Myocardial contractile function during postischemic low-flow reperfusion: critical thresholds of NADH and O$_2$ delivery

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Submitted 12 May 2003; accepted in final form 2 September 2003

NADH has been used to characterize mitochondrial energy states in isolated hearts and myocardial tissue (6, 8, 24, 28). Hypoxia and ischemia lead to blocking of the electron transport chain and accumulation of NADH due to inadequate D˙O$_2$ for continued oxidative phosphorylation (10). Previous studies in isolated and perfused beating hearts describe accumulation of NADH during ischemia and report resolution on reperfusion (33). However, NADH changes during incremental reperfusion that represents various states of D˙O$_2$ have not been studied. This is a critical issue after both in vivo regional and global ischemia due to a variable reperfusion flow to the ischemic myocardium. In previous studies, we noted the importance of the level of reperfusion flow on postischemic left ventricular (LV) function and myocardial bioenergetic recovery (14). Previous animal studies of conventional CPR demonstrated maximal blood flow rates achieved that ranged from 10 to 30% of prearrest levels (7, 15, 19, 25, 30). CPR-generated reperfusion after cardiac arrest represents a small fraction of normal physiological coronary artery flow (26, 27). This in part accounts for the very low resuscitation rates after cardiac arrest (3, 16). In the absence of good tissue or cellular markers, it is difficult to assess the adequacy of myocardium reperfusion. Characterizing the efficacy of very low-flow reperfusion after global ischemia remains an important question in resuscitation medicine.

In this study, we utilized tissue NADH levels as a real-time indicator of the postischemic return of mitochondrial function during variable levels of reperfusion flow (D˙O$_2$). NADH levels were noted to be a sensitive indicator of cellular D˙O$_2$ to the ischemic myocardium, and at higher reperfusion flows, NADH levels were inversely correlated with postischemic LV function. Our results help characterize postischemic reperfusion by demonstrating that any low-flow reperfusion (>1% of baseline) can improve the cellular NADH (redox state) toward an apparent critical threshold that is required for return of contractile function.

METHODS

Langendorff heart preparation. Male Sprague-Dawley rats (350–450 g body wt) supplied by Harlan (Indianapolis, IN) were used in accordance with guidelines of the National Institutes of Health (Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, Revised 1996) and the approval of the Ohio State University Laboratory Animal Resources Committee. Rats were anesthetized with pentobarbital sodium (50–65 mg/kg ip). The right superficial jugular vein was isolated, and 1,000 U/kg heparin was administered. The trachea was cannulated with a 16-gauge AngioCath attached to a...
was positioned in a glass temperature-controlled (37°C) Krebs-Henseleit buffer perfusion at 85 mmHg. The heart was quickly excised from the chest and transferred to the Langendorff apparatus with continued warmed Krebs-Henseleit buffer perfusion at 85 mmHg. The heart was positioned in a glass temperature-controlled (37.4°C) chamber. Coronary flow rates were continuously measured and monitored by an electronic flow meter (T206, Transonic Systems; Ithaca, NY).

LV function measurements. The LV balloon volume was inflated at the beginning of the experiment to yield a LV end-diastolic pressure of 5 mmHg. The balloon volume remained constant during the experiment so that changes in the LV end-diastolic pressure were due to changes in myocardial compliance. LV pressure was continuously sampled at a frequency response of 45 Hz and was digitally processed by a heart-performance analyzer (Digi-Med, Micro-Med; Louisville, KY). The following measures of LV function were derived by computer algorithm and stored in a spreadsheet: LV systolic pressure, LV diastolic pressure, heart rate, dP/dt, duration of relaxation time, LV pressure were continuously recorded during the various perfusion protocols. Real-time autofluorescence of NADH was recorded continuously in arbitrary NADH units during the various perfusion protocols. NADH autofluorescence during reperfusion was compared with baseline autofluorescence levels, thereby allowing each heart to serve as its own control. Each heart studied was limited to ≤4 intervals of ischemia-reperfusion to minimize the effects of stacking ischemic insults.

