Microcirculatory and mitochondrial hypoxia in sepsis, shock, and resuscitation

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Ince C, Mik EG. Microcirculatory and mitochondrial hypoxia in sepsis, shock, and resuscitation. J Appl Physiol 120: 226–235, 2016. First published June 11, 2015; doi:10.1152/japplphysiol.00298.2015.—After shock, persistent oxygen extraction deficit despite the apparent adequate recovery of systemic hemodynamic and oxygen-derived variables has been a source of uncertainty and controversy. Dysfunction of oxygen transport pathways during intensive care underlies the sequelae that lead to organ failure, and the limitations of techniques used to measure tissue oxygenation in vivo have contributed to the lack of progress in this area. Novel techniques have provided detailed quantitative insight into the determinants of microcirculatory and mitochondrial oxygenation. These techniques, which are based on the oxygen-dependent quenching of phosphorescence or delayed luminescence are briefly reviewed. The application of these techniques to animal models of shock and resuscitation revealed the heterogeneous nature of oxygen distributions and the alterations in oxygen distribution in the microcirculation and in mitochondria. These studies identified functional shunting in the microcirculation as an underlying cause of oxygen extraction deficit observed in states of shock and resuscitation. The translation of these concepts to the bedside has been enabled by our development and clinical introduction of hand-held microscopy. This tool facilitates the direct observation of the microcirculation and its alterations at the bedside under the conditions of shock and resuscitation. Studies identified loss of coherence between the macrocirculation and the microcirculation, in which resuscitation successfully restored systemic circulation but did not alleviate microcirculatory perfusion alterations. Various mechanisms responsible for these alterations underlie the loss of hemodynamic coherence during unsuccessful resuscitation procedures. Therapeutic resolution of persistent heterogeneous microcirculatory alterations is expected to improve outcomes in critically ill patients.

microcirculation; mitochondria; oxygen; shock; sepsis; resuscitation

Dysregulated oxygen transport to tissue in states of shock and resuscitation has always been a topic of controversy in clinical medicine. Fundamental aspects of oxygen transport variables at the level of the microcirculation and mitochondria have been central to this debate. Resolving these issues is crucial, because the clinical manipulation of the cardiovascular system to achieve adequate tissue perfusion and oxygenation is central to the diagnosis and management of critically ill patients. The clinical dilemma is most extremely encountered under conditions of sepsis, shock, and resuscitation, in which signs of hypoxia and reduced oxygen extraction persist despite the recovery of systemic oxygen delivery variables. Resolving this central clinical challenge requires a deeper understanding of the determinants of oxygen transport pathways under conditions of health, shock, and resuscitation.

Current evidence shows that the circulatory compromise resulting in an inability of the circulation to secure adequate oxygen transport to parenchymal cells is not due to alterations in systemic variables but rather to the failure of the microcirculation and mitochondria to transport and efficiently use oxygen. In the 1990s there was much debate concerning the origin of the oxygen extraction deficit in states of shock and sepsis. Based on the concept that a deficit of tissue oxygenation hindered oxygen delivery, Shoemaker et al. (83) proposed targeting supranormal levels of systemic oxygen delivery, which was monitored by using the recently introduced pulmonary artery catheter. However, this concept was soon abandoned when clinical trials found no difference in mortality or morbidity between septic patients targeted to receive supranormal oxygen delivery levels (e.g., 38). The enigma of clear signs of tissue hypoxia in the presence of normal or even supranormal systemic oxygen delivery remained. We proposed that the underlying reason for this condition was the following: the deficit in oxygen extraction was not caused by insufficient
systemic delivery of oxygen but rather by a pathological heterogeneity of microcirculatory perfusion resulting in functional shunting of the microcirculation, clinically manifesting as a reduction in oxygen extraction (52, 70).

The heterogeneity in oxygen pressures between different physiological compartments and continuously changing via complex regulatory mechanisms, have made the study of oxygen pressures challenging. In fact, even the definition of hypoxia in terms of its physiological impact has been a source of debate. This controversy has resulted in the definition of a more physiological concept related to hypoxia, termed dysoxia, a condition of hypoxia in which oxygen availability at the cellular level is inadequate to sustain the level of oxidative phosphorylation needed to produce ATP (70, 76, 77). Oxygen transport and utilization are highly dependent on the functional morphology and metabolism of organs, the underlying disease, the disease duration, and also of the type of therapy being administered (50). Insight into the variables related to oxygen transport to tissues is clinically highly relevant, especially for critical care medicine, because these variables determine the nature of resuscitation procedures, the optimal monitoring modality for guiding therapy, and importantly, the end points of resuscitation that must be reached.

