Hibernation in Noncontracting Mammalian Cardiomyocytes

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Background—The aim of the present study was to establish whether isolated neonatal mammalian cardiomyocytes were capable of downregulating energy-using processes other than contraction while maintaining metabolic stability when oxygen availability was reduced.

Methods and Results—Metabolic response of cardiomyocytes was investigated under moderate (5 to 6 μmol/L) and severe (2 to 3 μmol/L) forms of hypoxia. Cells exposed to oxygen concentrations of 5 to 6 μmol/L exhibited rates of oxygen consumption, which were decreased to 64% of normoxic rates. Rates of cellular energy usage were decreased because this reduced rate of oxygen consumption was not associated with either decreased intracellular ATP and phosphocreatine concentrations or a compensatory switch to glycolysis. When cells were exposed to oxygen concentrations of 2 to 3 μmol/L, rates of oxygen consumption decreased to 9% of normoxic rates. This decreased rate of oxygen consumption was associated with energetic stress, because a significant switch to glycolysis occurred and intracellular phosphocreatine concentrations were decreased by 40%. Rates of cellular energy usage were further decreased as indicated by stable intracellular ATP concentrations.

Conclusions—Our results suggest that isolated cardiomyocytes are capable of downregulating energy-consuming processes other than contraction when oxygen supply is decreased. Regions of myocardial tissue are also capable of downregulating metabolic activity during ischemia by shutting down contractile activity (myocardial hibernation). We suggest that metabolic downregulation associated with myocardial hibernation may not be exclusively due to reduced rates of contractile activity. Other energy-using processes (e.g., protein synthesis, mRNA synthesis, ion channel activity, and proton leak) may also be shut down. (Circulation. 2000;102:3124-3129.)

Key Words: hibernation ■ hypoxia ■ metabolism ■ myocytes ■ respiration

The term “hibernating myocardium” is used to describe clinical observations in which a region of intact myocardium remains viable through a decrease in contractile function when myocardial blood flow is reduced and that at reperfusion completely recovers contractile function.1 Myocardial hibernation with functional recovery has been demonstrated clinically in patients undergoing coronary artery bypass surgery2–5 and in various animal models.6–8 However, such metabolic downregulation has been measured directly only in animal models, by determination of rates of oxygen consumption and lactate output and of intracellular ATP and PCR concentrations.8–10

A similar response, “oxygen conformance,”11 has been observed in various isolated cell preparations, including adult rat hepatocytes11 and cardiomyocytes12 and embryonic chick cardiomyocytes.13–14 Budinger et al13 demonstrated that suspensions of isolated embryonic chick cardiomyocytes were capable of deceasing their rates of oxygen consumption and contractile activity at oxygen concentrations as high as ~63 μmol/L. The authors concluded that isolated embryonic chick cardiomyocytes responded to reduced oxygen availability by downregulating energy-using pathways associated with contraction.

The abilities of ischemic regions of the myocardium to undergo hibernation and of isolated cardiomyocytes to conform to hypoxic oxygen concentrations suggest that cardiomyocytes contain oxygen sensors involved in regulation of contractile activity. Studies of hibernating myocardium suggest that only contractile activity is downregulated during ischemia. Because contractile activity does not always immediately recover after revascularization,5,15 it seems likely that other energy-using processes may also be shut down. A study by Piper et al16 supports this suggestion, given that rates of energy consumption in quiescent adult rat cardiomyocytes were shown to be reduced under anoxic conditions. However, rates of ATP usage were measured only under anoxic conditions, and as a consequence intracellular ATP concentrations were not stable. The purpose of the present study was to determine whether isolated mammalian cardiomyocytes were capable of downregulating energy-using processes other than contraction while maintaining metabolic stability when exposed to moderate (5 to 6 μmol/L) and severe (2 to 3 μmol/L) forms of hypoxia. Our results show that rates of cellular energy usage were decreased under both forms of hypoxia because no compensatory switch to anaerobic gly-
colysis occurred and intracellular ATP concentrations remained stable. We suggest that contractile activity is not the only energy-using process downregulated during hypoxia in cardiomyocytes.

