondrial reactive oxygen species generation. Stowe et al. demonstrated that the cardioprotective effect of the mK_Ca channel agonist 1,3-Dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one (NS1619) requires superoxide radical generation during the preconditioning stimulus. Furthermore, preconditioning by NS1619 reduces mitochondrial calcium overload and mitochondrial reactive oxygen species production during the subsequent period of ischemia and early reperfusion. Such a reduction in mitochondrial calcium overload and reactive oxygen species generation has been suggested to prevent mPTP opening. Both, mK_ATP and mK_Ca channel activation trigger preconditioning independent from each other and by involvement of the mPTP.

Our results suggest that regulation of mitochondrial respiration is involved in helium-induced preconditioning: helium induced a reduction of the respiratory control index in ZL rats while it had no effect on mitochondrial function in ZO rats. Furthermore, the helium-induced effect on mitochondrial respiration was abolished before the onset of lethal ischemia. Based on these data we suggest that the prediabetic related blockade of helium-induced preconditioning is related to defects at the level of the mitochondria or mitochondrial potassium channels, respectively, or its upstream signaling cascade. It has been shown that mitochondrial uncoupling is capable of inducing cardioprotection: pharmacological uncoupling by 2,4-dinitrophenol or FCCP reduced infarct size in rat heart in vitro.
CHAPTER 6

The role of pro-survival signaling kinases in helium-induced preconditioning remains unclear. In the rabbit heart, the protective effect of helium was blocked by pharmacological inhibition of phosphatidylinositol-3-kinase, extracellular signal-regulated kinase, and 70-kDa ribosomal protein s6 kinase. Hausenloy et al. demonstrated for ischemic preconditioning that Akt and Erk1/2 were phosphorylated before and after the ischemic period compared to control group\textsuperscript{32}. In the current study, we tested time dependent phosphorylation of GSK-3beta, Akt and Erk1/2. We did not detect an effect of helium on Erk1/2 and Akt phosphorylation. GSK-3beta shows a decreased activity in Zucker lean helium treated rats after 15 minutes of ischemia compared with respective controls. The importance of Akt phosphorylation in the signal transduction of ischemic preconditioning was demonstrated by Tsang et al\textsuperscript{19}. In their study, preconditioning caused an increased Akt phosphorylation 5 min after the last preconditioning cycle, i.e. the same timing of tissue sampling as we used in the present study. However, to our knowledge, there is no evidence that “pro-survival kinases” activate mitochondrial \(K_{Ca}\) channels to regulate mitochondrial function. Recently, it was shown that adrenomedullin treatment prior to ischemia reduces infarct size via protein kinase A mediated activation of m\(K_{Ca}\) channels\textsuperscript{33}. This effect was independent of phosphatidylinositol-3-kinase. In the present study, we did not test whether protein kinase A is involved in helium preconditioning.

The results of the present study have to be interpreted within the scope of some limitations. First, our experiments were conducted in Zucker obese and Zucker lean rats. Zucker obese rats have a Leptin receptor mutation and develop obesity at an early age\textsuperscript{34,35}. The Zucker obese rat is described to be hyperphagic compared to lean littermates from an early age on and obese condition is evident at 5 weeks. In the present study, feeding of the Zucker obese rat was not different from Zucker lean rats. However, we did not measure differences in caloric intake. Second, in the present study we did not investigate the effect of helium on the mPTP directly. However, it has already been demonstrated that helium confers cardioprotection by prevention of mPTP opening\textsuperscript{5}. Therefore, the present study was designed to investigate the effect of helium on possible regulators of the mPTP (i.e. mitochondrial respiration, GSK-3beta phosphorylation). Third, we did not investigate other possible avenues of preconditioning like endothelial or inducible nitric oxide synthase and or potassium channels in the present study. It was shown that helium preconditioning is mediated by endothelial but not by inducible nitric oxide synthase\textsuperscript{36}. All these enzymes reported to be involved in helium preconditioning are located upstream of mitochondrial potassium channels and the mitochondria. Abolished mitochondrial uncoupling in the Zucker obese rat heart before lethal ischemia together with no effects on the expression of enzymes of the prosurvival cascade, suggest a blockade of cardioprotection in the prediabetic heart caused by yet unknown mechanisms.
Taken together, the present study demonstrates that the noble gas helium can induce pre- and postconditioning in the rat heart in vivo. The protective effect of preconditioning could be explained by mild mitochondrial uncoupling, an alteration that is capable to prevent mPTP opening. Furthermore, the protective potency of helium-induced preconditioning is completely abrogated in the Zucker obese rat, a widely used animal model for prediabetic conditions of state 2 diabetes. Whether this cardioprotection can be re-established in the prediabetic heart by further pharmacological intervention needs further investigation.
REFERENCES


Helium conditioning in the obese Zucker rat


Helium-induced cardioprotection of healthy and hypertensive rat myocardium in vivo

Gezina Oei, Ragnar Huhn, Andre Heinen, Markus Hollmann, Wolfgang Schlack, Benedikt Preckel, Nina Weber
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INTRODUCTION

Helium is an odorless and colorless noble gas that does not induce anesthesia and lacks hemodynamic side effects. Despite supposed inertness, organ protective properties for helium have been described in the heart, the brain and blood vessels. In experimental studies in healthy animals it was shown that helium induces early preconditioning (EPC), late preconditioning (LPC) and postconditioning (PostC) of the heart.

Reperfusion after acute myocardial infarction by thrombolysis, percutaneous coronary intervention or coronary artery bypass surgery is the best therapeutic strategy to reduce definitive myocardial cell loss. “Conditioning” results in a further reduction of cell damage after reperfusion of a tissue exposed to a prolonged episode of ischemia. Postconditioning is the most feasible kind of conditioning in the clinical arena, as ischemic events often have already occurred upon arrival of the patient in the hospital.

The majority of patients with coronary artery disease is aged and suffers from multiple co-existing (chronic) diseases. Pathological conditions such as hypertension and/or myocardial hypertrophy, hyperlipidemia, diabetes, insulin resistance, atherosclerosis, heart failure, but also aging cause intrinsic changes of the myocardium. This makes them more susceptible for tissue damage, and might also abolish the tissue salvaging effect of cardioprotective interventions such as ischemic or pharmacologic conditioning. An example is the less pronounced infarct size reduction after EPC in isolated hearts from hypertensive rats in comparison to healthy controls. In guinea pigs, anesthetic EPC by sevoflurane was abolished in larger/older hearts in comparison to smaller/younger hearts.

In this study we used the Spontaneously Hypertensive Rat (SHR), a rat model of essential hypertension in which cardiac hypertrophy can already occur at 13 weeks of age. We hypothesized that a possible protective effect of helium PostC can be enhanced by a combination with LPC and EPC through an additive or synergistic mechanism, enabling us to protect the hypertensive heart.

Protein kinase C isoform epsilon (PKC-epsilon) activation after pharmacologic preconditioning comprises phosphorylation and subcellular redistribution. Increased phosphorylation of PKC-epsilon after xenon and isoflurane preconditioning with three 5-min cycles was found 10 min after application of the last preconditioning stimulus. These results were supported by administration of PKC-epsilon –blockers, resulting in loss of phosphorylation. The increased phosphorylation after isoflurane and xenon preconditioning was positively correlated with infarct size measurements, however, the protective ischemic preconditioning protocol was not affected by administration of PKC-epsilon –blockers and did not result in increased phosphorylation of PKC-
Zatta and colleagues found increased phosphorylation of PKC-epsilon after ischemic postconditioning at 30 minutes (min) of reperfusion\(^\text{13}\). Involvement of glycogen synthase kinase-3 beta (GSK-3beta) in helium-induced preconditioning was shown by the use of the GSK-blocker SB 216763\(^\text{15}\). Increased phosphorylation of GSK-3beta after isoflurane postconditioning was found directly after the end of the protective stimulus at 15 min of reperfusion\(^\text{16}\). Use of volatile anesthetics as a postconditioning stimulus exerts important immediate effects during early reperfusion, possibly by affecting mitochondria, the end-target of many signaling transduction pathways\(^\text{17}\).

We hypothesized that—considering the well-known role of both PKC-epsilon and GSK-3beta in ischemic and pharmacologic conditioning—a cardioprotective effect of helium-induced conditioning involves phosphorylation and subcellular redistribution of PKC-epsilon and GSK-3beta. We postulated that a reduction of infarct size after application of a conditioning stimulus is positively correlated with activation of the above-mentioned signaling kinases and that whenever effects after helium-induced conditioning are found; they emerge immediately after the end of the stimulus.
CHAPTER 7

MATERIALS AND METHODS

Ethics statement

The study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and was performed in accordance with the requirements of the Animal Ethics Committee of the University of Amsterdam, The Netherlands. The study was approved by the Animal Ethics Committee of the University of Amsterdam, The Netherlands and received approval number 101141.

Materials

Helium was purchased from Linde Gas (Linde Gas Benelux B.V., Dieren, The Netherlands). For western blot analysis, we used phospho-GSK-3beta (Ser9) and total-GSK-3beta rabbit polyclonal antibody, phospho-PKC-epsilon and total-PKC-epsilon rabbit polyclonal antibody purchased from Bio Connect (Huissen, The Netherlands), anti-alpha-tubulin mouse monoclonal antibody purchased from Sigma-Aldrich (Saint Louis, Missouri, USA), voltage-dependent anion selective channel rabbit polyclonal antibody purchased from Calbiochem, Merck (Darmstadt, Germany). For creation of infrared western blots, we used Odyssey Blocking Buffer, Millipore Immobilon FL polyvinylidene fluoride Membranes, infrared Dye 800CW conjugated goat (polyclonal) anti-mouse, IRDye 680 CW conjugated goat (polyclonal) anti-rabbit, all purchased at LI-COR (Westburg, Leusden, The Netherlands). All other chemicals were purchased from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands).

Animal preparation

Animals had free access to food and water at all times before start of the experiments. Male Wistar Kyoto rats (WKY rats) and SHR (both aged 12-14 weeks) were anesthetized by intraperitoneal S-ketamine injection (150 mg/kg) and diazepam (1.5 mg/kg). Spontaneously hypertensive rats start to develop elevated blood pressures at the age of 3 weeks, and blood pressure continues to rise until the age of 20-28 weeks\textsuperscript{11}. Surgical preparation for infarct size experiments was performed as described previously\textsuperscript{18}. 
Helium conditioning in the hypertensive rat

Experimental Protocol: Infarct size measurements and hemodynamics

Rats from each strain (WKY rats and SHR) where assigned to one of four groups (Figure 1). Sample size analysis for our primary endpoint infarct size revealed a required number of rats being 8 per group. The input for the power calculation was based on similar studies from our laboratory, with an expected difference in mean of infarct size of 18%, an expected difference in standard deviation of 12%, a power of 80% and a type I error of 0.05. Complementary to the rats needed for a total of 64 successful experiments, we calculated 13 rats for the expected drop out of 20%. We therefore performed 68 successful experiments for this study. We had 9 dropouts due to failure of staining of the area at risk or fatal rhythm disorders.

All animals were left untreated for 20 min before the start of the respective experimental protocol. The aortic pressure signal from the fluid filled pressure catheter was digitized using an analogue-to-digital converter (Powerlab/8SP, ADInstruments Pty Ltd, Castle Hill, Australia) at a sampling rate of 500 Hertz and continuously recorded on a personal computer using Chart for Windows, version 5.0 (ADInstruments Pty Ltd). Regional ischemia of 25 minutes was induced by tightening the snare, followed by 120 min of reperfusion (verified by the disappearance of epicardial cyanosis) induced by loosening the snare. Animals were all mechanically ventilated with 30% oxygen and 70% nitrogen during the experiment, except during conditioning protocols EPC and PostC, when 30% oxygen and 70% helium was given. Frequency and pressure were adjusted to maintain carbon dioxide and pH at physiologic levels; approximately 50/min with positive end-expiratory pressures of 2-3 cm water column. Late preconditioning was induced by placement of the animals in a chamber for 15 min 24 hours before ischemia and exposing them to 30% oxygen and 70% helium.

After 120 min of reperfusion, the heart was excised in deep anesthesia and treated for further analysis as described previously. The area of risk and the infarcted area were determined by planimetry using SigmaScan Pro software (SPSS Science Software, Chicago, IL, USA).

