Measuring microvascular and mitochondrial oxygen tension: novel techniques for studying tissue oxygenation

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Chapter 5

Monitoring of Renal Venous PO₂ and Kidney Oxygen Consumption in Rats by a Near-Infrared Phosphorescence Lifetime Technique

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ABSTRACT
Renal oxygen consumption (\(\text{VO}_{2,\text{ren}}\)) is an important parameter that has been shown to be influenced by various pathophysiological circumstances. \(\text{VO}_{2,\text{ren}}\) has to be repeatedly measured during an experiment to gain insight in the dynamics of (dys)regulation of oxygen metabolism. In small animals, the classical approach of blood gas analysis of arterial and venous blood samples is only limitedly applicable due to fragile vessels and a low circulating blood volume. We present a phosphorescence lifetime technique that allows near-continuous measurement of renal venous \(\text{PO}_2\) (\(\text{vPO}_2\)) and \(\text{VO}_{2,\text{ren}}\) in rats. The technique does not rely on penetration of the blood vessel, but uses a small reflection probe. This probe is placed in close proximity to the renal vein for detection of the oxygen-dependent phosphorescence of the injected water-soluble near-infrared phosphor Oxyphor G2. The technique was calibrated in vitro and the calibration constants were validated in vivo in anesthetized and mechanically ventilated male Wistar rats. The hemoglobin saturation curve and its pH dependency were determined for calculation of renal venous oxygen content. The phosphorescence technique was in good agreement with blood gas analysis of renal venous blood samples, for both \(\text{PO}_2\) and hemoglobin saturation. The technique was used in four rats during endotoxin infusion (10 mg\(\cdot\)kg\(^{-1}\)\(\cdot\)h\(^{-1}\) during 1 h). Renal \(\text{vPO}_2\) reduced by 40% upon reduction in oxygen delivery to 30% of baseline, but \(\text{VO}_2\) remained unchanged. This study documents the feasibility of near-continuous, nondestructive measurement of renal \(\text{vPO}_2\) and \(\text{VO}_2\) by oxygen-dependent quenching of phosphorescence.

INTRODUCTION
The measurement of oxygen in blood within arteries and veins is mandatory for the study of oxygen supply and demand of organs. For example, the oxygen delivery (\(\text{DO}_2\)) to an organ is calculated by the arterial oxygen content of the blood per volume times the blood flow to the organ. To also determine the oxygen utilization (\(\text{VO}_2\)) by an organ, the venous oxygen content has to be known. Another example of a situation where knowledge of the venous oxygenation over time is necessary is monitoring of the “\(\text{PO}_2\)-gap,” i.e., the discrepancy between capillary \(\text{PO}_2\) and venous \(\text{PO}_2\) when the capillary \(\text{PO}_2\) drops below the venous \(\text{PO}_2\) (9, 22, 23). The latter occurs under certain pathophysiological conditions, such as sepsis and shock (5), and is a valuable parameter when studying resuscitation protocols and treatments.

Classically, the oxygen content of the blood is measured by taking blood samples for determination of blood gasses and the hemoglobin oxygen saturation. Although this is the most accurate way of measuring the oxygen content of the blood, it has several drawbacks when used in small animals like rats. Due to the small circulating volumes, repeatedly taking blood samples can rapidly lead to hypovolemia and hemodynamic alterations. Furthermore, the limited intraluminal space in the blood vessels prevents catheterization making repeated puncture mandatory. Since the latter is cumbersome and can easily lead to irreversible damage to the vessel, it is contraindicated in experiments where during a certain time course repeated measurements are needed.

The mentioned drawbacks of blood sampling in small animals have been recognized in the literature and have encouraged investigators to search for alternative methods. Recently, for example, Pakulla et al. (13) tested the possibility of using a continuous intravascular blood gas monitor in rats with a favorable outcome. However, the physical dimensions of such a catheter limit its use to the relatively large vessels (i.e., aorta and vena cava). For selective measurement of the \(\text{PO}_2\) in small veins of single organs, other techniques must be explored.