Additional experiments were done to assess NADH autofluorescence during “very low-flow” reperfusion rates (1–10% baseline flow) after global ischemia. To assess the effects of substrate manipulation during periods of very low reperfusion flow (1% of baseline), hearts were perfused after a 5-min period of global ischemia with reperfusion flow rates limited to 1% of baseline both with and without glucose (5.5 mM). NADH autofluorescence (n = 9 per group) was monitored throughout baseline flow, global ischemia, 1% of baseline reperfusion flow (both with and without glucose), and full-reperfusion flow.

Data analysis. NADH autofluorescence signals were digitized and recorded in arbitrary NADH units. The NADH signal is highly dependent on positioning of the fluorometer fiber-optic cable against the left ventricle. Owing to this variability, comparison of absolute levels of arbitrary NADH units between hearts was not done. Each heart was used as its own control, so that changes in NADH were normalized to baseline NADH during normal perfusion, variable percentages of baseline flow, and global ischemia. The percent NADH change based on these two fluorescence extremes (NADHbaseline, NADHmax) was used to compare the various reperfusion flow rates. LV function characteristics of RPP, DP, and dP/dtmax were expressed as a ratio of baseline function using each heart as its own control. All group values were expressed as means ± SE. Analysis was done using ANOVA and Tukey’s post hoc test (SYSTAT, version 10.2.01, Systat Software; Richmond, CA). P < 0.05 was considered statistically significant.

Differences between 1%-flow groups with and without glucose were analyzed. Each heart served as its own control with its percent NADH change measured both with and without glucose. The normality of the percent change for both groups as well as the actual differences for both groups were assessed using standard tests (Shapiro-Wilk and Anderson-Darling). We tested the following hypotheses: 1) the average percent change without glucose is zero; 2) the average percent change without glucose is zero; and 3) the percent change with glucose equals the percent change without glucose (i.e., the difference is zero).
Correlations of NADH to Do₂ were analyzed using linear regression. Correlation of NADH with LV function was performed using two linear regressions in which the interface between the two regressions was determined by minimizing the sum of the squared deviations. For this relationship, linear, logarithmic linear, and power functions were attempted but were eliminated due to poor correlations with the experimental data.

RESULTS

NADH fluorescence and variable reperfusion/Do₂. In the ischemic and reperfused heart, NADH changes were very rapid in response to alterations in Do₂ (Fig. 1). NADH quickly rose and approached a plateau during global ischemia. Partial levels of recovery toward the NADH baseline were observed with varied levels of postischemic reperfusion (n = 7 at each reperfusion flow rate). Additional recovery of NADH toward baseline was noted with reinstitution of full (baseline) flow after each low-flow reperfusion period. Significant changes in NADH levels were noted relative to the end of ischemia with reperfusion flows ranging from 12.5 to 75% of baseline levels tested (P < 0.01; see Fig. 1).

NADH exhibited a strong inverse relationship to reperfusion flow rate (Fig. 2). In this non-hemoglobin-perfused model, oxygen content of the perfusate is fixed and determined by the Po₂ in the saturated solution. As a result, Do₂ is a linear function of the perfusate flow rate (Do₂ ∝ perfusate flow rate). Therefore, the NADH-response curve relative to reperfusion flow and Do₂ is the same. Changes in NADH fluorescence relative to reperfusion Do₂ are not linear and are best described by a logarithmic relationship (Fig. 2). The greatest changes in NADH signal occurred during the lower extremes of Do₂ (Fig. 2). A strong correlation (r = 0.952) was noted between myocardial Vo₂ and Do₂ over the full range of reperfusion flows, which indicates the Do₂-dependent nature of the perfused heart model at all levels of reperfusion (Fig. 3).