Experimental Protocol: Molecular biology

For the involvement of the signaling kinases GSK-3beta and PKC-epsilon in helium-induced conditioning, we investigated the intervention groups with largest expected effect at key time points. Since activation of signaling kinases is most likely to be found in those groups with significant infarct size reduction, we investigated the PostC group in WKY rats and the EPC+LPC+PostC group in SHR. For this purpose, hearts were excised at the designated time points (see Figure 1).
Figure 1. Experimental protocols for infarct size measurements and molecular biology. EPC = early preconditioning, LPC = late preconditioning, PostC = postconditioning, Rep = reperfusion. Hemodynamic measurements: 1 = baseline, 2 = washout #3, 3 = 15 min ischemia, 4 = 24 min ischemia, 5 = 15 min reperfusion, 6 = 60 min reperfusion, 7 = 120 min reperfusion. WKY = normotensive Wistar Kyoto Rat, SHR = Spontaneously Hypertensive Rat.
Heart homogenization and fractionation

After excision, hearts were cut in four parts and frozen in liquid nitrogen and the tissue was kept in -80°C until further processing. For western blot assay investigating GSK-3beta and PKC-epsilon activation and distribution in the cell, tissue specimens were prepared for cellular fractionation (cytosolic and membranous fraction) according to literature. According to a cell fractioning protocol from our colleagues, we centrifuged heart homogenates at 1,000 g, 4°C, for 10 min to obtain a mitochondrial fraction for analysis of GSK-3beta in both WKY rats and SHR. This protocol was investigated in a study about myocardial hexokinase I and II content and activity after ischemic preconditioning, by measurement of the activity of citrate synthase (a mitochondrial marker), NADPH cytochrome-c reductase (a microsomal marker) and LDH (a cytosolic marker) in 3 different subcellular fractions. From these data, the 10,000 g pellet was designated as the mitochondrial fraction. Additionally, the distribution of the specific markers was unaffected throughout the ischemia-reperfusion protocol. The remaining supernatant was centrifuged again at 10,000 g, 4°C, for 15 min. The resulting pellet contained the mitochondrial fraction and was stored at -80°C until use for further Western blot assay.

Western blot analysis

After protein determination by the Lowry method, we prepared samples for western blot analysis as described previously. To prevent unspecific binding of the antibody we incubated membranes with Odyssey Blocking Buffer (LI-COR, Westburg, Leusden, The Netherlands) with 0.1% Tween (TBS-T) for 1 hour. Subsequently, the membrane was incubated over night at 4°C with the respective primary antibody phospho- or total GSK-3beta (1:5,000), phospho- or total PKC-epsilon (1:5,000), anti-alpha-tubulin (1:40,000) or voltage-dependent anion selective channel (1:10,000). After washing in fresh, cold TBS-T, the blot was subjected to the appropriate IRDye 800CW or IRDye 680 CW secondary antibody conjugated goat (polyclonal) anti-rabbit or anti-mouse for 1 hour at room temperature. Immunoreactive bands on the membrane were visualized by the two-channel laser system of the Odyssey system. The blots were quantified using the Odyssey IR Manager. Equal loading of the protein to the sodium dodecyl sulphatepolyacrylamide gel was ensured by Coomassie blue staining (Coomassie brilliant blue, Serva electrophoresis GmbH, Heidelberg, Germany) of each gel.
Statistical analysis of heart/body weight ratio, infarct size and hemodynamics

For statistical analysis we used SPSS Science Software version 18. For analysis of normal distribution of the data, the Kolmogorov-Smirnov test was used. Heart/body weight ratios were analyzed by students T-test and are shown as mean±standard error of the mean (S.E.M.). Infarct sizes were analyzed by one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test and are shown as mean±S.E.M. Differences were considered significant if P<0.05. Heart rate (in beats per minute) and mean aortic pressure (in millimeters of mercury) were measured at various time points during baseline, coronary artery occlusion and reperfusion. Significant differences over time in heart rate and mean aortic pressure between intervention groups and rat strains were investigated with a generalized linear univariate model.

Statistical analysis molecular biology

Western blot data in cytosolic, membranous or mitochondrial fractions were analyzed by student’s t test. In WKY rats, a comparison between control and PostC was made; in SHR we compared control and LPC+EPC+PostC groups at specific time points. Changes within and among groups were considered statistically significant if P<0.05.
RESULTS

Body and heart weight

In SHR, (n=33) both body weight (314±4 g) and heart weight (218±3 mg) were higher in comparison to bodyweights (293±3 g) and heart weights (188±4 mg) of WKY rats (n=33). The heart/body weight ratio was higher in SHR (0.70) than in WKY rats (0.64, P<0.05).

Hemodynamic variables

At baseline, mean aortic pressure and heart rate were significantly different between WKY rats and SHR, with a mean aortic pressure of 96±4 mmHg in WKY rats and 161±4 mmHg in SHR and a heart rate of 264±7/min in WKY rats and 372±6/min in SHR. During the course of the experiment, heart rate and mean aortic pressure in both SHR and WKY rats slowly decreased to similar levels. Exposure of WKY rats and SHR to helium conditioning protocols did not affect hemodynamics. For an overview of hemodynamics also see Table 1.
Table 1. Hemodynamics (heart rate and mean aortic pressure) during the experimental course. Data shown are mean ± S.D., a indicates P < 0.05 normotensive versus hypertensive rats at each time point. Time points are 1) baseline, 2) after 24 minutes of ischemia, 3) after 120 minutes of reperfusion.

<table>
<thead>
<tr>
<th>Hemodynamics</th>
<th>Baseline</th>
<th></th>
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<th>Reperfusion 120</th>
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<tbody>
<tr>
<td></td>
<td>WKY</td>
<td>SHR</td>
<td>WKY</td>
<td>SHR</td>
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<tr>
<td>Heart rate (BPM)</td>
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<td>CON</td>
<td>255 ± 36</td>
<td>370 ± 20</td>
<td>280 ± 55</td>
<td>361 ± 32</td>
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<tr>
<td>PostC</td>
<td>258 ± 33</td>
<td>371 ± 24</td>
<td>271 ± 33</td>
<td>346 ± 29</td>
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<td>LPC+PostC</td>
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<td>360 ± 30</td>
<td>261 ± 27</td>
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<td>CON</td>
<td>97 ± 18</td>
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<td>70 ± 14</td>
<td>132 ± 33</td>
<td>69 ± 18</td>
<td>78 ± 23</td>
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*Table 1. Hemodynamics (heart rate and mean aortic pressure) during the experimental course. Data shown are mean ± S.D., a indicates P < 0.05 normotensive versus hypertensive rats at each time point. Time points are 1) baseline, 2) after 24 minutes of ischemia, 3) after 120 minutes of reperfusion.*
Infarct size measurement

In WKY rats, helium PostC reduced infarct size compared to control (29±2 vs. 46±2% of area at risk, P<0.05, also see Table 2). The combination of helium LPC with PostC or the triple intervention EPC and LPC with PostC was similarly protective (30±3 and 32±2% of area at risk, with P<0.05 vs. control), but did not further reduce infarct size in comparison to PostC alone (P=0.99 and P=0.76, respectively). In SHR, only a triple intervention comprising helium EPC and LPC with PostC reduced infarct size in comparison to control (39±3 vs. 53±3% of area at risk, P=0.03), while helium PostC alone or in combination with helium LPC had no effect on infarct size (48±4 and 44±4% of area at risk, with P=0.69 and P=0.29, respectively). Pictures of representative myocardial slices are shown in Figure 2.

<table>
<thead>
<tr>
<th>Species</th>
<th>Groups</th>
<th>AAR as % of Total</th>
<th>IS as % of AAR</th>
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<tbody>
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<td>PostC</td>
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<tr>
<td>SHR</td>
<td>CON</td>
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<td>53 (3)</td>
</tr>
<tr>
<td></td>
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<td>LPC+PostC</td>
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<td></td>
<td>LPC+EPC+PostC</td>
<td>21 (4)</td>
<td>39 (3)*</td>
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</table>

Table 2. Infarct sizes. Area at risk (AAR) as % of total ventricular tissue and infarct size (IS) as percentage of AAR. Data are shown as mean±S.E.M., * indicates P < 0.05 in comparison to control.
Western blot experiments

In WKY rats, no differences in phosphorylation of GSK-3beta (Figure 3, panel A) and PKC-epsilon (Figure 3, panel B) in the cytosolic or membranous fraction were found between control and PostC at baseline, after 24 min of ischemia or 15 min of reperfusion. Data are shown as ratio of the phosphorylated target protein over the total amount of the target protein; equal protein loading was detected by alpha-tubulin. In SHR, no differences in GSK-3beta phosphorylation in the cytosolic or the membranous fraction were found between control and PostC at baseline or 15 min of reperfusion (Figure 4). Investigation of the mitochondrial fraction revealed no differences in phosphorylation of GSK-3beta in WKY rats between control and PostC (Figure 5, panel A) or in SHR between control and LPC+EPC+PostC (Figure 5, panel B), at baseline or 15 min of reperfusion. To assure equal protein loading, we detected the voltage-dependent anion selective channel – a constituent of the mitochondrial wall.
Figure 3. Phosphorylation of GSK-3beta and PKC-epsilon in WKY rats. Ratio of phosphorylated GSK-3beta over total amount of GSK-3beta and ratio of phosphorylated PKC-epsilon over total amount of PKC-epsilon in cytosolic and membranous fractions. Comparison between control and PostC at baseline, ischemia and reperfusion. All groups are n=6, except for one; this is shown in the figure with n=5. Data are shown as mean±S.E.M. Examples of representative western blots are shown above the graph and show the phosphorylated form, total protein and the internally used control tubulin. WKY = normotensive Wistar Kyoto Rat, SHR = Spontaneously Hypertensive Rat, EPC = early preconditioning, LPC = late preconditioning, PostC = postconditioning, GSK-3beta = glycogen synthase kinase-3 beta, PKC-epsilon = protein kinase C-epsilon.
Our main finding is that the hypertensive myocardium of young SHR can be protected against ischemia/reperfusion injury by a triple intervention of helium conditioning, while helium PostC as a single stimulus is not protective. This suggests the existence of a „threshold” in hypertensive myocardium, in which the combination of helium stimuli forms a stronger stimulus than each stimulus alone.

In healthy rabbit myocardium, the combination of ischemic LPC and EPC and ischemic LPC and sevoflurane-induced EPC led to a stronger infarct size reduction than each protective stimulus alone\textsuperscript{21,22}. These results suggest that application of a double preconditioning stimulus induces a stronger protective mechanism than a single preconditioning stimulus. In contrast, in dogs and rats it was shown that albeit protective when applied as a single stimulus, the combination of ischemic EPC and PostC did not provide extra infarct size reduction\textsuperscript{23,24}. The same was found for a combination of anesthetic conditioning with isoflurane: in young and healthy rats sevoflurane-induced

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Ratio_phosphoGSK3beta_over_totalGSK3beta.png}
\caption{Phosphorylation of GSK-3beta in SHR in cytosolic and membranous fractions. Figure shows the ratio of phosphorylated GSK-3beta over total amount of GSK-3beta in the cytosolic and membranous fractions in SHR. Comparison between control and EPC+LPC+PostC at baseline and reperfusion. All groups are $n=4$ and are measured in duplo. Data are shown as mean±S.E.M. SHR = Spontaneously Hypertensive Rat, EPC = early preconditioning, LPC = late preconditioning, PostC = postconditioning, GSK-3beta = glycogen synthase kinase-3 beta}
\end{figure}
EPC with PostC did not further reduce infarct size in comparison to sevoflurane EPC or PostC alone. This is in line with results in healthy animals from our study; the combination of LPC+PostC and EPC+LPC+PostC did not afford extra protection in comparison to PostC alone. It is possible that in healthy animals PostC alone triggered a maximum amount of protection.

Sato and colleagues investigated additive effects of ischemic conditioning in rats with exposure to 45- and 60-min coronary occlusions. After 45 minutes of coronary occlusion, ischemic LPC and PostC were found to be additive, however, PostC alone was not protective. Rats exposed to 60 minutes of ischemic injury could not be protected by LPC or PostC alone; only the combination of LPC and PostC led to a significant infarct size reduction. This observation supports the idea of a “threshold” of conditioning in animals with more severe injury due to extended exposure to ischemia. The inability to protect diseased or aging myocardium by a single stimulus of helium.
conditioning, e.g. EPC, was described in the prediabetic obese Zucker rat and in the aged Wistar rat\textsuperscript{3,27}. Intensifying the preconditioning stimulus in the prediabetic heart could not overcome the threshold for inducing an infarct size limiting effect\textsuperscript{3}. This suggests that the diseased myocardium needs to be stimulated with a combination of different conditioning stimuli at multiple time points.

It is generally accepted that hypertrophied hearts are more susceptible to ischemia/reperfusion injury than normotensive hearts\textsuperscript{6}. Rats in our study were aged between 12 and 14 weeks; SHR had higher heart rate and blood pressures as well as higher heart/body weight ratios than WKY rats. This is in line with literature in which increased heart/body weight ratio was described for 13-week old SHR\textsuperscript{10}. In animals with hypertension, the myocardium could be protected by ischemic preconditioning alone, showing that application of a single conditioning stimulus is sufficient to induce cardioprotection even in hypertensive myocardium\textsuperscript{28-30}. Regarding clinical practice, the value of the potentially dangerous ischemic stimulus is questionable, as application of additional ischemia might lead to additional tissue damage. Helium seems to be an interesting candidate as conditioning stimulus as it is easy applicable and harmless without proven side effects.