In small animals, nondestructive optical techniques to determine intravascular oxygenation could very well be a good alternative to the more invasive methods. The thin vessel wall and overall small proportions of the animal effectively counterbalance the in general small penetration depth of light. The use of phosphorescence lifetime measurement (25, 26) could be an attractive option. For almost two decades, oxygen-dependent quenching of phosphorescence has been used for quantitative oxygen
measurements in micro vessels (4, 18–20, 24, 29). However, until now it has not been used for oxygen measurements in larger vessels. An explanation can probably be found in the fact that the first widely used phosphor, Palladium-porphyrin, is excited with green light (10, 21). This excitation wavelength is in a part of the optical spectrum where the absorbance of blood is very high (16), preventing the use of Pd-porphyrin for oxygen measurements in full blood due to low excitation efficiency and low signal levels.

In recent years, however, Vinogradov and co-workers (2, 15, 17, 28) developed techniques to tune the quenching and optical properties of phosphorescent dyes. This has led to a new generation of phosphors for oxygen measurements with favorable spectral properties. Oxyphor G2, for example, is a water-soluble phosphor with excitation and emission wavelengths in the (near-)infrared spectrum (2). This allows much deeper tissue penetration because of the low absorbance of tissue in this so-called “tissue optical window” (12, 16). Recently, we demonstrated that Oxyphor G2 can be used to measure PO2 in dense, blood-rich tissue to a depth of several millimetres (6). The optical properties of Oxyphor G2 open the possibility to use this probe for oxygen measurements in full blood and therefore in large blood vessels.

The aim of this study was to test the possibility of measuring the PO2 inside a large blood vessel of a rat by using oxygen-dependent quenching of phosphorescence of the near-infrared phosphor Oxyphor G2. The following steps were undertaken. First, a frequency-domain phosphorimeter using a special probe with small tip for easy placement on a blood vessel was developed. Second, calibration experiments in vitro were performed to test the device. Third, the hemoglobin saturation characteristics of rat blood were determined for calculation of oxygen content from PO2 values. Fourth, the proposed method was evaluated by comparing phosphorescence lifetime measurements on the renal vein with blood samples drawn from the renal vein in a series of rats. Fifth, the practical use of the technique was demonstrated on the rat renal vein during an experimental protocol.

MATERIALS AND METHODS

Phosphorescence lifetime measurements. Oxyphor G2 [Pd-meso-tetra-(4-carboxyphenyl) tetra-benzo-porphyrin; Oxygen Enterprises, Philadelphia, PA] is a water-soluble phosphorescent dye that is injected into the circulation where it binds to albumin. The phosphor-albumin complex is well confined to the circulation (14) and emits phosphorescence with a wavelength ~800 nm (i.e., infrared light) if excited with light of a wavelength ~630 nm (i.e., red light). The lifetime of the phosphorescence is oxygen dependent (2) and is quantitatively related to the oxygen level by the Stern-Volmer relationship

\[ \text{PO}_2 = \frac{1}{\tau} - \frac{1}{\tau_0} \]

where \( \tau \) is the measured phosphorescence lifetime, \( \tau_0 \) is the phosphorescence lifetime at zero oxygen, and \( k_q \) is the quenching constant. The calibration constants \( k_q \) and \( \tau_0 \) are characteristic properties of the used phosphorescent compound and once determined the method needs no recalibration.

Phosphorescence lifetimes can be measured either in the time domain or in the frequency domain. In the time domain an actual decay of the light output can be observed, and the lifetime can be determined by applying a monoexponential fit procedure after taking the excitation pulse shape into account (11). In the frequency domain, the phosphor is excited by a modulated light source (27). The emitted phosphorescence shows the same modulation frequency as the excitation source. Since the phosphorescence emission is somewhat delayed compared with the excitation, the phosphorescence is detected with a phase shift. A longer phosphorescence lifetime results in a larger phase shift.