NADH fluorescence and LV function. Measures of LV function were inversely correlated with NADH fluorescence including DP (r = −0.755) and dP/dmax (r = −0.766). However, a better fit of LV function to NADH was found by using the LV functional parameter RPP and creating two intersecting linear regression lines (Fig. 4). During reperfusion at the lower extremes of Do₂ (1–25% of baseline), the NADH signal clearly returned toward baseline, which suggests improved tissue oxygenation, cellular redox state, and mitochondrial function (see Fig. 3). Relative changes in the NADH signal were greatest over these reperfusion flow rates. Despite this clear evidence of a metabolic response to low perfusion flow rates, no functional

![Fig. 1. NADH during variable reperfusion flow. NADH autofluorescence from a single experiment during full baseline flow followed by 5 min of variable reperfusion flow that represents 75, 50, 25, and 12.5% of baseline flow rate. NADH quickly peaks and plateaus during global ischemia, decreases during 5 min of variable reperfusion, and recovers to near-baseline levels with return of full flow (baseline flow). Postischemic NADH fluorescence decreases with increasing reperfusion flow rate.](http://ajpheart.physiology.org/)

![Fig. 2. Relationship of NADH fluorescence to reperfusion flow rates: oxygen delivery (Do₂) ∝ perfusate flow rate. For each postischemia reperfusion rate, n = 7 rats/group. Mean NADH fluorescence with 95% confidence interval is shown. Reperfusion flow rates denote percentages of baseline flow. NADH fluorescence is represented as the NADH plateau at ischemia divided by the NADH at baseline. A strong logarithmic correlation exists between NADH fluorescence and decreasing reperfusion flow rate (r = 0.961; P < 0.001).](http://ajpheart.physiology.org/)

![Fig. 3. Relationship of myocardial Do₂ to myocardial oxygen utilization (Vo₂) during variable postischemic reperfusion. Do₂ and Vo₂ (measured in μL of O₂/min · g of dry heart wt⁻¹) are closely correlated (r = 0.952; P < 0.001), which suggests the Do₂ dependence of the heart at reduced reperfusion levels.](http://ajpheart.physiology.org/)
contractile recovery was observed during reperfusion with 1–25% of baseline DO2. Conversely, at reperfusion DO2 levels >25% of baseline, there was return of contractile function in proportion to the level of DO2. Over this range of reperfusion DO2, NADH changes were much smaller (Fig. 4). An apparent threshold was identified (corresponding to a reperfusion DO2 of <25% of baseline and an NADH level >1.1 × baseline preischemia level) below which no recovery of LV contractile function was observed. Similar functional relationships were noted with DP and dP/dt\text{max}. With reperfusion at 100% of baseline, there was a rapid return of LV function and recovery of NADH to near-baseline levels after short-term ischemia.

To confirm the hypothesis that 25% of baseline flow provides adequate DO2 to maintain contractile activity of the perfused heart, additional hearts were perfused at 25% of baseline flow without prior global ischemia. Contractile function measured as the RPP immediately dropped and remained constant over the 2-h perfusion period at a mean of 26% of baseline RPP. NADH immediately increased and remained constant during the 2-h period.

**NADH fluorescence during very low-flow reperfusion.** Additional experiments (n = 6) utilizing “very low-flow” reperfusion rates of 1, 2, 6.25, and 10% of baseline flow were studied. An apparent threshold was noted at ~1% of baseline flow at which NADH increased during reperfusion. In contrast, reperfusion flows >1% of baseline universally demonstrated a decrease in NADH fluorescence. We hypothesized that this increase in NADH was due to sufficient substrate (glucose) delivery stimulating glycolysis and greater NADH generation but with insufficient oxygen to support oxidative phosphorylation.