In this study we used different timeframes for the helium conditioning stimuli. EPC comprised 3 short cycles of helium (5 minutes each, with wash outs of 5 min in between and a final washout episode of 10 minutes). LPC and PostC were induced by 15 min of helium administration. Each of these stimuli induced cardioprotection in previous studies from our laboratory\textsuperscript{2,3}. To reduce the amount of stress and thereby possible effects of released catecholamines on the preconditioning effects, animals were not instrumented for measurement of heart rate and blood pressure during LPC. Helium itself has previously been demonstrated to lack any relevant hemodynamic effect in a similar model\textsuperscript{3}.

In healthy animals, ischemic PostC activates PKC-epsilon by increased phosphorylation in comparison to control\textsuperscript{14}. PKC-epsilon is also involved in anesthetic EPC and LPC and in xenon EPC\textsuperscript{12,13}. Xenon EPC not only increases phosphorylation of PKC-epsilon, but also induces translocation from cytosolic to membranous fractions\textsuperscript{13}. In our study, increased phosphorylation or cellular redistribution of PKC-epsilon could not be found in groups exposed to cardioprotective helium conditioning protocols, suggesting that infarct size reduction after helium-induced conditioning is mediated by other mechanisms than PKC-epsilon modification.

Studies in rabbits described involvement of the GSK-3beta-mitochondrial permeability transition pore pathway in helium-induced conditioning\textsuperscript{4,15,31}. Phosphorylation of GSK-3beta was not investigated directly but blockers of the enzyme working directly or indirectly on the GSK-3beta-mitochondrial permeability transition pore pathway
were used. Although enzyme specific, blockers often appear to be unspecific especially when used in vivo. Moreover, a recent study in genetically modified mice suggested that GSK-3beta is not the key determinant in ischemic EPC and PostC\(^3\). This is in line with an earlier study from our laboratory showing that helium EPC was not mediated by an increase of GSK-3beta phosphorylation\(^3\). In the current study, we did not find increased phosphorylation of GSK-3beta in cytosolic, membranous or mitochondrial fractions after helium-induced conditioning in healthy and hypertensive animals.

Currently, the mechanism of action of helium-induced conditioning is not known. An overview of signaling kinases and targets that are considered to be involved in helium-induced cardioprotection are given in an overview article by Oei et al\(^1\). Many of these targets have been investigated indirectly, for example by use of specific blockers. A recent study in young rats showed involvement of protein kinase A (PKA) in helium-induced infarct size reduction: administration of the PKA blocker H-89 before helium preconditioning completely abrogated myocardial protection\(^33\). PKA-activity after helium preconditioning was directly investigated by performing western blot analysis of myocardial phosphorylated and total cyclic adenosine monophosphate response element binding protein (CREB), a marker for PKA-activity. Helium preconditioning did not increase phosphorylated CREB levels\(^33\). Mild mitochondrial uncoupling was found after helium-induced early preconditioning\(^27\), but an involved pathway was not found.

The first limitation of the study is the use of a triple intervention with two stimuli being applied before the index ischemia, which poses limitations for use in clinical practice. The primary hypothesis of this study however, concerned the possibility to potentiate a cardioprotective effect of postconditioning. In this study, hypertensive disease, required additional protective stimuli. Regarding poor applicability of preconditioning stimuli, further research in the mechanisms underlying poor cardioprotection in diseased subjects and development of more clinical applicable strategies is therefore needed.

The second limitation of the study is related to investigation of our molecular targets. After investigation of GSK-3beta and PKC-epsilon in WKY rats, we concluded that helium conditioning did not involve phosphorylation or translocation of both enzymes. Consecutively, a minimum amount of experiments was performed in SHR in the most protective group (triple intervention) after application of the last stimulus. It was postulated that when a triple helium intervention in SHR would involve the aforementioned targets, this effect should be found after application of the last intervention, i.e. 15 min of reperfusion. Immediate investigation of molecular target ERK 1/2 after preconditioning with xenon\(^34\) showed phosphorylation and translocation of ERK 1/2 directly after the first and second xenon stimulus. This effect could not be found after the third xenon-preconditioning stimulus, neither after 10 minutes of wash out before the start of the ischemic episode\(^34\) supporting our choice for direct
investigation of the molecular target after helium postconditioning.

The specific choice for GSK-3beta only, was based on results from an earlier study in which the involvement of protein kinase B (serine 473, threonine 308), extracellular signal-regulated kinase 1/2 and GSK-3beta in helium-induced preconditioning in healthy and diabetic rats was investigated. In this study, no differences in phosphorylation between control and helium preconditioning groups of the above mentioned kinases were found, except for the amount of phosphorylated GSK-3beta, which was significantly lower at the ischemia time point in the healthy group receiving helium preconditioning. Additionally, an important role of GSK-3beta in the development of hypertensive heart disease further contributed to this choice.

Despite the fact that a protective effect of helium conditioning under hypertensive circumstances is shown in this study, an explanation considering cell signaling lacks. This particular study limitation warrants more in-depth research in necrosis and apoptosis pathways under helium inhalation in vivo.

In summary, the present study shows that helium conditioning can protect the hypertensive myocardium. In contrast to the healthy heart, only a triple intervention of helium conditioning can reduce cell damage after ischemia/reperfusion, suggesting the presence of a threshold in the hypertensive heart. In this study we did not find enhanced phosphorylation of GSK-3beta or PKC-epsilon after helium conditioning. This result is remarkable and suggests that helium-induced conditioning is mediated through other mechanisms than ischemic and anesthetic conditioning.
REFERENCES


III

PART

Human models
Helium induces preconditioning in human endothelium in vivo

Kirsten Smit, Gezina Oei, Daniel Brevoord, Erik Stroess, Rienk Nieuwland, Wolfgang Schlack, Markus Hollmann, Nina Weber, Benedikt Preckel
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INTRODUCTION

Ischemic preconditioning (IPC) results in protection of organs against ischemia-reperfusion (I/R) injury by short, non-lethal periods of ischemia. Two phases are distinguished, an early phase of protection induced by a stimulus directly before I/R, known as classical or early preconditioning, and a second window of protection that arises 12-72 h after administration of the stimulus, which is known as late preconditioning. Next to ischemia, pharmacological agents (i.e., volatile anesthetics) can also induce preconditioning. The endothelium circumvents all blood vessels and serves as a first-line defence mechanism against organ and tissue injury. The protective functions of the endothelium include anti-coagulation, anti-inflammation, prevention of platelet activation, regulation of permeability and regulation of vascular tone. I/R elicits profound changes in the endothelial homeostasis, as attested by a significant suppression of endothelium-dependent vasodilation. Endothelial dysfunction is regarded as an independent risk factor for cardiovascular events and is a surrogate marker for monitoring the efficacy of therapeutic strategies.

Postischemic endothelial dysfunction can be attenuated by IPC, as was shown in a human forearm model using venous occlusion plethysmography. In this study, 20 min of forearm I/R resulted in a blunted vasodilatory response to acetylcholine, which could be restored by IPC. Animal studies demonstrated that the noble gas helium induces early and late preconditioning of the heart. Because helium is readily available, easy to administer and has no known side effects, it has the potential to become a perfect preconditioning agent.

Forearm I/R induces endothelial dysfunction by reducing vasodilation induced by increasing dosages of acetylcholine. Our primary hypothesis is that helium preserves postischemic endothelial function. There are markers of endothelial function present in plasma. Activated endothelial cells release nitric oxide products, inflammatory cytokines, adhesion molecules, regulators of hemostasis and microparticles. Microparticles are vesicles circulating in plasma which are derived from various cells in response to cell activation, injury or apoptosis. Endothelial microparticles have been used as a clinical and quantitative marker of endothelial cell dysfunction, and their presence is inversely associated with acetylcholine-induced vasodilation in coronary arteries. Our secondary hypothesis is that the underlying mechanism of helium preconditioning might be related to endothelial nitric oxide synthase production, circulating cytokines and adhesion molecules, or cell-derived microparticles released after I/R.
MATERIALS & METHODS

The Institutional review board of the Academic Medical Center, Amsterdam, The Netherlands approved the trial (registered at www.trialregister.nl with number NTR1124, site was last accessed March 16, 2012), which was conducted in accordance with the International Conference on Harmonization on Good Clinical Practice Guidelines and the Declaration of Helsinki. All subjects gave written informed consent.

Subjects

A total of 58 healthy volunteers were included (baseline characteristics and demographics are shown in Table 1). Volunteers abstained from caffeine, alcohol and smoking 12 h

<table>
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<th></th>
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<th>He-LPC</th>
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<td>5.8±1.5</td>
<td>5.7±1.0</td>
<td>6.3±1.9</td>
</tr>
</tbody>
</table>

Table 1. Demographics and baseline data of randomized volunteers. All data are expressed as mean ± SD. No statistical differences were observed between groups. BMI = body mass index, SBP = systolic blood pressure, DBP = diastolic blood pressure, ESR = erythrocyte sedimentation rate, Controls = control group without ischemia, I/R = ischemia/reperfusion group, He-EPC = helium early preconditioning group, He-LPC = helium late preconditioning group, IPC = Ischemic preconditioning group.
before onset of the experiment. All experiments were performed in a quiet, temperature-controlled (20-24°C) room. Fifty volunteers were randomized to one of five groups using randomization software (ALEA; NKI; Amsterdam, The Netherlands) provided by Clinical Research Unit of the Academic Medical Centre. An additional group of 8 volunteers received the endothelial nitric oxide synthase (eNOS) blocker N^G^-monomethyl-L-arginine (L-NMMA).

**Study design**

The study protocol is outlined in Figure 1. Forearm ischemia was induced by inflating a 12-cm wide blood pressure cuff placed on the non-dominant upper arm to a pressure of 200 mmHg for 20 min. Helium preconditioning was induced by administration of a helium mixture (Heliox: Helium 79%, Oxygen 21%, BOC, Mordon, United Kingdom) using a non-invasive delivery system (Helontix™vent, Linde Therapeutics, Eindhoven, The Netherlands) via a normal facemask with pressure support of 3 cm H2O. Volunteers were given three cycles of helium for 5 min, followed by 5 min of normal air breathing either directly (helium preconditioning, He-EPC) or 24 h (helium late preconditioning, He-LPC) before I/R (see Figure 1). The group receiving L-NMMA received a dosage of 0.4 mg min⁻¹ dL⁻¹ forearm tissue volume, and this dosage effectively blocked nitric oxide production in previous studies. L–NMMA was started 5 min before He-EPC and was continued during helium preconditioning (35 min in total). Ninety min after termination of this preconditioning protocol, FBF was restored to baseline values, indicative of normal eNOS activity before forearm I/R. Inflating the blood pressure cuff around the nondominant upper arm to 200 mmHg for 3 times 5 min interspersed with time 5 min reperfusion directly before I/R induced ischemic preconditioning.

**Assessment of endothelial function**

Assessment of vascular function was performed using venous occlusion plethysmography (EC4; Hokanson, Inc., Bellevue, WA). After local anesthesia with lidocaine 2%, the non-dominant brachial artery was cannulated under aseptic conditions using a 22 gauge needle. Bilateral forearm bloodflow (FBF) was measured with mercury-in-silastic strain gauges and expressed in ml/min/100ml forearm tissue volume. Forearm tissue was measured by water displacement. Endothelial function was assessed as described before, and measured in response to intraarterial infusion of the endothelium-dependent vasodilator acetylcholine (0.1; 0.5; 1.5; 5.0 μg/100ml forearm tissue volume/min, Novartis AG, Stein, Switzerland) and the endothelium-independent vasodilator sodium nitroprusside (6; 60; 180; 600ng/100ml/forearm tissue volume/min, AZH, The
Hague, The Netherlands). Each dose was given for 5 min, and intrabrachial infusion was kept constant at a rate of 90 ml/h. To reconfirm that our postischemic measurement was consistent, we repeated our postischemic baseline measurement twice within 10 min, after which we continued the measurement with infusion of acetylcholine and sodium nitroprusside.

Blood Samples

A venous cannula was inserted in the nonischemic arm to collect blood samples at baseline, after 10 min of reperfusion and after 3 h of reperfusion (at the end of the protocol) to allow activation of interleukins. Samples were centrifugated (1550g, 20 min, 20°C) within 15 min and aliquots were snap frozen in liquid nitrogen and stored at -80°C.