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lifetime can be calculated from the phase shift ($\Delta \phi$) by the following equation

$$\tau = \frac{1}{2\pi f} \tan(\Delta \phi)$$  \hspace{1cm} (2)

where $f$ is the modulation frequency. Phosphorescence lifetime measurements, in contrast to fluorescence lifetime measurements, require relatively low modulation frequencies in the order of a few hundred Hz to several kHz. This allows the construction of relatively simple and cost-effective measurement equipment.

**Description of the phosphorimeter.** The used phosphorimeter was especially designed for the application described in this paper. It is solely based on commercially available standard components, allowing a simple and easily reproducible construction. A schematic of the device is given in Fig. 1A. One of the most important parts of the device is its optical fiber, which has to be small to allow easy positioning on a single small blood vessel. We found a more than adequate solution in the Reflection Probe with Small Tip type FCR-7IR200-1,5X100-2 (Avantes World Headquarters, Eerbeek, The Netherlands). This probe is basically a branched fiber ending in a stainless steel cylinder of 10-cm length and 1.5-mm diameter around a bundle of seven 200-µm optical fibers in an arrangement shown in Fig. 1B. The central fiber is used as excitation path, while the surrounding six fibers serve as detectors. To prevent the fibers from becoming contaminated by phosphor, the tip of the probe was covered with a glass tube which was sealed at the end with a piece of microscopic coverslide.

**FIGURE 1 A:** schematic drawing of the frequency domain principle and the used phosphorimeter. APD, avalanche photo diode; amp, amplifier; DAQ, data acquisition; L1, collimator lens; F1, 800-nm band pass filter. B: schematic of the reflection probe and the fiber assembly within the probe.
The excitation light source is a focusable laser diode module operating at a wavelength of 635 nm (Lasiris DLS laser module, StockerYale, Salem, NH). The laser output is modulated by a sinusoidal voltage with a frequency of 2000 Hz which is generated by a data-acquisition board (PCI-MIO-16E1, National Instruments, Austin, TX). The laser bundle is focussed into the fiber branch containing the single central excitation fiber. The output of the other fiber branch is filtered by an 800-nm band pass filter and focused onto the cathode of an avalanche photo-diode. This diode is part of an avalanche photo-diode module (APD-module C5460, Hamamatsu, Hamamatsu City, Japan) containing a high-voltage generator, voltage controller, and a current-to-voltage converter with a 10-MHz bandwidth. The signal from the APD-module is amplified to a level that is adequate for analog-to-digital conversion by the data-acquisition board using an amplifier module from a NeuroLog system (Automate Scientific, San Francisco, CA). Both the modulation signal and the phosphorescence signal are sampled by the data-acquisition board and both are fed into a standard frequency analysis algorithm of the LabView Full Development System software (Version 6.1, National Instruments). This algorithm determines the frequency and phase of each signal. From the returned phases, the phase shift between both signals is calculated allowing subsequent lifetime and PO$_2$ calculation by equations 2 and 1, respectively.

**In vitro calibration.** In vitro calibrations were performed in a bicarbonate buffer containing 2% bovine serum albumin (Sigma, St. Louis, MO) and a final concentration of 10 µM Oxyphor G2. PO$_2$ values were regulated using a system consisting of gas flow controllers, an oxygenator, and a closed-loop recirculation system. A detailed description of this system can be found in a previous publication (12). Experiments were performed at 37°C. The pH value of the solution was checked during the experiments and remained at 7.4 ± 0.1.

**Hemoglobin saturation curves.** Rat hemoglobin saturation curves were constructed by pooling all blood samples drawn in rat experimental protocols in our laboratory over a 3-mo period. Blood samples were analyzed to determine blood gas values (ABL505, Radiometer, Copenhagen, Denmark), as well as hematocrit, hemoglobin concentration, and hemoglobin oxygen saturation (OSM 3, Radiometer). This resulted in a total number of 102 analyzed samples. The data set was subdivided in different pH ranges and subsequently analyzed by fitting Hill’s equation

$$HbO_2 = \frac{(PO_2)^n}{1 + (PO_2/P_{50})^n}$$

where HbO$_2$ is the hemoglobin oxygen saturation, PO$_2$ is the partial oxygen pressure, P$_{50}$ is the partial oxygen pressure at 50% saturation, and $n$ is the Hill’s coefficient. The fit was performed using the nonlinear Marquart-Levenberg fit procedure available in LabView.