To evaluate the significance of glucose at these very low reperfusion flow rates, additional experiments (n = 9 per group) were done to compare standard perfusate (5 mM glucose) with and without glucose at a flow rate of 1% of baseline flow after global ischemia. Changes in NADH fluorescence were expressed as a percent change from the global ischemia plateau. Reperfusion at 1% of baseline flow with glucose resulted in an increase in NADH of 3.4 ± 3.0% [ΔNADH/(NADH ischemia − NADH baseline) × 100%] above the global ischemic level compared with a decrease in NADH of −3.6 ± 5.4% in the nonglucose group (Fig. 5). The mean percent change for the 1% of baseline flow with glucose was 3.4% and was significantly different from zero (P = 0.0177). However, the average percent change for the 1% of baseline flow without glucose group was not significantly different from zero (P = 0.1289). The average percent difference between the two groups of 6.9% is significant (P = 0.0102). There was no difference in reperfusion DO2 (measured in μL O2/min · 100 g of dry wt) between groups (8.32 ± 2.88 with glucose vs. 9.36 ± 2.82 without glucose; P = 0.23).

**DISCUSSION**

In contrast with many ischemia-reperfusion studies where reperfusion constitutes restitution of full flow, extreme variation in reperfusion flow exists within the regionally reperfused in vivo heart and in particular during CPR conditions following the global ischemia of clinical cardiac arrest. We used NADH autofluorescence, a recognized indicator of redox state and mitochondrial metabolism (6, 8, 10, 24, 28), as a marker of mitochondrial VO2 and assessed a wide range of postischemic reperfusion flow rates. In this study, NADH fluorescence in the whole heart was noted to quickly rise to a steady state during short periods of global ischemia and to rapidly decrease to a lower steady state in proportion to the DO2. With the utilization...
of a variety of reperfusion flow levels that ranged from 1 to 100% of baseline flow, a strong inverse logarithmic relationship was noted between NADH concentration and \( \text{DO}_2 \). Spanning the full range of reperfusion flows, relative NADH fluorescence increased as \( \text{DO}_2 \) decreased.

Relative changes in NADH were greatest during reperfusion flow rates delivering 1 to 25% of baseline \( \text{DO}_2 \). Interestingly, during this same range of reperfusion \( \text{DO}_2 \), despite large decreases in the NADH concentration, there was no recovery of contractile function. The large changes in NADH with low reperfusion flows do indicate mitochondrial \( \text{VO}_2 \) at \( \text{DO}_2 \) levels below the apparent threshold for return of contractile function. In our model, this critical level of reperfusion \( \text{DO}_2 \) for return of contractile function was \( \sim 25\% \) of baseline \( \text{DO}_2 \).

Under conditions of very low-flow reperfusion, we identified another apparent \( \text{DO}_2 \) threshold (1% of baseline \( \text{DO}_2 \)) where reperfusion increased peak NADH fluorescence above that of global ischemia. This increase in NADH was blocked by removal of glucose from the perfusate. Under normal nonischemic perfusion conditions, fatty acids are the preferred energy source for the heart (4, 17, 18). However, under ischemic conditions, glucose becomes a dominant source for energy production as the ischemic heart shifts to anaerobic metabolism, thereby increasing both glucose uptake and glycogenolysis (21). The relative reliance on glucose as an energy source is directly related to the severity of the ischemic insult. As ischemia becomes severe, glucose extraction increases inversely with coronary flow (5, 31). Below a certain flow threshold, \( \sim 1\% \) of normal flow in this model, delivery of additional glucose under severely hypoxic conditions may allow for additional glycolysis, thereby resulting in further accumulation of NADH but insufficient oxygen for oxidation of NADH in the mitochondria. This increase in the cellular reduced redox state under very low-flow reperfusion states has not been described in vivo. Although these reperfusion levels are very small (\( \sim 1\% \) of normal flow), it is likely that these flow levels do exist during the variable reperfusion of the regionally ischemic myocardium and may occur during CPR-generated reperfusion.