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**Figure 1. Study protocol.** Endothelial function was established at baseline and was repeated after 20 minutes of forearm I/R except for controls. Treatment with helium preconditioning was administered directly before forearm I/R (He-EPC) or 24 h before I/R (He-LPC). Treatment of IPC was administered directly before forearm I/R. Controls = control group without ischemia, He-EPC = helium early preconditioning group, He-LPC = helium late preconditioning group, I/R = ischemia-reperfusion group, IPC = ischemic preconditioning group.
Flowcytometry of microparticles

Samples of frozen citrate plasma of subjects from control, I/R- and He-EPC group were analysed for circulating cell-derived microparticles. Samples were thawed and microparticles were isolated and incubated with annexinV and the cell-specific monoclonal antibody or isotype-matched control antibodies, and were analysed for 1 min in an automated cell sorter (FACSCalibur flow cytometer with CELLQuest 3.1 software (BD Immunocytometry Systems; San José, CA, for details see Supplemental Digital Content 1).

Enzyme-Linked Immuno Sorbent Assay (ELISA)

We used serum samples to determine levels of circulating interleukin (IL)-1beta, E-selectin, soluble vascular cell adhesion molecule-1, soluble intercellular adhesion molecule-1. Citrate plasma was used to determine levels of IL-6 and IL-8 (all kits from R&D systems, Minneapolis, MN). Samples were analysed by ELISA according to recommendations of the manufacturer.

Calculation & Statistics

All plethysmography results are presented as mean ± standard error of the mean (mean ± SEM). Demographic and cytokine data are presented as mean ± standard deviation (mean ± SD), microparticle data are presented as median and 25-75 percentiles.

As in previous studies (3) we planned to only perform a within group analysis. Therefore we performed a repeated measure ANOVA in each group, and compared the first measurements (responses to acetylcholine and sodium nitroprusside) with the respective postischemic measurements after I/R. We focussed on a group main effect and did not perform post hoc tests for each dosage. A probability value of P<0.05 was considered significant. FBF measurements were analysed using SPSS (version 16.0, Chicago, IL). The mean ratio of flow in the infused (measurement) arm/non-infused (control) arm was calculated (FBF measurement/control arm ratio). Baseline FBF measurement/control arm ratios were normally distributed, and comparison within groups of first and second measurement was performed by two-sided paired student’s t-test (one outlier (>2SD) in the IPC group was excluded before analysis). ELISA data were analyzed by one way ANOVA using Dunnett’s multiple comparison as post hoc test. Microparticle data were unequally distributed and Friedman’s test was used for within group analysis.
RESULTS

Demographic data of randomized volunteers are presented in Table 1, no statistical difference was observed between groups. All subjects tolerated the procedures without complications. Data of one patient were excluded because of violation of the preconditioning protocol, (IPC group n = 9, all other groups n = 10). Helium administration was well tolerated, and no effects on blood pressure and heart rate were observed.

Effect of forearm I/R on endothelial function

Forearm I/R resulted in a persistent postischemic hyperemia resulting in a significant increased baseline FBF at the start of the second measurement. The prolonged postischemic hyperemia was completely abolished after IPC and He-EPC, resulting in a similar baseline FBF at the start of the second measurement. However, after helium late preconditioning a non-significantly increased postischemic baseline FBF was observed (Figure 2).

Acetylcholine caused dose-dependent increases in FBF in all groups. In the control group, both the baseline and the second measurement showed similar responses to acetylcholine, illustrating the reproducibility of our methodology (Figure 3A). Forearm I/R significantly blunted the dose-dependent response to acetylcholine, reflecting postischemic endothelial dysfunction (p = 0.001, fig. 3B). The response to sodium nitroprusside remained unaffected after forearm I/R, and maximal increase of FBF was in the same range as maximal increase in response to acetylcholine (Supplemental Digital Content 2, Figure 1).

Effect of helium preconditioning on endothelial function

He-EPC prevented postischemic endothelial dysfunction by preserving the response to acetylcholine (p = 0.581 first vs. second measurement, Figure 3C). Even when administered 24 h before forearm I/R, helium late preconditioning preserved postischemic endothelial function (p = 0.165, fig. 3D). The protection by helium preconditioning was comparable to protection elicited by IPC, (p = 0.657, Figure 3E).
Figure 2. Baseline FBF measurement/control arm ratio of second measurement. Bar plot showing baseline FBF measurement/control arm ratio (mean ± SD) calculated by dividing FBF from the infused (measurement) arm/noninfused (control) arm. I/R caused significant postischemic hyperemia (*p=<0.05) compared to the first measurement, which was prevented by He-EPC, IPC, and He-LPC. Controls = control group without ischemia, FBF = forearm blood flow, He-EPC = helium early preconditioning group, He-LPC = helium late preconditioning group, I/R = ischemia/reperfusion group, IPC = ischemic preconditioning group.
Figure 3. Acetylcholine dose–response curves. All data are represented as mean ± SEM. A, Similar dose–response curves to acetylcholine for the first and the second measurement in controls. (P = 0.59). B, I/R of the forearm significantly blunted dose-dependent response to acetylcholine (P = 0.001), indicating postischemic endothelial dysfunction. Helium early (C, P = 0.581) and late preconditioning (D, P = 0.165) prevented postischemic endothelial dysfunction, as did ischemic preconditioning (E, P = 0.657). controls = control group without ischemia; FAV = forearm volume; He-EPC = helium early preconditioning group; He-LPC = helium late preconditioning group; I/R = ischemia-reperfusion group; IPC = Ischemic preconditioning group.
In the additional L-NMMA group (male/female 1/7, age 23 ± 5 yr, body mass index 21.7 ± 2.1, systolic and diastolic blood pressure 122 ± 19 mmHg and 69 ± 9 mmHg, respectively), L-NMMA significantly reduced FBF by 61 ± 8 %, indicating eNOS blockade during application of He-EPC. This blockade, however, did not block the protective effect of He-EPC as the response to acetylcholine was preserved ($p = 0.720$, Figure 4).

Figure 4. Acetylcholine dose-response curves after infusion of L-NMMA during helium preconditioning. FAV; forearm volume. Infusion of NG-monomethyl-L-arginine (L-NMMA) during helium preconditioning (He-EPC) did not block helium preconditioning, as postischemic endothelial function was still preserved.

Effect of I/R on circulating pro-inflammatory cytokines and adhesion molecules

We measured the levels of soluble vascular cell adhesion molecule-1, soluble intercellular adhesion molecule-1 and E-selectin. All baseline values were within the normal limits reported for healthy subjects. To investigate whether forearm I/R would affect these adhesion molecules, we measured the levels after 10 min of reperfusion and after 3 h of reperfusion. We observed no significant effects on the plasma levels of soluble vascular cell adhesion molecule-1, soluble intercellular adhesion molecule-1 and E-selectin.

The baseline levels of the pro-inflammatory cytokines IL-1beta and IL-8 were all below the lowest standard provided by the manufacturer. The levels of IL-1beta after 10 min and 3 h of reperfusion remained below the detection limit of the assay and no increases were observed. Forearm I/R did not affect systemic levels of IL-8 after 10 min or 3 h of reperfusion. In contrast, IL-6 increased in all groups in time without significant
differences between controls, I/R or preconditioning groups (Table 2).

<table>
<thead>
<tr>
<th>Target</th>
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<th>He-LPC</th>
<th>IPC</th>
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Table 2. Adhesion molecule expression and cytokine levels at 3 hours of reperfusion. Data are expressed as means ± SD. No statistical differences were observed between groups. Controls = control group without ischemia, He-EPC = helium early preconditioning group, He-LPC = helium late preconditioning group, I/R = ischemia-reperfusion group, IPC = ischemic preconditioning group. IL-1β = interleukin-1β, IL-6 = interleukin-6, IL-8 = interleukin-8, sICAM-1 = soluble intercellular adhesion molecule-1, sVCAM-1 = soluble vascular cell adhesion molecule-1.

Effect of I/R on circulating cell-derived microparticles

To investigate whether helium preconditioning or forearm I/R affected the release of endothelial microparticles, plasma samples from the control, He-EPC, and I/R group were analysed for the presence of microparticles (all groups n = 8). An example of the microparticles results is given in Supplemental Digital Content 2, Figure 2) Neither forearm I/R nor He-EPC affected the total levels of circulating microparticles in blood.

In line with earlier studies we found that the levels of microparticles originating from endothelial cells, i.e., microparticles binding antibodies directed against E-selectin (CD62e), VE-cadherin (CD144) or melanoma cell adhesion molecule (CD146), were very low and almost below the detection limit, except for samples from one volunteer in the I/R group who showed a strong increase of endothelial microparticles after I/R.

We further investigated the cellular origin of microparticles from platelets (CD61,
CD62p, CD63), lymphocytes (CD4, CD8, CD20), monocytes (CD14), granulocytes (CD66b) or erythrocytes (CD235a). We found a large variation in the baseline levels of erythrocyte-derived microparticles in all groups, reflecting large heterogeneity in our volunteers, which is possibly caused by mild hemolysis (Table 3). Forearm I/R resulted in a nonsignificant increase of systemic circulating erythrocytes derived microparticles (Table 3). Furthermore, we observed no effect of He-EPC or forearm I/R on microparticles exposing tissue factor, or microparticles derived from leukocytes. Because we focused on a group main effect of endothelial function and did not perform a power analysis for the effect of forearm I/R or He-EPC on microparticles, we cannot conclude that forearm I/R affects levels of systemic circulating microparticles, and that microparticles do not play a role in He-EPC on a systemic level. Since we did not investigate the levels of microparticles in the ischemic arm, we cannot exclude a possible effect on a local level.
### Table 3. Analysis of circulating microparticles at different timepoints. Data are presented as mean +/- SD. All values are corrected for isotope controls and represent *10^5 micrographs/ml. The percentage of positive CD62p particles was calculated by dividing particles positive for both CD62p and CD61 by the total amount of CD61-positive particles. No significant differences were observed between groups. Controls = control group, I/R = ischemia/reperfusion treated group, He-EPC = group treated with helium early preconditioning, Rep = Reperfusion time.

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<th>Controls Rep 3 hours</th>
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<th>I/R Rep 10 min</th>
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DISCUSSION

In this study, we show for the first time that inhalation of helium in humans prevents impairment of acetylcholine-induced vasodilation following I/R. A similar protection was observed 24 h after helium administration. Therefore, our data show that helium induces not only early but also late endothelial preconditioning in humans in vivo. This conclusion is supported by the improved postischemic FBF upon infusion of the endothelial-dependent vasodilator acetylcholine, while the response to the endothelial-independent vasodilator sodium nitroprusside was unaffected.

Forearm blood flow model

A time-control group was included in our study to demonstrate that the results were reproducible. In accordance with previous studies using the same model, ischemic preconditioning protected against endothelial dysfunction. Although an increased baseline FBF after ischemia (hyperemia) in the I/R group might interfere with postischemic response curve to acetylcholine, it is unlikely that this effect is responsible for the absent response to acetylcholine. The postischemic response to sodium nitroprusside (Supplemental Digital Content 2, Figure 1) demonstrates that vasodilation can still be achieved after I/R, indicating postischemic vasodilation was not maximal.

Acetylcholine results in calcium-mediated activation of eNOS via the endothelial muscarinergic receptor. Under physiological conditions, nitric oxide diffuses to the vascular smooth muscle cell layer and activates soluble guanylate cyclase eventually leading to cyclic guanosine monophosphate-mediated vasodilation and flow increase. Following I/R, Acetylcholine-induced vasodilation is reduced probably by a decrease in the release of nitric oxide which can be due to eNOS uncoupling, i.e., endothelial depletion of essential cofactors of eNOS like tetrahydrobiopterin and L-arginine. As a result of this uncoupling, scavenging of endothelial nitric oxide by increased reactive oxygen species can lead to production of peroxynitrite, which in turn can induce cellular injury and vasoconstriction. There could be a contribution of vascular smooth muscle cell dysfunction, however this is unlikely since sodium nitroprusside-induced vasodilation was unaltered by I/R.

Preconditioning by inhalational substances

Volatile anesthetics as well as the anesthetic noble gas xenon induce organ protection by preconditioning, as was shown in several in vitro and in vivo models. This organ
Helium preconditioning in human endothelium in vivo

Protective effect cannot be attributed to analgesic actions of these gases as the analgesic gas nitrous oxide did not precondition the rat heart in vivo. Noble gases without anesthetic properties induced preconditioning in rabbits in vivo and for the noble gas helium both the early and late phase of preconditioning have been demonstrated.

For volatile anesthetics, the translation to clinical I/R situations was made by showing the preconditioning effect of sevoflurane in patients undergoing coronary artery bypass graft surgery. Although previous studies demonstrated a late phase of preconditioning by gases, the present study shows for the first time that inhalation of helium induces early and also late endothelial preconditioning in humans.