**Animals.** The protocol of the present study was approved by the Animal Research Committee of the Academic Medical Center at the University of Amsterdam. Animal care and handling were performed in accordance with the guidelines for Institutional and Animal Care and Use Committees (IACUC). The experiments were performed in a total of 12 male Wistar rats (Charles River, The Netherlands) with a mean (±SD) body weight of 313 ± 38 g. The rats were kept in cages with water and food given ad libitum.

**Animal preparation.** Animals were anesthetized by an intraperitoneal injection of a mixture of ketamine (90 mg/kg), medetomidine (0.5 mg/kg), and atropine (0.05 mg/kg). Ketamine (50 mg•kg$^{-1}$•h$^{-1}$)
was infused intravenously to maintain anesthesia. To compensate for fluid loss, crystalloid solution (saline; 5 ml·kg⁻¹·h⁻¹) was administered continuously. A thermocouple probe placed in the rectum and a heating pad under the animal allowed controlling and maintaining body temperature at 37 ± 0.5°C.

The animal underwent a tracheotomy to enable mechanical ventilation with a mixture of 40% oxygen and 60% nitrogen. To diminish loss of fluid by the mechanical ventilation, a heat and moisture exchanger (Humid-Vent Micro, Gibeck, Sweden) was placed between the tracheal tube (Ch 6) and the ventilator. Measurements of the end-tidal PCO₂ allowed maintaining the arterial PCO₂ between 35 and 40 mmHg.

For intravenous administration of drugs and fluids, a polyethylene catheter (outer diameter 0.9 mm) was inserted into the right jugular vein. For continuous monitoring of arterial blood pressure and heart rate, a catheter of the same size was placed into the right carotid artery and connected to a pressure transducer. Two additional catheters were placed into both the right femoral artery and vein for withdrawal of blood and administration of fluid.

The left kidney was exposed after a flank incision, decapsulated, and immobilized in a Lucite kidney cup. In the following, the renal vein was isolated from the artery and the surrounding tissue and a 0.5 x 1.0-cm piece of aluminum foil was placed behind the vessel to prevent contribution of underlying tissue to the phosphorescence signal in the venous PO₂ measurement. For urine collection, the left ureter was ligated and cannulated. In animals receiving LPS infusion, a perivascular ultrasonic transient time flow probe (type 0.7 RB; Transonic Systems, Ithaca, NY) was placed around the left renal artery and connected to a flow meter (T206; Transonic Systems) to allow continuous measurement of renal blood flow (RBF). Throughout the entire experiment, the surface of the kidney was covered by saline-wetted cotton wool.

Hemodynamic and blood gas measurements. During the surgical and experimental procedures, systolic and diastolic arterial pressures were measured in the carotid artery. Mean arterial pressure was calculated as MAP (mmHg) = diastolic pressure + (systolic pressure - diastolic pressure)/3. The amplitude of the arterial blood pressure was calculated as pulse pressure (P_puls) = systolic pressure - diastolic pressure. For continuous measurement of the renal venous oxygen content, the optical fiber of the phosphorimeter was placed 1 mm above the surface of the isolated left renal vein. Arterial blood samples of 0.2 ml each were collected from the femoral artery catheter. Venous blood samples of 0.2 ml each were collected by means of repetitive venous puncture of the renal vein. Great care was taken not to damage the renal vein or its surroundings (especially the renal artery). The samples were used to determine blood gas values (ABL505, Radiometer), as well as hematocrit, hemoglobin concentration, and hemoglobin oxygen saturation (OSM 3, Radiometer).