A major complication that has limited investigation into the determinants of oxygen transport to the tissues has been the inadequacy of techniques to measure tissue oxygen pressures in vivo, especially in terms of the ability to map the heterogeneity of perfusion and oxygen pressure in the microcirculation as well as the energy state and oxygenation of the mitochondria. In the last few decades, however, significant advances in techniques used for quantitative measurement of oxygen pressure in vivo have occurred. These advances have resulted in further insight into the nature of oxygen availability and consumption in the microcirculation and in mitochondria. The use of these measurement techniques in animal models of shock and resuscitation revealed how alterations in oxygen transport are related to conditions of cardiovascular compromise. The translational application of these new insights marks the future of patient monitoring in intensive care. The clinical application of these insights will be based on an understanding of the relationship between systemic hemodynamic and oxygen derived variables and the perfusion and oxygenation of the microcirculation of the various organ systems. Based on this background, it is an opportune moment to review our current understanding of microcirculatory and mitochondrial oxygen transport and handling under conditions of shock, hypoxemia, and resuscitation.

IN VIVO METHODS FOR MEASURING MICROCIRCULATORY AND MITOCHONDRIAL OXYGENATION

In this section, we briefly review methods used to measure oxygen in vivo. Although several other methods exist, we have limited this discussion to techniques that have contributed to the understanding of the pathophysiology of oxygen transport to tissue in states of shock. Additionally, we have limited this review to quantitative techniques, although we included nicotine adenine dinucleotide (NADH) fluorescence for measuring the mitochondrial energy state because it has provided much insight into the heterogeneous nature of tissue oxygenation.

Oxygen Electrodes

The origin of tissue oxygen measurements was the development of the oxygen electrode by Clark (22). This technique has been central to tissue oxygenation research and arguably has resulted in many of the misconceptions surrounding oxygen delivery and utilization by parenchymal cells. Especially, perceived values of tissue PO$_2$ were extremely low in several organs (14, 18, 78). These findings have led to the general belief that large oxygen gradients between the microcirculation and tissue cells exist (e.g., 49, 88). A major limitation of the classical oxygen electrode has been its inability to sense the heterogeneity of oxygen pressures present in vivo. To address this shortcoming, multiarray surface oxygen electrodes and serial measurements using needle electrodes penetrating the tissue in a step-wise fashion were developed (e.g., 18, 94). A second and more important limitation of oxygen electrodes is their limited catchment area, as their depth of oxygen detection does not exceed 15 $\mu$m (48). Insertion of the oxygen electrode into tissue also results in microtrauma, and the oxygen electrode is therefore measuring a compartment of interstitial fluid directly surrounding the electrode that displays poor oxygen solubility, resulting in relatively low oxygen values. However, it can be argued that oxygen electrodes measure the interstitial oxygen tension distinct from the microcirculation. This distinction was made in a rat model of sepsis in which interstitial and microcirculatory PO$_2$ ($\mu$PO$_2$) were measured by a fluorescence quenching-based oxygen electrode and the phosphorescence quenching of an intravascular Pd-porphyrin dye, respectively (32). Both compartments showed similar responses to states of shock, but the interstitial measurement displayed lower oxygen levels than the microcirculatory measurement. Although this method represents an improvement over the Clark-type electrode, fluorescence quenching electrodes lack the ability to measure the heterogeneity of oxygen pressures without having to repeatedly puncture the tissue (18). This limitation masks important information on oxygen profiles in the case of compromised oxygenation, an effect demonstrated in the study by Evans and coworkers (1). Using fluorescence quenching electrodes, they were not able to demonstrate large changes in tissue PO$_2$ in models of acute kidney injury. However, hypoxia-sensitive histological staining with pimonidazole hydrochloride showed a heterogeneous pattern of hypoxia that characterized the heterogeneous nature of oxygenation in reperfusion-injured kidney (1).

