Chapter 20
Myocardial Microcirculation and Mitochondrial Energetics in the Isolated Rat Heart

J.F. Ashruf and C. Ince

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

Keywords Microcirculation • Mitochondrial redox state • NADH fluorescence • Sepsis • Hypertrophia

1 Introduction

Oxygen is transported to the cells via the microcirculation, which is composed of terminal arterioles, capillaries and venules. Cellular ischemia or hypoxia results in an increase of the mitochondrial NADH/NAD^+ ratio (redox state), which can be measured using NADH-fluorimetry [1]. Using this technique, it was found that

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there are areas in the healthy myocardium that are more vulnerable to dysoxia; these areas are reproducible in time and place and originate at the capillary level and are therefore closely associated with the architecture of the microcirculation. These weak units show the largest relative reduction in flow (independent of absolute flow levels) during compromising conditions, with dysoxia initially developing at the venous end of the capillary [2, 3]. Myocardial hypertrophia, work and sepsis have a profound effect upon the distribution of oxygen in the microcirculation as is shown by measuring the mitochondrial redox state under these conditions.

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

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

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

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

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

2 Distribution of Oxygen to Myocardium in Normal and Hypertrophic Rat Hearts

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

NADH fluorescence photography of the surface of Tyrode-perfused rat hearts, Barlow et al. [1] found that lowering coronary perfusion results in patchy NADH fluorescence. Steenbergen et al. [6] found that this heterogeneity in the hypoxic state occurred under ischemic as well as during high-flow hypoxic conditions.

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

Hypertrophia is known to be associated with a decreased muscle mass to vascularization ratio and thus a change of myocardial perfusion when compared to normal hearts [9–11]. Hulsmann et al. [12] found that hypertrophic Langendorff rat hearts displayed ischemic areas during normoxic perfusion whereas normal heart under the same circumstances did not. The relative hypoperfusion in these areas was shown to be caused by acidosis and oxygen free radicals (OFR), since measures taken to prevent acidosis (by stimulating fatty acid oxidation) and OFR production (by adding super oxide dismutase to the perfusate) dramatically improved local hypoxia. In a further study [7] it was shown that perfusion is altered at the arteriolar and arterial level in hypertrophia.

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

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

Another factor influencing this ratio is the type of substrate used in the perfusate: pyruvate increases the ratio more than glucose [4]. In our study of the effect of cardiac work on the NADH/NAD+ ratio, we observed that during perfusion with glucose as a substrate, the work-related decrease of the ratio was much slower than during perfusion with pyruvate as a substrate. An explanation for this might be that

pyruvate enters the Krebs cycle directly, thus reducing NAD^+ relatively quickly, whereas glucose first enters the glycolysis, which is a slower process when compared to the Krebs cycle and speeds up more slowly in case of increased workload. However, more investigations are needed to explain this observation.

3 Distribution of Oxygen to Myocardium in Septic Rat Hearts

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

Indeed, in a recent study by us (unpublished data, to be submitted) Langendorff perfused hearts from septic rats develop hypoxic areas which appear larger than the heterogeneous areas during reperfusion and embolization with microspheres of 5.9 μm diameter [2, 8]. This agrees with the finding that autoregulation is disturbed [14]; autoregulation is situated at the arterial and arteriolar level and we found that the hypoxic areas were larger than those elicited by embolization of capillaries and are comparable in size to those found in hypoperfused areas in hypertrophy and during embolization of arterioles and arteries [7, 8]. The result of these effects of sepsis on the microcirculation can be describes as shunting of the microcirculation [15], which means that certain portions of the myocardial capillary network remain hypoperfused and other portions receive a higher than needed (for adequate oxygenation) capillary flow. We also found that mitochondrial functioning in septic hearts appeared to be disturbed, as the development of hypoxia during interrupted perfusion was slower than in normal control hearts.

4 In Vivo Evaluation of Hypoxia and Ischemia

NADH videofluorimetry has been shown to be an effective method to assess tissue hypoxia in numerous studies ex vivo. Clinical applicability however requires in vivo application of this measurement and therefore we performed several studies to assess the possibility of in vivo application of NADH videofluorimetry on various organs (heart, intestines, skeletal muscle) [16–21]. It was shown, for instance, that with this technique it was possible to detect local hypoxia in vivo in myocardium induced by selective ligation of coronary arteries in rat heart

[18]. However, in vivo NADH fluorescence is disturbed by movement, hemodynamic and oximetric effects. A method was developed to compensate for these factors by means of utilizing the NADH fluorescence/UV reflectance ratio, making it possible to monitor the mitochondrial redox state of intact blood-perfused myocardium [20].

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

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

5 Recent Developments

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

A major disadvantage of the use of NADH video fluorimetry for obtaining information about mitochondrial bioenergetics and oxygenation is that it is a non-quantitative technique relying on relative changes in fluorescence signals. Recently we introduced a new method for a quantitative measurement of mitochondrial pO_2 (mit pO_2) values. The method is based on the oxygen dependent decay of delayed fluorescence of endogenously present mitochondrial protoporphyrin IX (P_pIX) [23]. Proof of concept measurements were initially performed by Mik et al. in single cells and later validated in vivo in rat liver and recently also in the heart [24, 25]. Deconvolution of the decay curves allowed mit pO_2 histograms to be generated and showed a heterogeneous distribution of mit pO_2 values. Ischemia-reperfusion injury was shown to induce mit PO_2 values with hypoxic as well as hyperoxic values [24].

One of the remarkable measurements of the mit pO_2 values was the finding of mit pO_2 values much higher (20–30 mmHg) than previously thought (less than 5 mmHg) and secondly the finding of markedly heterogeneous mit pO_2 values, similar to the distributions of microvascular pO_2 measurements using P_pIX quenching of phosphorescence we had measured in heart [23]. Indeed (early) observations of the heterogeneous state of myocardial and mitochondrial energy states underscores the importance of heterogeneity of oxygen delivery and utilization [3]. It is expected that the combination of NADH fluorimetry and mit pO_2 measured by P_pIX phosphorescence will provide new information concerning the transport and consumption of oxygen by the heart.

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