In contrast to our data showing helium preconditioning in the human endothelium, another study investigating helium preconditioning in human endothelium found that helium provided modest anti-inflammatory effects, but did not protect against I/R. There are several differences between the two studies. First, postischemic reactive hyperemia was used to assess endothelial function, which is less reliable to measure endothelial function compared to infusion of a vasodilator like acetylcholine. Second, the lack of a distinct preconditioning protocol could be the reason why helium failed to induce preconditioning, since the noble gas was applied continuously before, during and after ischemia. Previous clinical studies demonstrated that the preconditioning protocol plays a major role in volatile-anesthetic induced organ protection in humans: protection could only be evoked by a distinct and repetitive stimulus. Another difference between our study and the study from Luchinetti et al. is the helium concentration used to induce endothelial protection (50% compared to 79%). The minimal required concentration of helium to induce preconditioning in humans is unknown, and could be above 50%, although in experimental studies in rats a concentration of 30%-70% helium was sufficient to induce late preconditioning whereas 10% was not.

Possible mechanisms of protection

Although the mechanisms of helium induced preconditioning are not fully clarified, some mediators have been identified and are discussed in a recent review about the possible effects of helium in different organs. One experimental study showed that administration of the nonselective nitric oxide synthase inhibitor N-nitro-L-arginine methyl ester during helium preconditioning abolished cardioprotection in rabbits. Data from this study suggest that helium preconditioning is mediated by nitric oxide generated by eNOS in vivo. In order to investigate the possible role of eNOS in HeEPC, we administered L-NMMA during helium breathing. Our data (see Figure 4 and Supplemental Digital Content 2, Figure 3) demonstrate that in our experiments eNOS blockade administered during helium preconditioning does not abolish endothelial
protection. There are some limitations to our L-NMMA administration protocol: we only administered L-NMMA during helium preconditioning, and stopped infusion before the start of I/R. We cannot rule out from the current data that a prolonged infusion of L-NMMA might be able to block the helium preconditioning effect. We carefully considered our administration protocol for L-NMMA, and administration during forearm ischemia and reperfusion could have altered the postischemic damage. This is in line with another study in which infusion of L-NMMA was continued during acetylcholine infusion, resulting in a decreased response to acetylcholine even in the control group\textsuperscript{31}. Experimental data have shown that L-NMMA, given during ischemia and reperfusion, attenuated postischemic endothelial dysfunction in the Langendorff perfused heart\textsuperscript{32} making it impossible to compare results of these groups with groups not receiving L-NMMA.

Although it is very unlikely, L-NMMA might have induced preconditioning by itself, thereby overcoming a blockade of the helium preconditioning effect. However, this preconditioning effect of L-NMMA has never been demonstrated in animal studies before. Although blood flow was significantly reduced by L-NMMA, we did not observe any aspects of forearm ischemia caused by L-NMMA in the physically non-active study situation.

Endothelial injury may lead to the increased expression of inflammatory cytokines and adhesion molecules resulting in increased adhesion and migration of leukocytes. One of the cytokines that mediates endothelial dysfunction is tumor necrosis factor-alpha\textsuperscript{33}, which stimulates the production of IL-6. IL-6 is of vital importance to induce ischemic late preconditioning\textsuperscript{34} and increased levels of IL-6 are associated with poor prognosis in patients with heart failure\textsuperscript{35}.

Although we did not perform a power analysis of the effect of forearm I/R on cytokines, the current data suggest that forearm I/R does not affect the systemic levels of IL-1beta, IL-6, IL-8, soluble vascular cell adhesion molecule-1, soluble intercellular adhesion molecule-1 and E-selectin after 15 min or 3 h of reperfusion. However, we cannot exclude a local contribution of these cytokines to the endothelial dysfunction after I/R.

Another mechanism of endothelial dysfunction is the presence of circulating endothelial microparticles, which proved to be an independent risk factor for impaired endothelial vasodilation\textsuperscript{36,37}. Microparticles from patients with acute myocardial infarction selectively impaired nitric oxide production and caused severe endothelial dysfunction shown by impairment of acetylcholine-induced vasodilator response in isolated vessels\textsuperscript{11}. We showed that forearm I/R did not significantly affect the amount of circulating microparticles derived from platelets and erythrocytes. Microparticles derived from endothelial cells and leukocytes were at or below the detection limit, also
after I/R. It is unknown whether forearm I/R causes microparticles release, and it is possible that a local increase of microparticles, possibly endothelial microparticles, in the venous outflow tract was missed because of systemic dilution.

**Study limitations**

Since we collected our blood samples from the control arm, only systemic effects of cytokines and microparticles could be investigated. There are no previous studies in healthy volunteers that investigated cytokine release or its time course after forearm I/R. We therefore cannot exclude the possibility that there is cytokine involvement at other time points than those investigated in our present study.

In this study we focused on group main effects of forearm I/R and helium inhalation on endothelial function, statistical analyses between groups were not performed. We also cannot exclude effects of helium on cytokines and microparticles in a larger study population. However, previous studies found significant differences in similar sized study populations. Inhalation of helium has been shown to affect ventilation parameters in patients with chronic obstructive airway disease. Volunteers inhaled helium via a non-invasive ventilation machine and changes in ventilation parameters (e.g., breathing frequency) were not observed. We did not measure arterial partial pressure of oxygen during helium inhalation. However, significant changes in oxygen tension after inhalation of Heliox containing 21% oxygen is not to be expected in healthy volunteers. In addition, helium inhalation was stopped at least 5 min before forearm I/R, and because helium rapidly diffuses, a significant effect on oxygen tension during forearm I/R is most unlikely. We did not investigate the direct effect of helium on forearm bloodflow without I/R.

Helium is a nonanesthetic, nontoxic gas without any hemodynamic side effects that can easily be applied to patients. This inhalational gas could be a perfect instrument to induce preconditioning in patients subjected to clinical I/R situations, *i.e.*, coronary artery bypass graft surgery. However, whether helium preconditioning can protect patients with comorbidities like atherosclerosis, hypertension or diabetes mellitus still has to be investigated.
SUPPLEMENTAL DIGITAL CONTENTS

Supplemental Digital Content 1 - Methods Microparticles

Collection of blood samples
Venous blood samples were taken from the non-ischemic arm at baseline, after 10 min of reperfusion and after 3 hours of reperfusion. Cells were removed by centrifugation (1550 g, 20 min, 20°C) and aliquots of 250 μl plasma were snap frozen in liquid nitrogen within 30 min after withdrawal and stored at -80°C until performing the assay.

Antibodies
To establish the origin of the microparticles, we performed a triple labeling on each sample using different fluorochromes (Fluorescein isothiocyanate (FITC)-labeled IgG₁, phycoerythrin (PE)-labeled IgG₂, and allophycocyanin (APC) conjugated annexin V). These antibodies were used to analyse the origin of the circulating microparticles: CD61-PE (exposed on thrombocytes), CD63 (expressed by activated platelets), CD14 (mostly found on macrophages), CD4 (from T-cells), CD8 (also from T-cells), CD20 (from B-cells), glycophorin A-FITC (CD235a) from erythrocytes, CD144-FITC exposed on endothelial cells, CD62e-PE (E-selectin exposed on activated endothelial cells)

Isolation of microparticles
A sample of 250 μL of frozen plasma was thawed on melting ice for 1 h and centrifuged for 30 min at 18,890g at 20°C to pellet the microparticles. After centrifugation, 225 μL of the supernatant were removed. The pellet was resuspended in 225 μL phosphate-buffered saline (PBS) containing citrate, after which samples were centrifugated again and supernatants were removed again. The pellet containing microparticles was resuspended in 75 μL PBS-citrate for the final concentration.

Flowcytometry
Five microliters of the microparticle suspension was diluted in 30 μL CaCl₂ (2.5 mmol/L)-containing PBS. Then 5 μL APC-labeled annexin V were added to all tubes plus 5 μL of the cell-specific monoclonal antibody or isotype-matched control antibodies (total volume: 50 μL). The samples were incubated in the dark for 15 min at room temperature. After incubation, 900 μL of calcium-containing PBS were added to all tubes (except to the annexin V control, to which 900 μL of citrate-containing PBS were added). Samples were analyzed for 1 min in a fluorescence automated cell sorter (FACS Calibur) with CellQuest software (Becton Dickinson, San Jose, CA, USA). Both
forward scatter (FSC) and sideward scatter (SSC) were set at logarithmic gain.

Microparticles were identified on the basis of their size and density and on their ability to bind cell-type specific CD antibodies and annexin V. Microparticles positive for Annexin V, CD62e-PE and Cd144-FITC were considered to be derived from activated endothelial cells.
Supplemental Digital Content 2

![Graphs showing results of Nitroprusside infusion](image)

Figure 1. Results of Nitroprusside infusion
Controls = control group without ischemia, He-EPC = helium early preconditioning group, He-LPC = helium late preconditioning group, I/R = ischemia-reperfusion group, IPC = Ischemic preconditioning group. FAV = Forearm volume, FBF = Forearm blood flow. All data are represented as mean±SEM, no significant statistical differences were observed between first and second FBF measurement.
Figure 2. Example of microparticle results
These are the results of the microparticle analysis of a volunteer from the helium early preconditioning group, at timepoint 10 minutes of reperfusion. Microparticles positive for CD 61-FITC are shown in panels B and D, and microparticles positive for CD62p-PE are shown in panels A and C. Microparticles positive for CD61-FITC that are also positive for CD62p-PE (which indicates that these particles are derived from activated platelets) are shown in panel B, and are 4.37% (FITC; Fluorescein isothiocyanate, PE, phycoerythrin).
Figure 3 Sodium nitroprusside dose-response curve. Results of helium early preconditioning group with NG-monomethyl-L-arginine (L-NMMA) infusion (L-NMMA + He-EPC). FAV; Forearm volume; FBF; Forearm blood flow. All data are represented as mean±SEM, no significant statistical differences were observed between first and second FBF measurement.
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Effects of helium and air inhalation on the innate and early adaptive immune system in healthy volunteers ex vivo
INTRODUCTION

Helium, a colorless, odorless and tasteless gas with a lower density than air, was first used in patients with respiratory diseases. Recently it was shown that beside volatile anesthetics (e.g. isoflurane, sevoflurane, desflurane) and the anesthetic noble gas xenon, the non-anesthetic noble gas helium also reduces ischemia-reperfusion injury when administered before (preconditioning) or after (postconditioning) organ ischemia. Experimental studies in rabbits and rats suggested that helium conditioning protects myocardial and neuronal tissue against ischemia/reperfusion damage. In a forearm model of ischemia-reperfusion injury in humans, helium preconditioning protected the endothelium against ischemic damage. This suggests that helium can be a therapeutic agent against ischemia-reperfusion injury. However, before using helium as a “conditioning” agent in clinical settings, any negative effect on other organ systems such as the immune system should be ruled out.

Host immunity is classically divided into the innate and the adaptive immune system. The innate immune response involves monocytes, neutrophils, dendritic cells and macrophages but also parenchymal cells such as epithelial and endothelial cells. Upon danger sensing, secretion of cytokines and chemokines results in monocyte and neutrophil migration to inflamed tissues and antigen presentation. This initial and aspecific cascade induces secondary antigen specific events known as the adaptive immune system involving T- and B-lymphocytes. Volatile anesthetics and xenon exert immunomodulatory effects by affecting endothelial expression of adhesion molecules and the secretion of cytokines and chemokines as well as lymphocytes.

We investigated whether helium breathing in healthy volunteers affects the ability of the immune system to respond to ex vivo stimulation of whole blood. For the innate arm, we measured the proinflammatory cytokines tumor necrosis factor-alpha (TNF-alpha), interleukin-1beta (IL-1beta) and interleukin-6 (IL-6) and chemokine interleukin-8 (IL-8) after stimulation with lipopolysaccharide (LPS) and lipoteichoic acid (LTA). To assess effects of helium on the adaptive immune system, interferon-gamma (IFN-gamma) and interleukin-2 (IL-2) production after T cell receptor specific stimulation was determined.
METHODS

The study was approved by the ethical committee of the Academic Medical Centre, Amsterdam (www.trialregister.nl/NTR2152) and was conducted in accordance with the International Conference on Harmonization on Good Clinical Practice Guidelines and the Declaration of Helsinki. After written informed consent, twelve healthy, non-smoking, male volunteers (age 22-35) were included and were asked to use no caffeine or alcohol containing drinks, and not to exert heavy physical exercise twelve hours before the experiment. Volunteers did not use any medication influencing the immune system, or were known to have any condition that could influence the immune system. A second group of 6 volunteers (age 19-31) inhaled 60 min of helium and air.

Experimental design

Experiments were conducted in a crossover design in a quiet room with standardized circumstances. All participants underwent two experimental cycles: once with 30 or 60 minutes of heliox (79%He/21%O₂, BOC, Mordon, UK) inhalation using a non-invasive delivery system (Helontix™vent, Linde Therapeutics, Eindhoven, The Netherlands) via a normal face mask with pressure support of 3 cm H₂O, and once with air inhalation, with two weeks in between cycles. Venous blood was sampled at baseline (T0), at 25 min of inhalation (T1), or 1 (T2), 2 (T3), 6 (T4), or 24 h after inhalation (T5), respectively. C-reactive protein (CRP), leukocyte and lymphocyte counts were determined in ethylenediaminetetraaceticacid–anticoagulated blood, and heparin-anticoagulated blood was used for incubation with immune stimulants.