Quenching of Pd-Porphyrin Phosphorescence

The realization that it was important to measure oxygen in a noninvasive way led Wilson and coworkers (93) to develop oxygen-dependent quenching of phosphorescence for the in vivo measurement of microcirculatory PO$_2$ ($\mu$PO$_2$). The oxygen-sensing Pd-porphyrin dye binds to albumin, which confines the dye to the intravascular compartment after intravenous injection. Excitation of the dye by pulsed light and measurement of the time constant of the phosphorescence decay enables the quantitative measurement of oxygen pressures based on the Stern-Volmer relationship. We demonstrated that this technique indeed specifically measures $\mu$PO$_2$ in an intravital study (86). The primary advantage of this time-resolved measurement is that it is independent of the concentration of the dye (85). This feature facilitates the noninvasive and quantitative measurement of oxygen pressures.
in vivo. Furthermore, this technique is especially sensitive to conditions of hypoxia because the measurement becomes more sensitive as PO2 decreases. Additionally, the catchment area is several orders of magnitude larger than that of oxygen electrodes (between 300 and 500 μm penetration as opposed to less than 15 μm for oxygen electrodes). Over time, this technique has been developed further by several groups (e.g., 41, 64, 66, 100) and its application has provided much detailed insight into the nature of oxygen transport to tissue in vivo.

An important step forward in the phosphorescence technique was the development of a near-infrared dye (Oxyphor G2) that displays deeper penetration because of its excitation properties (31). The two excitation peaks allow dual-wavelength phosphorimetry to measure μPO2 in superficial and deep tissue compartments independent of one another (54). We applied this technique in several studies to measure renal cortical and medulla μPO2 in different states of hemodynamic compromise (e.g., 54, 59). Recently, we established the catchment depths of the multi-wavelength approach for its application to the heart (8). In this optically dense tissue, the catchment depth ranged from 160 (blue excitation) to 350 μm (red excitation).

A unique feature of the phosphorescence quenching technique is that it inherently provides information about the heterogeneity of oxygen levels. In cases of a homogenous distribution of oxygen, the signal displays a single monoexponential lifetime that can be converted to the PO2 according to the Stern-Volmer relationship after correction for the excitation pulse (64). However, in cases of a heterogeneous oxygen distribution within the measurement volume, the signal displays a distribution of different lifetimes. Mathematical deconvolution of this lifetime distribution enables the retrieval of the oxygen distribution (12, 55) at high temporal resolution.

In addition to the evident advantage offered by the phosphorescence technique for quantitative measurement of μPO2 in vivo, there are a number of shortcomings of this technique that must be considered. First and foremost, this technique requires the injection of a Pd-porphyrin dye that limits its use to experimental animals. Additionally, this measurement method is limited to the surfaces of organs where the penetration depth of the optical catchment volume of ~500 μm. A further limitation is that the photo-oxidation of hemoglobin (Hb) can affect the measurement of oxygen availability, although this effect is considered to be negligible (41). A final important limitation of this technique is that technically complex phosphorimeters are needed to make reliable measurements. These phosphorimeters require the careful integration of complex electronics, optics, and mathematical algorithms to implement the Stern-Volmer relationship if quantitative PO2 measurements are to be performed. Reliable, validated equipment is not commercially available and investigators have had to develop and validate their own devices (e.g., 85).

NADH Fluorescence for Measurement of the Mitochondrial Energy State

Normal mitochondrial function is essential for metabolic energy production and is a key factor in maintaining cellular integrity and activities. Oxidative phosphorylation depends on the sufficient supply of oxygen to the mitochondria. Within the respiratory chain, NADH is oxidized, a process that comes to a halt because of the lack of oxygen. The mitochondrial PO2 levels must drop below a few millimeters Hg to make this happen (61, 101). Because NADH shows autofluorescence and its oxidized form (NAD+) does not, measuring NADH autofluorescence is a method used to detect mitochondrial activity (21) and, thus, a lack of oxygen in mitochondria (82). This technique has been used to study the heterogeneous nature of microcirculatory oxygenation, especially in isolated rat heart (51).

Major limitations of this technique include that it is not quantitative and relies on the detection of emitted fluorescence induced by light excitation at 360 nm. Despite this limitation it has been of interest to investigate the heterogeneous nature of dysoxia because it is well suited for imaging modalities. In addition the excitation light is heavily absorbed by Hb in blood, which during hemodynamic changes, causes unwanted variations in the intensity of excitation light in tissues and thus the intensity of the emitted fluorescence independent of the levels of NADH (63). This effect, which has been referred to in the literature as the hemodynamic effect, limits the utility of this technique in vivo. However, this effect can be partially compensated for by simultaneously measuring the reflected excitation light (23). In addition to this hemodynamic effect, the movement of tissue surfaces (e.g., the heart) can modulate the amount of excitation light presented to the tissue. This effect can also be compensated for by measuring the reflected excitation light (17).