**Methods**

**Isolation of Cardiomyocytes**
Neonatal cardiomyocytes were isolated from the hearts of 1-day-old Sprague-Dawley rats by use of a method described previously. 17

**Oxygen Consumption Measurements**
Cells were resuspended in a Tris-buffered Krebs-Henseleit solution (137 mmol/L NaCl, 5.4 mmol/L KCl, 1.0 mmol/L NaH₂PO₄, 0.8 mmol/L MgSO₄, 2.0 mmol/L CaCl₂, 5.0 mmol/L Tris, 5.5 mmol/L glucose, 10 μmol/L verapamil, and 50 μmol/L NiCl₂; pH 7.4 at 37°C), and rates of oxygen consumption were measured in either the perfusion (flow-through) system or the closed-cell chamber. 19

**Analytical Methods**
Intracellular concentrations of ATP and phosphocreatine (PCr) were measured by capillary electrophoresis as described previously. 17 Lactate content in samples of perfusate from the flow-through system were measured bioluminescently as described previously. 20 Rates of lactate accumulation in samples from the closed-cell chamber were assessed spectrophotometrically by use of a method described previously. 18

**Calculation of ATP Turnover**
Rates of ATP turnover were calculated by determining yield of ATP from rates of oxidative phosphorylation (assuming a P:O ratio of 2.58). 21 Anaerobic glycolysis (assuming 1 mol of ATP is produced per mole lactate) and ATP and PCr depletion.

**Measurement of Glucose Uptake**
Rates of glucose uptake were determined in the closed-cell chamber by calculating rates of glucose oxidation, lactate production, and glycogen accumulation under normoxic and anoxic conditions. Rates of glucose oxidation were measured by the method of Guppy et al. 22 Rates of lactate production were measured by use of a method described previously. 23 Intracellular glycogen concentrations were measured as described previously. 24 –25

**Measurement of Glucose Uptake in the Presence of Fatty Acids**
Rates of glucose uptake were additionally measured in the presence of palmitate 130 μmol/L and oleate 81 μmol/L under normoxic conditions. The fatty acid mixture was made up in medium 199 according to the method of Guppy et al. 26

**Measurement of Lactate Oxidation**
Rates of lactate oxidation were measured in the presence of 1 mmol/L lactate in the closed-cell chamber under normoxic conditions. Cell suspension (2 mL) was preincubated with L-[U-C¹⁴]lactate (2 μCi) for 30 minutes. Rates of ¹⁴CO₂ production were determined according to the method of Guppy et al. 22

**Respiratory Inhibition With Myxothiazol**
Rates of oxygen consumption were measured in the presence of myxothiazol in the closed-cell chamber. Myxothiazol was incrementally added to the cell suspension until the rate of oxygen consumption was inhibited by 95% to 98%. A final myxothiazol concentration of 6 μmol/L was typically required to achieve this level of respiratory inhibition.

**Results**

**Neonatal Cardiomyocytes as an Experimental Model for Studying Differences in Normoxic and Hypoxic Cardiac Energy Metabolism**
We measured rates of glucose oxidation in the presence and absence of fatty acids and measured rates of lactate oxidation to determine whether isolated suspensions of neonatal cardiomyocytes respond similarly to the whole heart with respect to fuel usage. Rates of glucose uptake in the presence of fatty acids (0.41±0.04 nmol·min⁻¹·10⁻⁶ cells) were 66±13% lower than rates measured in the absence of fatty acids (2.1±1.3 nmol·min⁻¹·10⁻⁶ cells; P<0.05; n=3). When provided with lactate as an alternative fuel to glucose, isolated cardiomyocytes oxidized lactate at a rate of 0.10±0.02 nmol·min⁻¹·10⁻⁶ cells. These results suggest that isolated suspensions of neonatal cardiomyocytes represent a valid model for the study of cardiac energy metabolism, because cells were capable of oxidizing lactate and rates of glucose uptake were significantly inhibited when measured in the presence of fatty acids. 28

**Effect of Extracellular Oxygen Concentration on Oxygen Consumption**
Isolated neonatal cardiomyocytes decreased their rates of oxygen consumption over a range of oxygen concentrations in the flow-through system (Figure 1).