Whole blood stimulation

After sampling, heparinized whole blood (0,5 ml) was diluted with an equal volume of RPMI-1640 (Invitrogen, Breda, the Netherlands) serving as control (CON), or RPMI-1640 containing LPS (Ultra pure LPS from Escheria coli 0111:B4, InvivoGen, San Diego, United States) in a final concentration of 200 ng/ml⁻¹, RPMI-1640 containing LTA (Purified LTA from Staphylococcus aureus, InvivoGen, San Diego, United States) in a final concentration of 20 μg/ml⁻¹, or RPMI-1640 containing T-cell stimuli anti-CD3/anti-CD28 (TCS; murine monoclonal antibodies CLB-T3/3 against the CD3 molecular complex and CLB-CD28/1 against the T-cell differentiation antigen CD28, provided by dr. R. van Lier, Academic Medical Centre, Amsterdam, The Netherlands) in a final concentration of 10 μg/ml⁻¹, respectively. Incubation was done in aliquots of 0,5 ml
in sterile tubes (Sarstedt, Etten-Leur, the Netherlands) at 37°C for 0, 2, 4 or 24 hours, all performed in duplicate. After incubation, plasma was prepared by centrifugation at 1200 RPM for 10 minutes at 4°C. Plasma was stored at -20°C until further analysis.

Established in our research institution by the laboratory of van der Poll, the use of whole-blood cultures now is a widely used method to screen for influences of treatments on the immune response\(^1\)\(^4\). The whole blood induced cytokine production by specific bacterial antigens has important advantages. In this system, cell populations that are important for the defense against pathogenic organisms (e.g. neutrophils, monocytes, and lymphocytes) and soluble factors like complement, antibodies, and other serum components can interact thus resembling the in vivo situation. LPS is a major constituent of the cell wall of Gram-negative bacteria, LTA a constituent of the cell wall of Gram-positive bacteria. Using both stimuli thereby covers a broad range of microbial agents and the resulting activation of monocytes and neutrophils induces synthesis of proinflammatory cytokines such as TNF-alpha, IL-1beta and IL-6 and chemokine IL-8\(^1\)\(^5\)\(^-\)\(^7\). These cytokines and chemokines are in turn able to activate T lymphocytes.

In contrast to pro-inflammatory cytokines that will peak within a few hours after exposure to antigens, the T-cell mediated response usually peaks later and can be monitored by the production of the typical cytokines that reflect T cell function, among which IFN-gamma and IL-2. We studied T-cell function by specific activation of the T cell receptor through application of a combination of antibodies directed against CD3 and CD28\(^1\)\(^8\)\(^-\)\(^9\).

**Cytokine and chemokine measurement by multiplex bead-based immunoassays**

Plasma TNF-alpha, IL-1beta, IL-6, IL-8, IFN-gamma and IL-2 concentrations were measured simultaneously by cytometric bead array (CBA), a flow cytometry based fluorescence detection of antibody-coated beads (‘Human Inflammatory Cytokine Kit’ and ‘Human Th1/Th2 cytokine kit’, BD Biosciences, Breda, the Netherlands). For measurement and analysis of cytokines, we used the fluorescent activated cell sorter FACSCalibur (BD Biosciences, Breda, The Netherlands) with BD FACSComp and BD CellQuest software.

**Statistical analysis**

Normal distribution of the data was tested with the Kolmogorov-Smirnov test. As data were not normally distributed, differences between helium and air groups were tested by the Wilcoxon test for paired measurements and considered significant if \(p < 0.05\). All data in figures 1 to 4 are shown as mean ± SEM. P-values of all tests at each time point for each parameter are given in table 2. In this table the amount of experiments in the
helium and the air group as well as the amount of pairs included in the Wilcoxon test for paired measurements are given.
RESULTS

Helium inhalation does not influence leukocyte and lymphocyte counts

At baseline, there was no difference in C-reactive protein between the 30 min inhalation groups; 1.6 ± 0.4 (mg/l) and 2.3 ± 0.6 (mg/l) in the helium and air group respectively (p>0.05). In the 60 min inhalation group, there was no difference at baseline in C-reactive protein either: 0.92 mg/l in the helium group versus 0.82 mg/l in the air group. Table 1 shows leukocyte and lymphocyte counts; no differences could be detected between heliox and air inhalation at baseline, 2 or 24 hours after inhalation (p>0.05), for both the 30 min and the 60 min inhalation group.

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<tr>
<td></td>
<td>Heliox</td>
<td>Room air</td>
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<td>T0</td>
<td>5.55 ± 0.29</td>
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<tr>
<td>T3</td>
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Table 1. Leukocyte and lymphocyte counts. Data are shown as mean ± SEM, no statistic differences between groups (p>0.05).

Ex vivo stimulation of whole blood with LPS, LTA or TCS significantly increased TNF-alpha, IL-1β, IL-6, IL-8, IFN-gamma and IL-2 in comparison to incubation with RPMI alone

After 2, 4 and 24 hours of incubation with LPS, the amount of TNF-alpha was significantly higher in comparison to incubation with RPMI alone, also see Figure 1. After 2, 4 and 24 hours of incubation with LPS or LTA, the amount of TNF-alpha, IL-1β, IL-6 and IL-8 (pg/ml) was significantly higher in comparison to incubation with RPMI alone (data not shown). After 2, 4 and 24 hours of incubation with TCS, the
amount of IFN-gamma, and IL-2 was significantly higher in comparison to incubation with RPMI alone (data not shown), indicating that the used immune agents were able to induce an adequate immune response.

Thirty or 60 min of helium inhalation did not affect the amount of TNF-alpha, IL-6, IL-1beta and IL-8 (pg/ml) after 0, 2, 4, and 24 hours of incubation of whole blood with LPS in comparison to room air at all time points (p>0.05). Figures 2 (TNF-alpha, IL-6) and 3 (IL-1beta and IL-8) show cytokine levels after 4- and 24-hour stimulations with LPS.

Helium inhalation for 30 min does not influence inflammatory cytokine and chemokine levels in whole blood after ex vivo incubation with LTA, in comparison to inhalation of air.

After 0, 2, 4 and 24 hours of incubation with LTA the amount of TNF-alpha, IL-1beta, and IL-8 (pg/ml) did not differ between heliox and air groups (p>0.05). The amount of IL-6 after 24 hours of incubation with LTA was similar after heliox or air inhalation at baseline (T0), 1 (T2), 2 (T3), 6 (T4) or 24 (T5) hours after inhalation.
(p>0.05), but differed significantly after 25 minutes of helium inhalation compared to air (T1). Figure 4 shows cytokine levels after 4 and 24 incubation with LTA, data at 0 and 2 hours are not shown.

**Helium inhalation for 30 min does not influence levels of inflammatory cytokines IL-2 and IFN-gamma excreted in whole blood after ex vivo incubation with T-cell stimuli anti-CD3/anti-CD28, in comparison to inhalation of room air.**

After 0, 2, 4 and 24 hours of incubation with anti-CD3/antiCD28, the amount of IFN-gamma and IL-2 (pg/ml) was not affected by helium inhalation in comparison to room air at T0, T1, T2, T4, or T5 (p>0.05), except for statistically different IFN-gamma levels at two time points. After 4 hours of incubation, the amount of IFN-gamma 1 hour after 30 min of helium inhalation (T2) was statistically higher in comparison to air inhalation. After 24 hours of incubation, the amount of IFN-gamma 24 hours after air inhalation was significantly different in comparison to helium inhalation. IFN-gamma and IL-2 levels after 30 min of helium/air inhalation are shown in Figure 5.
Figure 2. TNF-alpha (panel A-D) and IL-6 (panel E-H) levels in plasma after 4 and 24 hours incubation with LPS. Time points on y-axis represent blood sampling time points shown in the experimental protocols above. Panels A, B, E and F show 30 minutes of inhalation of helium and air; panels C, D, G and H 60 minutes. Data shown are means +/- SEM. Experimental protocol is shown above; T0: baseline, T1: at 25 min inhalation, T2: 1 h after inhalation, T3: 2 h after inhalation, T4: 6 h after inhalation, T5: 24 h after inhalation.
Figure 3. IL-1β (panels A-D) and IL-8 (panels E-H) levels in plasma after 4 and 24 hours incubation with LPS. Time points on y-axis represent blood sampling time points shown in the experimental protocols above. Panels A, B, E and F show 30 minutes of inhalation of helium and air, panels C, D, G and H 60 minutes. Data shown are means +/- SEM. Experimental protocol is shown above; T0: baseline, T1: at 25 min inhalation, T2: 1 h after inhalation, T3: 2 h after inhalation, T4: 6 h after inhalation, T5: 24 h after inhalation.
Figure 4 TNF-alpha (panel A-B), IL-6 (panel C-D), IL-1beta (panel E-F) and IL-8 (panel G-H) levels in plasma after 4 and 24 hours incubation with LTA. Time points on y-axis represent blood sampling time points shown in the experimental protocols above. Panels show 30 minutes of inhalation. Data shown are means +/- SEM. Experimental protocol is shown above; T0: baseline, T1: at 25 min inhalation, T2: 1 h after inhalation, T3: 2 h after inhalation, T4: 6 h after inhalation, T5: 24 h after inhalation.
Figure 5 IL-2 (panel A-B) and IFN-gamma (panel C-D) levels in plasma after 4 and 24 hours incubation with TCS anti-CD3/28. Time points on y-axis represent blood sampling time points shown in the experimental protocols above. Panels show 30 minutes of inhalation. Data shown are means +/- SEM. Experimental protocol is shown above; T0: baseline, T1: at 25 min inhalation, T2: 1 h after inhalation, T3: 2 h after inhalation, T4: 6 h after inhalation, T5: 24 h after inhalation.
DISCUSSION

Our study suggests that prolonged inhalation of helium does not affect the ability of the innate and early adaptive immune system to respond to immune stimuli LPS, LTA or anti-CD3/anti-CD28 ex vivo. TNF-alpha, IL-1beta, IL-6, IL-8, IFN-gamma and IL-2 levels did not differ at various time points before and after helium inhalation compared to air inhalation. These results are of interest for a broad field, as the use of helium-oxygen mixtures for respiratory disease or with the purpose of cell protection against ischaemia/reperfusion injury in the critical care unit and the operating theatre might expand.

Two in vivo studies using a forearm model of ischaemia-reperfusion injury investigated the protective effect of helium inhalation on endothelial function and additionally looked at systemic immune parameters. Our results are in line with results found in the first study, in which similar concentrations of helium/oxygen were used. This study showed that application of three 5-minute cycles of helium (79%He/21%O₂) interspersed with 5 min of air breathing before the ischemic episode induces preconditioning in human endothelium. To investigate the influence on the innate immune system, venous blood was collected at the non-injured arm for analysis of systemic levels of adhesion molecules sVCAM-1, sICAM-1, E-selectin, and proinflammatory cytokines IL-1beta and IL-8 at 10 minutes and 3 hours after reperfusion. No effects of forearm ischaemia/reperfusion or helium conditioning could be found on all of these parameters, suggesting no net effect of helium on the investigated immune parameters.

In contrast, Lucchinetti and colleagues found immunomodulatory effects after 35 minutes of helium breathing in a concentration of 50%He/50%O₂. Venous blood was taken from the injured arm at baseline, and after 5, 10 and 30 minutes of reperfusion to investigate proinflammatory markers on leukocytes. A decrease of CD11b on monocytes at 10 and 30 minutes of reperfusion and a decrease of ICAM-1 on monocytes at 5 minutes of reperfusion was found under helium inhalation in comparison to control. Although no effects on other markers were found, these findings implicated a net negative effect of helium inhalation on systemic immune parameters. However, differences in duration and concentration of the inhaled helium/oxygen mixtures make a direct comparison between the aforementioned studies difficult. Another difference is the proximity of the induced tissue damage (forearm ischemia-reperfusion) and the point of blood sampling for analysis of immune parameters. In the latter study, blood collection took place from the injured arm – closer to the site of injury - in contrast to the first study in which blood collection took place from the non-injured arm. It might be possible that the immunomodulatory effect of helium can only be found locally or that helium exerts...
anti-inflammatory effects only when tissue damage is present. An example is the anti-inflammatory effect of helium in comparison to nitrox breathing that was found in a pig model of acute lung injury. Anti-inflammatory effects were shown in lung tissue as a reduction of pro-inflammatory cytokine IL-8 and myeloperoxidase, a measure for neutrophil activity. The big difference with the current study is the lack of tissue damage at the time of helium breathing: stimulation of the immune system is done after helium breathing and blood sampling. This provides an objective way of assessing the immunomodulatory characteristics of this noble gas per se.