Calculation of renal DO₂, VO₂, and O₂ER. Renal oxygen delivery was calculated as DO₂,ren (ml/min) = RBF * arterial oxygen content (1.31 * Hb * SaO₂) + (0.003 * PaO₂). Renal oxygen consumption was calculated as VO₂,ren (ml/min) = RBF * (arterial - renal venous oxygen content difference). Renal venous oxygen content was calculated as (1.31 * Hb * vHbO₂) + (0.003 * vPO₂). Renal venous hemoglobin saturation (vHbO₂) was calculated from the renal venous PO₂ (vPO₂) using Hill’s equation. The oxygen extraction ratio (in percentage) was calculated as (VO₂,ren/DO₂,ren) * 100%.

Experimental protocol. To get a broad range of different renal venous PO₂ values, eight animals were ventilated with variations in the fractional inspired oxygen content (FiO₂). Therefore, FiO₂ of 0.2, 0.4, and 1.0 were adjusted with a flow meter. After each change in FiO₂, the animals were allowed to adapt for 15 min before the renal vein was punctured for collection of a venous blood sample. The puncture was performed by using a 27-gauge cannula. Hemolysis due to the small diameter of the needle was excluded by preceding centrifugation of several blood samples. The LPS protocol was performed in four animals. Thirty minutes after stabilization from surgery, the animals received a 1-h
infusion of LPS (10 mg/kg, serotype 0127:B8; Sigma-Aldrich, Zwijndrecht, The Netherlands) to induce endotoxemia.

**RESULTS AND DISCUSSION**

To test the technical performance of the described device, an in vitro calibration was performed. The results from measurements in a 10 µM Oxyphor G2 solution in 2% bovine serum albumin are shown in Fig. 2. Figure 2A shows the phosphorescence phase shift relative to the excitation light as function of the applied PO$_2$ in the sample solution. The reciprocal lifetime shows excellent linearity to PO$_2$ over the physiological PO$_2$ range, as shown in Fig. 2B. Calibration constants were determined to be $k_q = 267$ mmHg$^{-1}$s$^{-1}$ and $\tau_0 = 252$ µs. Those calibration constants are in agreement with previously reported values (2, 6).

To calculate the venous oxygen content from the vPO$_2$ (venous PO$_2$) reading, one has to be able to make a sound estimation of the hemoglobin saturation. However, the relationship between the PO$_2$ and the saturation is influenced by other factors of which in our case the pH value of the blood is the most important. Figure 3A shows the results from 102 different blood gasses measured in samples from rats. The raw data are categorized in four different pH ranges. Figure 3B shows the corresponding fits of Hill's equation to the four data sets. The values for the Hill coefficients obtained from the nonlinear fitting procedure are given in the table (Fig. 3C). For pH values above 7.15 the influence of pH variations is relatively small and the effect of pH on HbO$_2$ calculation is overall well below 10%. However, for pH values below 7.15 the pH-introduced deviation of calculated HbO$_2$ (i.e., at $P_{50}$) can be as large as 40%. Although this potential for error should always be kept in mind when applying this technique, it is important to consider that the blood gasses with pH <7.15 were drawn from rats in an exceptionally bad state just before the termination of a severe sepsis protocol. Over a broad range of pathophysiological conditions conversion of vPO$_2$ to vHbO$_2$ will not be severely affected by this pH dependency. In this context, it is also important to note that the quenching constants of Oxyphor G2 are pH independent (2).
FIGURE 3 A: hemoglobin saturation vs. PO₂ determined by blood gas analysis in fresh drawn blood samples. B: result of fitting Hill's equation to the data. The corresponding values for recovered P₅₀ and n coefficient are provided in the table.