**Effect of Moderate Hypoxia (5 to 6 μmol/L) on Rates of Oxygen Consumption, Glycolytic Activity, and Cellular Energy State**
Reduced rates of oxygen consumption could be maintained for 1 hour when neonatal cardiomyocytes were maintained at 5 to 6 μmol/L in the flow-through system (Figure 2). Rates of oxygen uptake at 5 μmol/L were 64±2% of rates at 100 μmol/L and remained decreased after 1 hour, at 66±2% of reoxygenated values (P<0.05). Calculated rates of lactate oxidation were 95% to 98% inhibited by 6 μmol/L myxothiazol in the closed-cell chamber (Figure 3).
out at 5 μmol/L were significantly higher than rates at 100 μmol/L (1.2±0.3 versus 0.9±0.2 nmol·min⁻¹·10⁻⁶ cells at 5 and 100 μmol/L, respectively; P<0.05), whereas rates of lactate output after 1-hour incubation were not significantly higher than reoxygenated rates (1.1±0.2 versus 1.0±0.2 nmol·min⁻¹·10⁻⁶ cells at 6 and 100 μmol/L, respectively). Rates of ATP turnover at 5 μmol/L were 68±2% of rates at 100 μmol/L and after 1 hour of hypoxia were 69±2% of reoxygenated rates (P<0.05; Figure 2).

Results from experiments in the closed-cell chamber revealed that magnitude of metabolic downregulation was dependent on duration of hypoxic exposure. Oxygen concentration in the closed-cell chamber decreased at a rate proportional to the rate of oxygen consumption by the cells contained within it. This process allowed measurements to be taken more rapidly: oxygen concentration usually decreased to 100 μmol/L before being decreased to 5 μmol/L. Rates of ATP turnover were not significantly different than results presented above (data not shown).

In experiments with the flow-through system, cellular rates of oxygen consumption at 3 μmol/L were 13±2% of rates at 100 μmol/L (P<0.05), and after hypoxic incubation for 1 hour, rates remained decreased, at 9±2% of reoxygenated rates (P<0.05; Figure 4). Rates of lactate output were significantly higher under hypoxic conditions (Figure 4). Rates of ATP turnover at 3 μmol/L were 36±2% of rates at 100 μmol/L, and after incubation for 1 hour at 2 to 3 μmol/L, rates remained decreased, at 40±2% of the reoxygenated value (P<0.05; Figure 4).

Rates of oxygen consumption at 2 μmol/L were 64±3% of rates at 100 μmol/L when measured in the closed-cell chamber (P<0.05; n=3). This degree of shutdown in oxygen consumption was significantly less than when measured at 2 to 3 μmol/L in the flow-through system (P<0.05; n=3 for closed-cell experiments and n=4 for flow-through experiments).

Intracellular ATP and PCr concentrations were measured to establish whether decreased rates of ATP turnover were associated with reduced rates of ATP usage. No significant differences existed in intracellular ATP and PCr concentrations between normoxic and hypoxic conditions with the flow-through system (Figure 3). When experiments were performed in the closed-cell chamber, ATP and PCr concentrations did not change significantly between 100 and 5 μmol/L. Rates of oxygen consumption and degree of metabolic downregulation were also measured in the absence of contractile inhibitors and were not significantly different than results presented above (data not shown).
Figure 5. Relationship between extracellular oxygen concentration and intracellular concentrations of ATP (●) and PCr (○). Measurements were taken as described in Figure 2. *P<0.05 vs metabolite concentrations at initial oxygen concentration of 100 μmol/L; n=4.