Quenching of Delayed PpIX Fluorescence

Until recently researchers lacked a technique to measure PO2 at the mitochondrial level in situ. This shortcoming was resolved by our development of the PpIX technique. PpIX is the final precursor of heme in the heme biosynthetic pathway and is synthesized in the mitochondrial matrix (72). We discovered that PpIX has oxygen-dependent optical properties that highly resemble phosphorescence specifically a delayed fluorescence (68). The lifetime of delayed PpIX fluorescence inversely correlates with the amount of oxygen according to the Stern-Volmer relationship. Because delayed PpIX fluorescence shares the advantages of lifetime properties with phosphorescence quenching, it is equally suited for PO2 measurements in vivo (67). The PpIX technique has been used to measure mitochondrial PO2 (mitoPO2) in the liver (67), the heart (9, 65), the skin (45, 46), and the buccal mucosa (47).

Importantly, deconvolution of the lifetime decay curves reveals heterogeneity of mitochondrial PO2 levels. A detailed review of the technique can be found elsewhere (69). In addition to quantitatively measuring mitoPO2, the PpIX method also enables mitochondrial respirometry in vivo (47). To this end, dynamic PO2 measurements are performed during the local occlusion of the microcirculation, from which the oxygen disappearance rate can be determined. The oxygen disappearance rate is directly related to mitochondrial oxygen consumption. By using this approach, an endotoxin-induced oxygenation-independent reduction in mitochondrial oxygen consumption in rats was demonstrated in vivo (47).

There are number of limitations of this technique that need to be considered. The most important of these is that the concentration of endogenous PpIX is too low to perform reliable measurements of mitoPO2. The method used to overcome this limitation is to increase the amount of endogenous...
PpIX via the administration of its precursor 5-aminolevulinic acid (ALA). ALA thereby increases mitochondrial PpIX to levels that are sufficiently high for the application of the delayed fluorescence decay method. Although ALA is non-toxic compound, the application of high intensity excitation light can induce photo toxicity (67).

**HETEROGENEITY IN \( \mu \text{PO}_2 \) AND MITOPO\(_2\)**

The classical view of the transport of oxygen from the arteries, via the microcirculation to the tissue cells, has been described by the model of Krogh in which the oxygen pressures progressively decrease as the oxygen in red blood cells travel from the arterioles to the capillaries to the tissues via diffusion, as described by Fick’s law (71). Research performed by Duling and Berne (30) and others, however, identified that oxygen also diffuses to cells and other compartments of the microcirculation directly from the arterioles. Metabolic signaling derived from erythrocytes and parenchymal cells in addition to sheer stress mediated microvascular tone controls microvascular blood flow in a heterogeneous microcirculatory network in such a way as to optimize microvascular blood flow to meet regional cellular oxygen demands (37).

The mitochondria have always been regarded as the primary oxygen sink in tissue, but the presence of anatomical and functional shunting pathways has complicated its measurement. Functional shunting in the microcirculation increases in the presence of inflammation (52). The production of oxidative and nitrosative radicals alter (micro)vascular regulation and microcirculatory flow becomes impaired. Under these conditions, it is unclear the extent to which cells and mitochondria suffer from oxygen deprivation in light of the potential compensatory downregulation of oxygen consumption and cellular hibernation. This effect is of specific relevance to resuscitation medicine in which it is uncertain whether conventional therapies aimed at restoring the macrocirculation are effective in enhancing oxygen transport pathways or actually exert the opposite effect by impeding oxygen transport at the microcirculatory level (15, 16). Appreciation of the heterogeneous nature of oxygen transport and shunting pathways plays a key role in understanding the effects of conventional resuscitation methods on the microcirculation (52).