The main rationale behind the present study was to investigate whether the use of helium gas against organ ischemia/reperfusion injury may have detrimental effects on the immune response. Ischemic and pharmacologic conditioning protocols described in the literature normally do not exceed a total of 30 minutes of intervention. Therefore, 30 minutes of helium inhalation resembles a clinically relevant time frame of gas application. Investigation of the innate immune response after 60 min of helium and air inhalation was done in an additional group, to rule out that prolonged inhalation did exert effects. Furthermore, a concentration of 79% helium is the maximum from a clinical point of view, although a variety of lower helium concentrations have also been used. Higher percentages of helium lead inevitably to hypoxic gas mixtures. It is highly unlikely that lower concentrations have detrimental effects on the immune system, when a higher dose does not.

In this study, we mainly focused on pro-inflammatory components of the immune system. However, in some cases it is not so clear whether cytokines exhibit purely pro- or anti-inflammatory actions, such as IL-2. This cytokine has pro-inflammatory effects, but might play an anti-inflammatory role in diabetes. The finding that no effects on pro-inflammatory components were found suggests that no net-effect of helium inhalation on the immune response exists. It has to be noted that clinical outcome of infections is the result of a balance in quantity and time course of pro- and anti-inflammatory components. Therefore, several other anti-inflammatory components of the immune system, as well as other constituents of the innate and early adaptive immune system still need further investigation. In our study we did not investigate cytokine production in fractionated blood leukocytes. Instead, we investigated total amounts of cytokines in whole blood, as this model represents a condition in which many of the physiologically present cellular interactions remain intact. Given the fact that leukocyte counts did not differ between groups at the different time points either, we consider the lack of difference in cytokine levels between groups a good reflection of the unaltered immune status after helium inhalation.

Whole blood stimulation is a widely known model used for various goals. In a recent study it was shown that ex vivo stimulation of whole blood with pathogenic Leptospira
induced a cytokine response\textsuperscript{27}. The whole blood stimulation model was also used in another study to show that erythromycin infusion in healthy volunteers reduces IL-8 production after ex vivo stimulation with Streptococcus pneumoniae\textsuperscript{28}. Stress-related suppression of cytokine production after whole blood incubation with LPS was shown in a study in which 20 male, healthy volunteers were exposed to bungee jumping\textsuperscript{29}. In this study it was shown that bungee jumping was associated with higher epinephrine, norepinephrine and cortisol levels, but also with increased leukocytes. Nevertheless, the amount of TNF-alpha and IL-8 levels after ex vivo stimulation with LPS was decreased\textsuperscript{29}.

Despite the evidence for the applicability of our used model, a limitation of the study is the difficulty of proving absence of an effect while 3 significant differences between helium and air inhalation were found. However, with a total of 168 tests being performed in total, 8 can be significant by chance alone. Furthermore, significant findings at solely one time point of one cytokine after one stimulation type are not likely to reflect a clinically relevant effect of the intervention. As can be seen in the figures, lines of helium and air inhalation intersect at random time points, suggesting that even when an effect seems to be there it does not persist over time.

A second limitation of the study concerns the investigation of healthy, male volunteers only. The target population for helium-induced organ protection often suffers from comorbidity, which might be of influence on the innate immune response to ex vivo stimulation. We have chosen to use a model in which possible confounders by comorbidities were excluded. From literature it is known that sex differences exist in immune defense capacity and cytokine production\textsuperscript{17}. To minimize the influence of this possible confounder we have chosen to investigate males only.

The results of the present study indicate that inhalation of helium for 30 and 60 minutes does not affect leukocyte counts and does not have detrimental effects on the ability to evoke an adequate immune response in healthy volunteers after ex vivo whole blood stimulation with LPS, LTA and anti-CD3/CD28. These findings have implications for the use of helium as a conditioning agent in clinical practice, as it seems unlikely that helium affects innate immunity.
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<th>A (TNF-α, 30 min Hc, 4h LPS)</th>
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Table 2. Overview of all p-values (p), number of helium-air pairs included in the Wilcoxon test at each time point (n)*, number of experiments in the helium group (n He), and the air group (n Air). The table contains values for all figures included in the manuscript (Figure 1 to 4). * n-numbers vary in some cases due to technical problems with cytokine and chemokine measurements.

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LIST OF ABBREVIATIONS

LPS = lipopolysaccharide; LTA = lipoteichoic acid; TCS = T-cell stimuli (anti-CD3/anti-CD28); TNF-alpha = tumor necrosis factor-alpha; IL-1beta = interleukin-1beta; IL-6 = interleukin-6; IL-8 = interleukin-8; IFN-gamma = interferon-gamma and IL-2 = interleukin-2.
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Helium inhalation and the immune system


Summary

Gezina Oei
In this thesis, three main research questions have been addressed and answered. Firstly we showed that hypoxia induces late preconditioning in vivo (chapter 3). Secondly we proved that helium induces pre- and postconditioning (chapter 4-7). Because of clinical applicability we focused on helium postconditioning and found that application of a 15-minute episode of helium seems to be the most protective stimulus (chapter 4). We investigated the influence of helium conditioning on various aspects and constituents of reperfusion injury and pathways (chapter 4-8). Thirdly we showed that a combination of various helium conditioning stimuli protects the diseased myocardium against ischemia/reperfusion injury (chapter 7).

In Chapter 2 the physical and chemical properties of helium gas are described, and we explained the possible mechanisms by which helium could exert its biological effects. We then set out the organ protective properties, and showed that helium protects the heart, brain and neuronal tissue, lung, immune system and blood vessels. Due to its safety, we suggested that helium should be used in various clinical settings such as acute cardiovascular disease and cardiac surgery.

In chapters 3, 4 and 5 we investigated hypoxia- and helium-induced conditioning in healthy male rats. In Chapter 3 we showed that late preconditioning induced by hypoxia (16%, 12% and 8% oxygen) reduces infarct size in rats, and is as effective as sevoflurane-induced early preconditioning. We demonstrated that continuous administration of sevoflurane and propofol do not induce cardioprotection, but both could not abrogate hypoxic preconditioning either. Hypoxic preconditioning seemed to be mediated by PKCA and involved up-regulation of hypoxia inducible factor-1α dependent genes heme oxygenase-1 (HO-1) and vascular endothelial growth factor (VEGF).

Chapter 4 describes the difference between 15-, 30- and 60-minute helium postconditioning protocols. This chapter shows that the 15-minute postconditioning protocol seems to represent a break-even point at which longer administration of helium abrogates protection. The protective effect of 15-minutes of helium postconditioning seemed to be unrelated to the extent of the cytokine burst, suggesting that the I/R-induced up-regulation of innate immunity was not tempered by 15 min of helium. In contrast, 30 and 60 minutes of helium during reperfusion showed a trend towards higher expression of pro-inflammatory cytokines.

The infarct sparing effect of 15 minutes of helium during reperfusion was shown in haematoxyline-eosine stained histological slices in Chapter 5. In this chapter the different contribution of cell death and cell survival pathways are highlighted. A
differential contribution of necrosis, apoptosis and autophagy to final infarct size is described as well as the influence of helium postconditioning on particular pathways. Helium postconditioning seemed to up-regulate genes employed in anti-apoptotic and autophagic pathways.

In chapters 6 and 7 it is shown that signal transduction in diseased rats is different from their healthy counterparts. Moreover, it’s more difficult to reduce infarct size by pre- and postconditioning in the diseased rat.

Diabetic Zucker rats have been used in Chapter 6 to show that pre- and postconditioning is abrogated under diabetic circumstances, even when the preconditioning stimulus is increased from 3 cycles of 5 min helium 70% to 6 cycles of helium 70%. In healthy rats, mild mitochondrial uncoupling and a reduction in phosphorylation of glycogen synthase kinase-3 beta (GSK-3beta) was found after the ischemic episode in the helium-preconditioning group. These effects were not seen in diabetic rats. In this chapter we also show that helium conditioning did not exert any effects on extracellular signal-regulated kinases 1/2 (ERK1/2) and Akt/Protein kinase B.

Infarct size reduction by postconditioning alone could not be induced in the Spontaneously Hypertensive Rat (SHR) in Chapter 7, in contrast to healthy control animals that could be protected by postconditioning. The combination of helium postconditioning with helium-induced late preconditioning or postconditioning with late- and early preconditioning did not afford additional protection. In hypertensive animals, only the combination of helium-induced early- and late preconditioning and postconditioning exerted an infarct size sparing effect. Chapter 7 also describes the lack of involvement of protein kinases GSK-3beta and protein kinase C-epsilon (PKC-beta) in helium-induced conditioning.

Human models are used in chapters 8 and 9 to investigate helium-induced endothelial protection (chapter 8) and the safety of helium inhalation regarding the innate immunity response (chapter 9).

In Chapter 8 we show in a forearm model of ischemia/reperfusion that helium inhalation increased the blunted vasodilator response to acetylcholine after ischemia/reperfusion. This protective effect on endothelium was found after helium-induced early and late preconditioning and ischemic early preconditioning; helium and ischemic early preconditioning equally protected the endothelium. Helium did not influence the plasma levels of cytokines, adhesion molecules and microparticles.
In Chapter 9 we show that 30 and 60 minutes of helium inhalation do not affect the ability of the innate immune system to respond to pathogens. Ex vivo stimulation of whole blood with different immune stimuli resulted in similar levels of pro-inflammatory cytokines with and without helium inhalation. We concluded that helium can be safely used in patients with infections, as it does not influence the immune response.
General discussion
Conditioning of the heart by helium

In rats, fifteen minutes of helium postconditioning (PostC) reduced myocardial infarct size in comparison to ischemia/reperfusion, as we showed in this thesis in chapter 4, 5, 6 and 7. These findings are in line with results from studies done in our and other laboratories, showing that helium induces early preconditioning (EPC) in rabbits and rats\(^1\)\(^-\)\(^7\). In all of these studies it was demonstrated that application of short cycles of helium before the index ischemia reduces infarct size. Experiments with sevoflurane-induced preconditioning (PC) in guinea pigs showed that 2 cycles of 5 minutes of 1.4 vol% sevoflurane decreased infarct size to a greater extend than 1 cycle of 15 minutes of 2.8 vol% sevoflurane\(^8\).

These data show that the way the conditioning protocol is carried out is quite important. While PC has been induced mostly by repetitive short cycles, this is different for PostC. Sevoflurane-induced PostC in rats was achieved by both a short episode of inhalation for 2 minutes\(^9\) but also after prolonged administration of 15 minutes\(^10\). In this thesis, the 15-minute helium PostC protocol proved to be effective. On the contrary, prolonged administration of helium during reperfusion did not induce protection. This is very different from the expectations one could have from a pharmacological point of view; it sounds logic that a stronger protection arises after continued inhalation of a protective substance. With helium PostC this was not the case, as infarct sizes returned to control levels after 30 and 60 minutes of helium PostC (chapter 4). A similar observation was done in chapter 3 with sevoflurane. While 3 five-minute cycles of sevoflurane reduced infarct size, continuous administration with sevoflurane did not exert myocardial protection.

As prolonged administration of helium did not result in enlarged protection, it was hypothesized that multiple stimuli might still be protective. Pagel and colleagues\(^2\) earlier investigated the application of 1, 3 and 5 five-minute cycles of helium prior to index ischemia, and found that infarct sizes were reduced from 44% in control to 35%, 25% and 20% respectively. These findings were confirmed by the same group in another study\(^6\). Apparently, more cycles of PC increase the level of protection. This phenomenon was applied in the Zucker rat in chapter 6; both 3 and 6 five-minute cycles were used to protect the diabetic heart, unfortunately without success. It seems that simply repeating ‘parts’ of one type of conditioning is not strong enough to convey protection in the diseased heart.

A combination of PC and PostC resulted in increased protection in rats and rabbits\(^9\)\(^,\)\(^11\)\(^,\)\(^12\). In rats it was shown that sevoflurane EPC combined with sevoflurane PostC reduced infarct size in a stronger way than sevoflurane EPC alone\(^9\). In this study, sevoflurane-induced EPC was applied by 2 five-minute cycles of sevoflurane with a
10-minute washout before ischemia; sevoflurane-induced PostC was executed by a two-minute episode at the onset of reperfusion. We hypothesized that a combination of different conditioning protocols would therefore be needed to induce infarct size reduction in the hypertensive myocardium in chapter 7. We combined helium PostC with helium LPC and also applied a triple intervention by the combination of LPC and EPC plus PostC. In healthy animals this did not enhance protection in comparison to PostC alone, but all interventions did reduce infarct size in comparison to control. In the hypertensive animals, only the triple intervention reduced infarct size, suggesting an increased threshold to protection in the diseased myocardium.