Figure 6. Effect of oxygen and myxothiazol on rates of ATP turnover. *P<0.05 vs rates at 100 μmol/L; n=11 for measurements at 100 μmol/L, n=7 for measurements at 6 μmol/L, n=4 for measurements at 3 μmol/L, and n=3 for measurements in the presence of myxothiazol.

No significant difference in ATP concentrations existed between normoxic and hypoxic conditions (Figure 5). Intracellular PCr concentrations decreased significantly when oxygen concentrations were decreased to 2 to 3 μmol/L and with reoxygenation recovered to 96±13% of initial measurement (Figure 5).

Rates of Glucose Uptake Under Normoxia and Anoxia
Rates of glucose uptake were measured in the closed-cell chamber so that we could determine whether increased rates of lactate output under hypoxia were metabolically significant. Normoxic rates of glucose uptake were 2.1±1.3 mmol·min⁻¹·10⁻⁶ cells compared with 0.9±0.1 mmol·min⁻¹·10⁻⁶ cells under anoxia. Lactate production accounted for a significantly increased proportion of glucose uptake when oxygen concentrations were reduced from normoxia to anoxia: 21±7% and 99±1%, respectively (P<0.05; n=3). Intracellular glycogen accumulated at a rate of 0.9±0.8 mmol·min⁻¹·10⁻⁶ cells under normoxic conditions and was degraded at a rate of 0.5±0.1 mmol·min⁻¹·10⁻⁶ cells under anoxic conditions.

Effects of Myxothiazol on Rates of ATP Depletion
The closed system was used to determine whether the mechanism by which cardiomyocytes downregulated energy usage at 2 to 3 μmol/L was dependent on energetic stress. Respiratory rates of cells maintained under normoxic conditions (100 μmol/L) were inhibited by 95% to 98% with myxothiazol. Rates of ATP turnover were calculated and compared with rates at oxygen concentrations of 100, 5, and 3 μmol/L (Figure 6). Rates of ATP turnover in the presence of myxothiazol were 84% lower than rates at 100 μmol/L.

Discussion
The present study is the first to suggest that energy-using processes other than contraction can be downregulated by isolated mammalian cardiomyocytes over a range of oxygen concentrations (2 to 70 μmol/L). Cardiomyocytes held in suspension generally are assumed not to exhibit contractile activity. However, evidence suggests that stirring stimulates contractile activity in suspensions of cardiomyocytes. For this reason, all experiments were performed in the presence of the contractile inhibitors verapamil (10 μmol/L) and NiCl₂ (50 μmol/L). Effects of Different Degrees of Hypoxia (5 to 6 μmol/L Versus 2 to 3 μmol/L) on Oxidative Phosphorylation, Glycolytic Activity, and Cellular Energy State
We found that isolated noncontracting neonatal cardiomyocytes decreased rates of oxygen consumption in response to reduced oxygen availability. Rates of oxygen consumption were decreased at oxygen concentrations ≤70 μmol/L. Our results compare well with others that used contracting embryonic chick cardiomyocytes in which rates of oxygen consumption were decreased at oxygen concentrations as high as ~63 μmol/L. In contrast to our findings, several studies have shown that rates of mitochondrial oxygen consumption by isolated adult rat cardiomyocytes do not begin to decrease until extracellular oxygen concentrations are much lower: ~0.6, 32 ~6, 33 ~12, 12 and 20 μmol/L. In these studies, adult cardiomyocytes were exposed to only brief periods of hypoxia. Research has suggested that metabolic downregulation is a time-dependent effect, and a previous study has demonstrated that isolated hepatocytes display oxygen conformance only when exposed to hypoxia for >2 hours. Exposure of adult cardiomyocytes to such a brief period of hypoxia may therefore explain why these cells did not exhibit oxygen conformance at oxygen concentrations as high as 70 μmol/L. For this reason, we performed studies with 2 different experimental systems. In the closed-cell chamber, cardiomyocytes were exposed to hypoxia for only brief periods (<1 minute), whereas in the flow-through system, cells were subjected to hypoxia for substantially longer periods (~60 minutes). Our results show that the magnitude of metabolic shutdown at 2 to 5 μmol/L was significantly greater when experiments were performed in the flow-through system as opposed to the closed-cell chamber. These findings support the suggestion that metabolic downregulation is a time-dependent effect and may help to explain the differences in oxygen conformance between adult and neonatal cardiomyocytes.
A potential source of error in our measurements could have been introduced by presence of either intracellular oxygen gradients or oxygen gradients within our experimental setup. However, we do not believe that either of these factors have affected our results, because intracellular oxygen gradients have been measured in resting adult cardiomyocytes and were found to be <0.2 μmol/L. Rates of oxygen consumption by platelets have been measured in the flow-through system and did not decrease until oxygen concentrations fell to <5 μmol/L. These results suggest that significant oxygen gradients do not exist within neonatal cardiomyocytes or within our experimental system.