It has long been known that there is a marked heterogeneity of blood flow not only at the level of the microcirculation (57) but also between different layers and compartments of organs systems. The function of the microcirculation adapts to the metabolic needs of the tissues in terms of structure and density, and this process is disrupted during shock and sepsis (2, 40). NADH videofluorimetry in the isolated rat heart has provided much insight into the heterogeneity of microcirculatory perfusion of the myocardium, in which states of hypotension are characterized by heterogeneous oxygenation (Fig. 1). Analysis of the patchy areas in the heart enabled the functional descrip-

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**Fig. 1.** NADH fluorescence imaging in an isolated Tyrode-perfused rat heart at different perfusion pressures illustrates the heterogeneous nature of microcirculatory oxygenation in hypotension. NADH fluorescence images are seen in A to D, where patchy fluorescence patterns develop corresponding to the step-wise reduction in coronary pressure shown in E. As can be seen, hypotension is associated with the appearance of patchy weak microcirculatory units indicating the heterogeneous nature of hypotensive shock (high fluorescence indicates high NADH fluorescence associated with hypoxia). [Borrowed with permission from Ashruf et al. (4).]
tion of the nature of heterogeneity as the presence of anatomically defined weak microcirculatory units (WMU; 51, 52). The vulnerable WMUs are the first areas of the microcirculation to become dyoxic (e.g., during tachycardia, sepsis; 6, 51) and are the last to recover after an episode of hypoxemia (Fig. 1; Refs. 51, 99). The size of WMUs, at least in the heart, is on the order of 300 μm². We found that WMUs consist of capillary units, as demonstrated in our study of isolated rat hearts in which we matched the WMU patterns elicited by reperfusion to the patterns elicited by administrating microspheres to embolize the exact same WMU areas at the level of the capillary vessels (57). Much larger weak units are observed under conditions of acidosis and hypertrophy and can be reproduced via embolization with microspheres of 15 μm in diameter (5). Layec et al. (58) found in healthy human subjects that the intrinsic capacity for mitochondrial ATP synthesis in vivo is primarily limited by convective microcirculatory flow, not by mitochondrial capacity. These findings underscore the importance of microcirculatory flow in promoting mitochondrial function. Alternatively, reduced mitochondrial function can cause enhanced oxygen delivery by altering metabolic and vascular pathways (60).

The heterogeneity in oxygen pressure can be studied via phosphorescence quenching. Several authors have used deconvolution algorithms to calculate a distribution of lifetimes to generate PO2 histograms (12, 41, 55). Using this approach, we performed many animal studies of the response of the heterogeneity of μPO2 to various states of shock and resuscitation. Using μPO2 histograms we were able to detect the presence of microvascular hypoxic areas in the kidney cortex during endotoxemia (55). Importantly the average μPO2 in these experiments did not change, emphasizing the functional importance of measuring the heterogeneity of oxygen pressures in vivo. Combining renal phosphorimetry with laser speckle imaging of microcirculatory perfusion in the cortex during fluid resuscitation, we demonstrated that μPO2 alterations are a consequence of altered microvascular perfusion despite normal renal arterial blood flow (59). This study demonstrated the septis-induced loss of coherence between regional and microcirculatory blood flow and oxygen demand despite apparently adequate fluid resuscitation.

In addition to the intrinsic heterogeneity of oxygen pressures within the microcirculation itself, there are also differences in oxygen pressures between various anatomical compartments of organ systems. The most studied organs in this respect are the heart, kidney, and intestines in which there are higher μPO2 levels in the epicardium, the renal cortex, and the intestinal serosa compared with the endocardium, medulla, and mucosa, respectively. We used multifiber phosphorimetry to investigate the response of the different exposed organ surfaces to states of shock and resuscitation (e.g., 84, 92, 95). To penetrate the deeper layers of a solid organ, two-photon excitation phosphorimetry can be used. This approach revealed a difference of 20 mmHg between the renal cortex and medulla (66), confirming the results of oxygen electrode measurements (32). The functional morphology of the microcirculation and the heterogeneous distribution of endothelial receptors, which mediate vascular tone in various organ systems, renders some organs more vulnerable to states of shock than others. In hemorrhagic shock for example, the heart μPO2 is better preserved than the intestinal μPO2 (95). Additionally, during hemodilution the heart is much more resistant to states of anemia than other organs such as the kidney and the intestines (92).