The percentage of the helium gas administered in the studies of this thesis also deserves some attention; 70% in the animal studies versus 79% in the clinical studies. From a clinical point of view, 79% helium in a mixture with 21% oxygen is the maximum amount of helium that should be administered, unless it is desirable to administer a hypoxic gas mixture. As it is often preferential to increase the administered level of oxygen to patients somewhat above 21%, the 70% helium percentage use in the animals seems to be justified. There is only one argument against the use of 70% helium and it is of pharmacologic nature. One could argue that a more pronounced effect of helium conditioning could be found when a stronger dose is used.

**Mechanisms of helium-induced conditioning**

The signaling pathways that are supposed to be involved in helium-induced EPC are set out in chapter 2. Simplified, the so-called survival kinases and mitochondria were suggested as major players. Survival kinases are proteins in the cell that function as switches; they can switch on and off and thereby influence the next protein in the pathway. By posttranslational modification such as phosphorylation they turn to the active or passive state and subsequently influence another protein. A whole set of signaling kinases ultimately affects an end target, which could be any organelle or receptor within a cell. An example of an end target that determines whether cell survival or death takes place, are the mitochondria.

By use of blockers directed against specific kinases, phosphatidylinositol 3-kinase (PI3K), extracellular signal-regulated kinase 1/2 (ERK1/2), 70-kDa ribosomal protein s6 kinase (p70s6K) and glycogen synthase kinase-3beta (GSK-3beta) were found to be involved in helium EPC. Beside these kinases, three mitochondrial channels were also investigated and found to be involved in helium EPC; the mitochondrial permeability transition pore (mPTP), the mitochondrial adenosine triphosphate-regulated potassium channel (mK$_{ATP}$), and the mitochondrial calcium-sensitive potassium channel (mK$_{Ca}$). Stress-induced formation of the mPTP in particular is thought to play an important role.
in helium conditioning. Cardioprotective effects of helium EPC were abrogated after simultaneous maintenance of a metabolic alkalosis during reperfusion, a condition that causes opening of the mPTP\(^5\). Protection was restored again after co-administration of the mPTP formation inhibitor cyclosporin A\(^5\).

In this thesis, both the involvement of pro-survival kinases and mitochondria were investigated. In chapter 6 we investigated phosphorylation of Akt, ERK1/2, and GSK-3beta, all proteins that belong to the group of survival kinases and of which ERK1/2 and GSK-3beta were found to be involved in helium-induced early preconditioning\(^1,2\). In chapter 6 we demonstrated that Akt and ERK1/2 were not involved in helium EPC, as phosphorylation levels were not higher in comparison to control animals. Nevertheless, a reduced phosphorylation of GSK-3beta was found during the index ischemia in animals that were exposed to helium EPC. In contrast, in chapter 7 we found no differences in GSK-3beta phosphorylation level after helium PostC in comparison to control.

Taken together it seems that involvement of the above-mentioned survival kinases have only been shown in experiments that were performed with non-specific blockers. The more specific investigations on a biochemical level of these proteins could not confirm these data. In chapter 6 it is proposed that helium EPC causes mild mitochondrial uncoupling during the index ischemia, a state that prevents mPTP opening. This was shown by measurement of different respiratory states of the isolated mitochondria and calculated as the respiratory control index.

Additional to the investigations on the effect of helium conditioning on mitochondria and survival kinases, we also investigated other mechanisms of helium conditioning. In chapter 4 we looked at the effect of helium postconditioning on the protein- and mRNA levels of inflammatory cytokines in the myocardium. Both the myocardium at risk -i.e. downstream of the ligated coronary artery- and adjacent healthy tissue were investigated, but in neither type of tissue we found signs of involvement of innate immunity in helium conditioning. This implies that helium conditioning does not exert its protective effects by attenuating the hyper acute cytokine burst. Instead, we suggest in chapter 5 that helium PostC affects a whole set of genes that are involved in cell death and cell survival, all related to necrosis, apoptosis and/or autophagy. We show that helium conditioning induces a relative up-regulation of multiple genes that are employed in autophagy and against apoptosis. It is possible that a short episode of helium inhalation during reperfusion results in a higher level of autophagy, which enables cells to get rid of damaged proteins and organelles that accumulate during ischemia and the first minutes of reperfusion. For cellular survival it is of vital importance that these products can be disposed outside of the cell. The combined stimulation of autophagic cell processes and anti-apoptotic pathways could have resulted in the reduction of cell damage that has been observed in all studies of this thesis.
Limitations of experimental models and translation to the clinic

In this thesis well-established in vivo and in vitro models have been used to investigate helium conditioning. Animal models in particular always pose ethical and moral dilemmas for the involved research team as well as the scientific community in general. However, we still need animal models to keep up the scientific solutions and developments in medicine. An example of a disease that cannot be investigated without the use of animals is coronary heart disease, for which we used the myocardial regional ischemia/reperfusion model. Although well established and widely used, there are some disadvantages with this model. The most profound problem with this model is the origin and development of the phenomenon myocardial ischemia. In humans, risk factors such as obesity, diabetes and hypertension influence the severity and time frame of disease development and consequently also affect the window of protection.

In general, five objections can be distinguished when considering these types of animal experiments and might explain why translation of successful experimental therapies to the clinic is scarce\(^\text{15}\): (1) patient populations are heterogeneous while experimental studies are often conducted in male subjects receiving standardized diets and live in controlled environments; (2) the duration of index ischemia is relatively shorter in animal experiments compared to clinical situations, which enlarges the possibility of salvaging strategies; (3) experimental animals are young and healthy whereas patients are old with comorbidities; (4) patients often use multiple pharmacologic treatments a priori that affect the final myocardial injury; (5) in animal studies function of other organs than the heart remain uninvestigated while treatments could be detrimental for these other organs and preclude clinical use. In the last years, several reviews on this issue have been written and set out the problems researchers were faced with when testing new therapeutics in clinical trials, such as ischemic and anesthetic conditioning\(^\text{14,15}\). A recent review article addressed this issue and analyzed 16 ischemic PC, 12 ischemic PostC, 25 remote PC and 13 pharmacologic conditioning trials\(^\text{16}\). Of the 66 clinical trials that have been conducted, not a single one was large enough and could show a profound benefit of cardiac conditioning. It has to be noted that in clinical trials, infarct size measurement is still a subject of debate, as it is difficult to find a marker that shows minimal natural variability. This is the reason why experimental studies have an advantage; infarct size measurement by TTC-staining is a very reliable method.

A small step towards the translation of helium as an organ protective agent to the clinic has been made in this thesis. In chapter 8 and 9 human male and female volunteers were used to investigate helium in humans in vivo. In both studies, a relatively small number of healthy, young subjects were used and exposed to helium inhalation. Despite
their contribution to a better understanding of the effects of helium in vivo, these studies clearly targeted a non-realistic population considering the lack of comorbidity and young age. Nevertheless, these studies are a crucial first step towards larger clinical trials in which human subjects are used.

**Future research**

This thesis has answered questions but inevitably also raised questions and left some problems unsolved. In conjunction with the last paragraph, it should be emphasized that a clinical trial with sufficient patient should be performed in order to find out whether helium can be used as an adjunct therapy to reperfusion therapy and in which modality it should be used. This thesis has provided answers considering timing and duration of the helium conditioning protocol in rats, but it is unknown whether these protocols can be applied in patients in the same form.

Before such a clinical trial would be conducted it would be wise though, to further investigate certain issues and questions that were raised in this thesis. These issues all relate to the underlying mechanism of helium conditioning and our insufficient understanding of its cardioprotective effects. One of the most important points comprises pathways that are involved in prevention of apoptosis and execution of autophagy. As mitochondria are likely to be important players in helium conditioning as well, one could imagine that the clearing of defective mitochondria from cells in order to keep it healthy and functional might play a role.

To conclude, helium-induced conditioning seems to be for the better, but so far this only holds true for healthy animals and healthy volunteers. It remains a question whether helium-induced conditioning can also be applied in sickness, i.e. in individuals with concomitant disease or comorbidity. Future research should therefore include investigations in experimental models of disease and eventually the target patient population, i.e. the diabetic-, hypertensive- or obese aged patient.
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PART

Appendices
Acknowledgement/ Dankwoord
Een proefschrift zonder dankwoord is als een OK zonder OK-borrel: eigenlijk wil je er niet aan beginnen, maar uiteindelijk eindig je er toch. Dit proefschrift is tot stand gekomen door de adviezen, steun en hulp van velen, aan hen richt ik graag dit dankwoord: Dankjewel!

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Appendix

Publications
Appendices


Research portfolio of the candidate
Appendices

GRANTS/PRIZES

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CONFERENCE/ABSTRACTS


Appendices


Appendix

About the author
Gezina Oei studied Health Care Policy and Management (Erasmus University Rotterdam, 2001-2004). She graduated after a thesis on international law and fixed prices of health care products in the Netherlands, and an internship at the Board of Directors of the University Medical Centre Utrecht, implementing a quality measurement tool.

Subsequently she studied Medicine (University of Amsterdam, 2004-2007), after which she started a combined MD/PhD. Her research activities were conducted at the Laboratory of Experimental Intensive Care and Anesthesiology (L.E.I.C.A; Prof. Dr. M.W. Hollmann, Prof. Dr. B. Preckel and Dr. N.C. Weber). Mw Oei chose a senior internship cardiology at the Department of Cardiology at the Onze Lieve Vrouwe Gasthuis Hospital Amsterdam (Dr. G.A. Somsen) and a senior internship in critical care medicine at the Department of Intensive Care at the Academic Medical Centre Amsterdam (Prof. Dr. M.B. Vroom).

After finishing her clinical internships in August 2011, she continued the work on her PhD thesis and shortly worked as a resident at the Department of Cardiology at the Jeroen Bosch Hospital in Den Bosch (Dr. M.C.G. Daniels). In January 2012 she started her residency at the Clinical Department of Anesthesiology, Academic Medical Centre Amsterdam (Dr. C. Keijzer and Prof. Dr. W.S. Schlack). Currently Mw. Oei works as a resident Anesthesiology at the Onze Lieve Vrouwe Gasthuis Hospital Amsterdam (Dr. P. de Haan).

When Gezina is not busy working, she likes to spend her time on sports, seeing friends, reading and traveling.
Appendix

Short Dutch summary/ Korte Nederlandse samenvatting
Een onderbreking van de bloedtoevoer naar het hart resulteert eerst in omkeerbare schade (ischemie) en vervolgens in onomkeerbare schade (necrose, celdood) in de hartspiercellen. Door de onderbreking van bloedtoevoer krijgen de cellen geen zuurstof en moeten de cellen overgaan tot een alternatieve stofwisseling waarbij schadelijke stoffen zich ophopen en de cellen uiteindelijk uit elkaar vallen. De functie van de hartspier komt hierdoor in gevaar. Een dergelijke onderbreking van de bloedtoevoer komt meestal voor bij patiënten met ziekte van de kransslagaders van het hart (bijvoorbeeld een hartinfarct), maar doet zich ook voor tijdens hartoperaties waarbij de toevoer van bloed tijdelijk onderbroken wordt.

Het herstellen van de bloedstroom (reperfusie) is essentieel voor het behoud van de cellen, maar levert op zich zelf ook schade op; ‘reperfusieschade’. Al jaren wordt gepoogd de reperfusieschade te verminderen door het hart te beschermen of te ‘conditioneren’. Deze bescherming zou werken door het hart voor de ischemische episode te laten wennen aan de onderbreking van bloed- en zuurstofftoevoer: ‘preconditionering’. Belangrijker voor de klinische praktijk is de methode waarbij tijdens de reperfusie een beschermende prikkel toegediend kan worden (‘postconditionering’); immers kan bij patiënten met een hartinfarct niet voorspeld worden wanneer dit zal gaan plaatsvinden.

Helium is een kleurloos, reukloos, edelgas zonder anesthetische werking, wat organen kan beschermen tegen ischemie/reperfusie schade. In dierstudies wordt aangetoond dat inademing van helium voor of na een ischemische episode de infarctgrootte van het hart kan verminderen. In dit proefschrift wordt beschreven hoe lang en wanneer het gas toegediend moet worden om de ischemie/reperfusie schade te verminderen. Tevens is onderzocht hoe helium op cellulair en moleculair niveau zijn beschermende werking uitoefent. Het werkingsmechanisme van helium omvat veranderingen op eiwit en gen niveau, die uiteindelijk gezamenlijk het beschermende effect teweegbrengen.

Deze mechanismen blijken te veranderen wanneer sprake is van onderliggende aandoeningen zoals suikerziekte en hoge bloeddruk. In dit proefschrift wordt tevens beschreven dat helium inademing door de mens de binnenbekleding van de bloedvaten (de endotheelcellen) kan beschermen, en dat het immuunsysteem hierbij niet beïnvloed wordt. Toekomstige studies in cellen en dieren zullen het mechanisme van helium-geïnduceerde bescherming van het hart verder moeten ontrafelen. Klinische studies zullen moeten uitwijzen of patiënten van deze bijzondere eigenschap van helium kunnen profiteren in de toekomst.