Cardiomyocytes responded to oxygen concentrations of 5 to 6 μmol/L by decreasing their rates of oxygen consumption by 35%. This decreased rate of oxygen consumption could be maintained for ≥1 hour and was fully reversible on reoxygenation. Cellular viability can be maintained when aerobic ATP production is decreased if anaerobic ATP production is increased or if cellular energy usage is reduced. Our results indicate no compensatory switch to anaerobic glycolysis despite rates of ATP turnover that were reduced by 32%. Because intracellular ATP and PCR concentrations remained stable, we conclude that rates of ATP usage must have been reduced to match rates of ATP production.

The second part of the present study examined how cardiomyocytes responded to a more-severe degree of hypoxia (2 to 3 μmol/L). Cardiomyocytes exposed to oxygen concentrations of 2 to 3 μmol/L exhibited rates of oxygen consumption that were decreased to 9% of rates at 100 μmol/L. These decreased rates of oxygen consumption were associated with stable ATP concentrations, decreased PCR concentrations, and a significant switch to anaerobic ATP production. Our results show that isolated cardiomyocytes respond to near-anoxic oxygen concentrations by downregulating energy-using processes other than contraction and that this downregulation is associated with energetic stress as indicated by decreased PCR concentrations and increased lactate concentrations.

Energetic Stress Provides the Signal for Metabolic Downregulation at Oxygen Concentrations of 2 μmol/L

The present results suggest that energetic stress may act as a signal to downregulate energy usage when oxygen concentrations fall to less than ~3 μmol/L. We created energetic stresses under normoxic oxygen concentrations by inhibiting rates of oxygen consumption by 95% to 98% with myxothiazol, an inhibitor of electron flow through complex III in the mitochondria. If cells were not capable of downregulating metabolic activity under these conditions, then ATP stores would be rapidly degraded and death would ensue. In the presence of myxothiazol, cells remained viable and further decreased rates of ATP turnover.

Summary

The present results show that cardiomyocytes are capable of responding to reduced oxygen availability and that this response is dependent on severity of hypoxia. Cardiomyocytes responded to oxygen concentrations of 5 to 70 μmol/L by downregulating energy-using processes. This metabolic downregulation was not associated with energetic stress. At oxygen concentrations less than ~3 μmol/L, cardiomyocytes were capable of further reducing rates of energy usage. However, this additional downregulation was dependent on the presence of energetic stress.

Because isolated suspensions of cardiomyocytes seem capable of shutting down energy-using processes other than contraction and in view of clinical studies that show that contractile activity does not always immediately recover after revascularization, metabolic downregulation associated with myocardial hibernation seems unlikely to be due to decreased rates of contractile activity exclusively. Other energy-consuming processes that may be downregulated include protein synthesis, mRNA synthesis, ion-channel activity, and proton leak. Shutting down these energy-using processes when oxygen supply is reduced may provide a means of conserving ATP and thus making it available for processes necessary for survival. Mechanisms by which changes in oxygen concentration are sensed in regions of hibernating myocardium and in isolated suspensions of cardiomyocytes are unclear but may involve cytochrome c oxidase or various intracellular signaling molecules, including nitric oxide and reactive oxygen species.

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References


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