Whether the μPO2 levels are consistent with the heterogeneous μPO2 distribution or are more evenly distributed at a low PO2 to drive diffusive oxygen transport has been an open question. Our development of in vivo mitoPO2 measurements using delayed PpIX fluorescence and its application to the liver (67) and the heart (65) revealed mitoPO2 distributions to be highly heterogeneous (Fig. 2). Strikingly, mitoPO2 in general was higher than conventionally thought (49, 88) and direct comparison between mitoPO2 and μPO2 revealed that the diffusion gradient in the liver is small (13). Differences in microcirculatory architecture and cell size likely account for the different mitoPO2 distributions between the liver and the heart, in which the mitoPO2 distributions are approximately normal instead of bimodal in distribution. In the healthy heart a significant portion of mitochondria function at a low PO2 level. Interestingly, in hypertrophied right ventricular failure mitoPO2 appears to be increased (9). The latter finding is surprising because myocardial hypertrophy leads to increased diffusion distances that could be expected to result in decreased mitoPO2 because of large diffusion gradients. Mitochondrial downregulation might account for this contrasting finding.

The understanding that cellular oxygen consumption might actually depend on the available amount of oxygen, even at PO2 levels in the physiological range, is a recent paradigm shift. Classical studies in isolated mitochondria and cells have shown that oxygen consumption is unaffected by oxygen until PO2 drops below 2-3 mmHg (56, 61, 101). Moreover, more recent studies in intact cell, organs and even humans have demonstrated that mitochondrial metabolism is regulated by oxygen via multiple pathways under normal physiology. A study in healthy volunteers exposed to normoxia and hypoxia during exercise showed that metabolic efficiency was directly related to mitochondrial respiration demonstrating that mitochondrial metabolism actively adapts to achieve optimal power production (79). In an in vitro study we showed that increased work directly influences myocardial mitochondrial bioenergetics (6). A key player in the adaptive response to hypoxia is the expression of the transcription factor, hypoxia inducible factor (HIF) and its activation in numerous adaptive cellular responses, including a switch to glycolytic metabolism, the activation of EPO, and the stimulation of angiogenesis. Several factors related to tissue hypoxia have been implicated in HIF activation. These factors include oxygen tensions, oxygen consumption, reactive oxygen species, and nitric oxide (10, 20, 42, 43). The adaptive response of cells to hypoxia in terms of HIF-1 activation is a rapidly evolving field, and the reader is referred to the many excellent reviews on the subject (34, 81). Of particular interest from the perspective of the subject matter of this paper, however, is the adaptive response of oxygen consumption by mitochondria to states of low oxygen pressures. Recent studies in intact cells have identified a cellular mechanism, referred to as “oxygen conformance of metabolism,” by which oxygen consumption is downregulated when cells are subjected to moderate oxygen deprivation for an extended period of time (80, 89). Interestingly, oxygen conformance occurs at PO2 levels as high as 70 mmHg (75, 89). Our finding of high in vivo hepatic mitoPO2 in the range of 20 to 30 mmHg, in combination with the generally observed μPO2 in the range 30 to 40 mmHg, suggests that oxygen...
Fig. 2. Effect of ischemia-reperfusion on mitochondrial PO2 (mitoPO2) histograms in rat liver in vivo measured by the delayed PpIX fluorescence method shows a heterogeneous mitoPO2 distribution after reperfusion. A: ischemia-reperfusion protocol and times of measurement at baseline (B), at 15 (I 15) and 25 (I 25) min of ischemia and at 5 (R 5) and 15 (R 15) min after reperfusion. B: average mitoPO2 (C–G). Distributions of mitoPO2 at time points B (C), I 15 (D), I 25 (E), R 5 (F), and R 15 (G). Error bars indicate the SD (n ± 3). [Borrowed with permission from Mik et al. (67).]
conformance is a cellular regulatory mechanism that is active under physiological conditions rather than simply a cellular adaptation to pathologic hypoxia (67). These concepts are directly relevant to resuscitation medicine, because future research will investigate the conditions under which such conformance is sustained or lost during shock and resuscitation.

MICROCIRCULATORY HETEROGENEITY AND THE LOSS OF HEMODYNAMIC COHERENCE DURING SHOCK

Experimental findings of ours and others have identified that microcirculatory hypoxia can remain unresolved in resuscitated states of shock despite the recovery of systemic variables. An example of such a condition is shown Fig. 3. In this example, brain cortical μPO2 was measured during endotoxic shock followed by fluid resuscitation. As shown, fluid resuscitation was ineffective in restoring brain cortical μPO2 despite increasing cardiac output to levels above baseline. The loss of coherence between the macro- and microcirculation was accordingly demonstrated under this condition of septic shock and explains the origin of a deficit in oxygen extraction. Therefore to fully reveal the heterogeneous nature of shock and to identify the loss of hemodynamic coherence, clinical monitoring techniques aimed at the microcirculation are required to identify the heterogeneity in perfusion.