**Significance of NADH accumulation.** In this study, NADH was used as an indicator of mitochondrial function and cellular redox state. In previous studies of skeletal muscle bioenergetics, NADH fluorescence using spectrophotometry was identified as an excellent indicator of cellular \( \text{P}_2 \) levels. In experiments on skeletal muscle, the degree of NADH decrease (NADH oxidation) shifts with oxygen availability during muscle hypoxia. As cellular hypoxia caused decreases in muscle performance, NADH was increasingly reduced rather than oxidized with increasing workloads (22).

Myocardial NADH accumulation may have important implications for reactive oxygen species (ROS) generation during ischemia and reperfusion. ROS are believed to be a primary mechanism for reperfusion injury and myocardial dysfunction after ischemia. During early reperfusion, with the reintroduction of oxygen, there is a burst of ROS production (34, 35). Significant contribution of accumulated NADH as a mechanism for this ROS reperfusion burst has recently been suggested (23). Increased NADH levels during ischemia can serve as a trigger to increase ROS production via NADH oxidase activity on both xanthine dehydrogenase and xanthine oxidase (11, 12, 29). Some authors believe that increased concentrations of NADH will preferentially inhibit xanthine dehydrogenase and shift the influx of purine-generated electrons through xanthine oxidase with consequent increased ROS production (12, 23). As a potential mechanism for increased ROS production, increased NADH may serve as an indirect measure of ROS generation at reperfusion.

This work highlights some important limitations of the perfused heart as a model for studying in vivo global myocardial ischemia and reperfusion. Oxygenated perfusate lacks the oxygen-carrying capacity of a blood-perfused system. Owing to the absence of hemoglobin, oxygen-delivery capabilities of the buffer solution roughly approximate 10% of that seen in normal oxygenated blood. Under normal perfusion conditions in the perfused heart, coronary vasculature autoregulatory properties allow sufficient flow to prevent ischemia. Evidence for adequate perfusion in this model under full-flow conditions includes the preservation of function, the lack of NADH increase, and the lack of increased lactate production or creatine kinase release over prolonged periods of time. However, unlike the normal blood-perfused heart in which significant reductions in coronary blood flow may be tolerated without evidence of ischemia (13, 20, 32), small reductions in flow of <25% in the Langendorff model result in accumulation of NADH and reduction in function. The linear relationship of myocardial \( \text{DO}_2 \) to myocardial \( \text{VO}_2 \) at flow levels <75% of baseline flow indicate that there is very little oxygen reserve at baseline-flow levels in this model. Thus reductions of flow in the non-blood-perfused heart correspond to proportionally greater reductions of flow under in vivo conditions. Under in vivo conditions, hemoglobin interference is possibly the most important confounding factor in assessing NADH fluorescence. As this is a non-blood-perfused model, this major limitation was not a factor.

In summary, NADH is a sensitive and instantaneous indicator of myocyte \( \text{VO}_2 \) in the whole heart under conditions of reperfusion. Using this tool, we observed that low reperfusion levels of \( \text{DO}_2 \) result in oxidation of NADH, presumably in the mitochondria, at \( \text{DO}_2 \) levels that are insufficient to support recovery of contractile function. In the perfused heart, postischemic reperfusion \( \text{DO}_2 \) levels >1% of baseline decrease NADH (oxidation). However, after short-term global ischemia, a threshold reperfusion \( \text{DO}_2 \) level of 25% of baseline is needed before the return of measurable contractile activity can occur. At very low reperfusion levels of <1% of baseline in this model, a paradoxical increase in NADH (reduction) was observed, which suggests that substrate as well as oxygen may be important determinants of myocardial NADH levels at very low reperfusion flows.

**ACKNOWLEDGMENTS**

The authors thank Allen Blumberg and Mersiha Hadziahmetovich for important assistance in this study.

**GRANTS**

This research was supported by grants from the Emergency Medicine Foundation and Wyeth-Ayerst (to M. G. Angelos), the American Heart Association, Ohio Affiliate (to M. G. Angelos), and National Heart, Lung, and Blood Institute Grant HL-53333 (to T. L. Clanton).

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