<table>
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<th>P₅₀ value</th>
<th>n-coefficient</th>
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<tr>
<td>≥ 7.35</td>
<td>32.68</td>
<td>2.12</td>
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FIGURE 4 A: vPO₂ determined in vivo with phosphorescence quenching vs. the PO₂ determined by blood gas analysis in samples drawn from the renal vein. The solid line is the line of equality. B: vHbO₂ calculated from phosphorescence lifetime measurements vs. HbO₂ determined by blood gas analysis.
The in vivo performance of the phosphorimeter in measuring real \( vPO_2 \) was checked by taking venous blood samples for a direct comparison between \( vPO_2 \) and blood gas values. Figure 4A shows \( vPO_2 \) measured by the phosphorescence technique vs. the \( PO_2 \) measured by blood gas analysis in blood samples drawn from the renal vein at the corresponding time points. The solid line is the line of equality (slope = 1) and it is clear that the phosphorescence technique allows excellent quantification of renal vein \( PO_2 \). This approach also allows for in vivo calibration of the quenching constants and we found in vivo \( k_q = 276 \, \text{mmHg}^{-1}\text{s}^{-1} \) and \( \tau_0 = 262 \, \mu\text{s} \), in very good agreement with the in vitro values. To our knowledge, this is the first in vivo calibration experiment and it shows that the previously reported in vitro calibration constants are indeed applicable in vivo. Figure 2B shows the calculated \( vHbO_2 \) values vs. \( HbO_2 \) determined by blood gas analysis. For the conversion of \( vPO_2 \) to \( vHbO_2 \), we used equation 3 with \( P_{50} = 36.9 \) and \( n = 2.26 \), determined by an overall fit of the data in Fig. 3A after exclusion of the \( pH < 7.15 \) values. Those values for \( P_{50} \) and \( n \) are in agreement with previously used values by other groups (1, 3). The solid line is again the line of equality and, despite the theoretical limitations, there is good agreement between the calculated \( vHbO_2 \) and the measured \( HbO_2 \). 

**FIGURE 5** Effects of a 1-h continuous infusion of LPS on hemodynamic parameters and renal oxygen supply and demand. A: effects on mean arterial pressure (MAP). B: effects on renal blood flow (RBF). C: effects on renal venous oxygen tension. D: effects on renal oxygen delivery and oxygen consumption. E: effect on renal oxygen extraction ratio. Given are means ± SD (\( n = 4 \)).
To demonstrate the feasibility of our method in practice, it was applied in a number of rats receiving a short continuous infusion of endotoxin. Figure 5 shows the mean and standard deviation of DO\(_2\), VO\(_2\), and O\(_2\)ER measurements in four rats. LPS infusion induces a transient reduction in MAP together with a reduction in RBF (to ~30% of the baseline value). The drop in RBF causes an analog drop in oxygen delivery. The VO\(_2\) is calculated from the vPO\(_2\) measurements and despite a drop in vPO\(_2\) and increased oxygen extraction the VO\(_2\) did not change. Overall, the measurement is very steady and reproducible and this experiment showed that the VO\(_2\) in the kidney is highly independent from the DO\(_2\). Even a drop in DO\(_2\) to 30% of the baseline value did not lead to a decrease in VO\(_2\). Two studies where our approach did detect changes in renal VO\(_2\) were recently published and concerned fluid resuscitation in normotensive endotoxemia (7) and normovolemic hemodilution (8).

In summary, this study demonstrated the feasibility of using the near-infrared phosphor oxyphor G2 to measure PO\(_2\) and oxygen content in the renal vein of the rat. The concept of the measurement is not limited to the renal vein and should equally well work in any other average-sized blood vessel. The method has the advantage that it allows quantitative, real-time, and near-continuous monitoring of intravascular oxygenation. Furthermore, over standard blood sampling it has the advantage that there is no risk of damaging the blood vessel or depletion of blood upon repeated measurements. Because of the good agreement in renal venous oxygen content measurements between the phosphorescence technique and a blood gas analyzer, it is now possible to monitor renal oxygen consumption online. In an experimental study where Oxyphor G2 is already used for measurement of microvascular PO\(_2\), it extends the possibilities of the phosphorescence quenching technique. For example, combining this new approach with the recently published dual-wavelength phosphorescence technique (6), which allows near-simultaneous measurement of cortical and outer medullary microvascular PO\(_2\), oxygen-dependent quenching of Oxyphor G2 becomes a powerful means for comprehensive assessment of renal oxygenation and oxygen consumption.

REFERENCES