To this end we developed and clinically introduced handheld microscopy in the 1990s to directly observe microcirculatory alterations in critically ill patients. During the past few decades these handheld microscopes have been developed technologically; the first generation devices were based on orthogonal polarized spectral (OPS) imaging (62) followed by a second generation side stream dark field (SDF) devices (39). Recently a third generation computer-controlled image sensor-based device called Cytocam-incident dark field (IDF) imaging has been developed (7). These handheld microscopes use green light for illumination because it is maximally absorbed by Hb in cells, allowing their movements to be observed as flowing dark globules. The optical methods incorporated in handheld microscopes, specifically OPS, SDF, and IDF imaging, refer to the different optical methods needed to view the microcirculation via epi-illumination (i.e., illumination from the top). Conventional intravital microscopes illuminates the tissue from below (trans-illumination). The optical problem that must be resolved in epi-illumination mode is that surface reflections of the illumination light must be filtered out to enable the observation of the microcirculation below the tissue surface. In OPS imaging, polarized light illumination is applied and observation made after crosspolarization of the reflected light, thereby filtering surface reflections. In SDF and IDF imaging, dark field peripheral illumination is used to observe the central field of view that also filters out surface reflections. A more comprehensive review of these techniques can be found elsewhere (11).

During the last decade, handheld microscopy, predominantly applied sublingually, has been extensively used to investigate the heterogeneous nature of microcirculatory alterations in clinical states of cardiovascular compromise, shock and resuscitation and has provided insight into the clinical relevance of microcirculatory alterations (e.g., 28). The most consistent reports of the presence of heterogeneous microcirculatory alterations and loss of hemodynamic coherence have been found in clinical studies of sepsis. Irrespective of the recovery of systemic variables such as blood pressures and cardiac output by using conventional resuscitation procedures, the persistence of microcirculatory sublingual alterations was found to be related to increased morbidity and mortality (25, 91) with heterogeneous microcirculation being a key consistent finding (33). We confirmed the significance of microcirculatory alterations in a large international multicenter study of the incidence of microcirculatory alterations in which we identified that microcirculatory alterations in combination with tachycardia was associated with increased mortality (99). Studies of other types of shock and cardiovascular compromise have reported the loss of systemic and microcirculatory coherence, showing this finding to be a common occurrence. Examples in addition to sepsis include traumatic hemorrhagic shock (96), heart failure (26, 27), and malaria (44). A main area of research concerns the inability of fluid therapy to resolve the disparity between macro- and microcirculation (e.g., 53). Handheld microscopy was used to identify patients for which fluid resuscitation was not successful despite successful targeted normalization of cardiac output in such cases as sepsis and malaria (44, 73).

Based on these insights, we identified four types of microcirculatory alterations underlying the loss of hemodynamic coherence, in which the recovery of the systemic macrocirculation does not lead to a parallel improvement in the microcirculation. 1) The microcirculatory flow is heterogeneously distributed such that areas of perfusion are adjacent to areas of obstruction. Such a profile is consistent with red blood cell hemorheological abnormalities in combination with endothelial cell dysfunction, which are characteristic of inflammatory states of shock such as septic shock (e.g., 25, 33); 2) Hemodilution, in which the dilution of blood causes a loss of red blood cell-filled capillaries leading to an increase in oxygen diffusion distances (e.g., 53). 3) Inappropriate manipulation of the macrocirculation (e.g., excessive use of vasoconstrictors or increased venous pressures) causes tamponade or the constric-

![Figure 3](image-url). Fluid resuscitation after endotoxemic shock fails to correct brain cortical microcirculatory PO2 (μPO2) despite normalized cardiac output, illustrating loss of hemodynamic coherence between the macrocirculation and microcirculation in this porcine model of fluid resuscitated septic shock. Jugular vein PO2 is shown together with cardiac output and brain μPO2 measured by the quenching of Pd phosphorescence technique. Shock is induced by infusion of endotoxin (LPS). After a delay (shock phase) fluid resuscitation using Ringer lactate is administered, resulting in increased cardiac output. The brain cortical μPO2 however, remains depressed despite recovery of cardiac output.

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tion of microcirculatory flow (15, 16, 29, 97). 4) Edema limits oxygen diffusion increasing the diffusion distances due to tissue swelling (e.g., Ref. 44). Current studies are underway to test the hypothesis that under conditions of loss of hemodynamic coherence, microcirculatory guided therapy will result in improved outcome.

A final unresolved question in relation to oxygen transport to tissue in shock and resuscitation is the concept of persistent mitochondrial dysfunction despite normalization of microcirculatory perfusion and oxygenation. This condition, originally termed cytopathic hypoxia, was based on oxygen electrode measurements in combination with CO₂ tonometry in animal models of sepsis (35, 94). Although this concept was recently abandoned (36), we have shown that these findings could alternatively be explained by methodological considerations related to oxygen electrodes, the heterogeneity of μPO₂ between the intestinal serosa and mucosa, and the sepsis-induced heterogeneity of iNOS in the intestines (3).

In addition to alterations in microcirculatory function, various adaptive mechanisms, or the failures of these mechanisms could cause a reduced ability of the mitochondria to utilize oxygen to produce ATP, thereby contributing to the defect in oxygen extraction observed in states of sepsis and shock. A possible candidate may be the inhibitory effects of nitric oxide on oxidative phosphorylation that can be caused by the greater affinity of nitric oxide than oxygen to cytochrome c, the terminal actor of the respiratory chain, because of the availability of excess nitric oxide in states of inflammation (24). A possible rescue pathway may involve mitochondrial biogenesis, although PPARγ-coactivator-1a, which is essential to this process, may also be affected by inflammatory mediators (87, 98). Based on the possibility that mitochondrial adaptation pathways may be activated and that the primary insult underlying oxygen limitation leads to microcirculatory dysfunction in states of shock, it appears to be more appropriate to first resolve microcirculatory abnormalities, which can be directly observed at the bedside, and then investigate the extent to which mitochondrial dysfunction may persist in terms of a deficit in oxygen extraction. Nevertheless, there are ample evidence of mitochondrial alterations in states of shock and sepsis (19), and if mitochondrial dysfunction in the presence of normal microcirculatory oxygen transport exists, independent of microcirculatory dysfunction, then this would have a dramatic impact on the current understanding of resuscitation medicine, which is entirely focused on promoting oxygen transport to tissues via convection.

In addition to these basic questions of pathophysiology, the effect of the impairment of oxygen transport pathways on parenchymal cell function and, more importantly, whether these alterations are reversible are unknown. If so, further investigations must identify which alterations mediate any irreversibility. Clearly, the answers to these questions are expected to have a significant impact on the treatment strategies applied to shock patients. However, equally important to understanding the mechanisms underlying these issues is the development of suitable tools at the bedside to enable monitoring and the verification of treatment success at the microcirculatory, cellular, and mitochondrial level. Tools for the observation of the microcirculation and even for the direct measurement of mitochondrial oxygenation and respiration are continuously being developed. Recent advances indicate the feasibility of monitoring the determinants of oxygen transport pathways at the microcirculatory and mitochondrial levels.

DISCLOSURES
In the last 2 years, Dr. Ince has received honoraria and independent research grants from Fresenius-Kabi, Bad Homburg, Germany; Baxter Health Care, Deerfield, IL; and AM-Pharma, Bunnik, The Netherlands. Dr. Ince has developed SDF imaging and is listed as inventor on related patents commercialized by MicroVision Medical (MVM) under a license from the Academic Medical Center (AMC). He has been a consultant for MVM in the past, but has not been involved with this company for more than 4 years now, but he still holds shares. Braedius Medical, a company owned by a relative of Dr. Ince, has developed and designed a handheld microscope called CytoCam-IDF imaging. Dr. Ince has no financial relation with Braedius Medical of any sort, i.e., never owned shares or received consultancy or speaker fees from Braedius Medical. Dr. Mik is listed as inventor on patents related to the PpIX technology for mitochondrial oxygen measurements held by the Academic Medical Center Amsterdam (AMC) and the Erasmus Medical Center Rotterdam (ErasmusMC), The Netherlands. Furthermore, Dr. Mik is founder and shareholder of Photonics Healthcare, a company that holds exclusive licenses to these patents. Photonics Healthcare is aiming to develop a patient monitor for mitochondrial oxygenation and metabolism based on the PpIX technology.

AUTHOR CONTRIBUTIONS
Author contributions: C.I. conception and design of research; C.I. prepared figures; C.I. and E.G.M. drafted manuscript; C.I. and E.G.M. edited and revised manuscript; C.I. and E.G.M. approved final version of manuscript.

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Review

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