Hypoxia Causes Downregulation of Protein and RNA Synthesis in Noncontracting Mammalian Cardiomyocytes

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Abstract—The aim was to identify energy-consuming processes, other than contraction, downregulated during moderate hypoxia (≈5 μmol/L, 0.5% O2) and severe hypoxia (<0.5 μmol/L, <0.05% O2) in isolated neonatal cardiomyocytes. The metabolic response of cardiomyocytes to moderate and severe hypoxia was assessed by measuring rates of energy consumption and energetic status of cells maintained under these conditions. We found that the rates of energy production were decreased during both forms of hypoxia. Decreased rates of energy production under moderate hypoxia were associated with reduced energy wastage through a downregulation of proton leak in the mitochondria. Cellular protein synthesis and RNA synthesis, major energy-consuming pathways, were downregulated only during severe hypoxia, when oxygen concentrations were low enough to induce energetic stress (quantitatively defined as being any situation in which phosphocreatine concentrations had fallen by ≥40%). Our results suggest that energetic stress is the signal responsible for this downregulation. (Circ Res. 2002;90:777-783.)

Key Words: cardiomyocytes □ protein synthesis □ RNA synthesis □ Na+,K+-ATPase activity □ proton leak

Regional myocardial hypoperfusion does not necessarily lead to irreversible cell damage. Indeed, clinical observations have revealed that regions of myocardium are capable of reversibly downregulating contractile activity when faced with ischemia.1,2 Myocardial hibernation, the term used to describe this energy-conserving response first recognized by Rahimtoola,3 has also been demonstrated experimentally in a variety of animal models.4,5 These studies collectively suggest that hibernating cardiomyocytes maintain metabolic stability by reducing their rates of energy utilization to levels that match rates of energy production. It is not clear whether contraction is the only process downregulated during myocardial hibernation or whether any of the other energy-using processes are also shut down.

The response of isolated contracting cardiomyocytes6,7 to hypoxia (≈8 to ≈29 μmol/L, 0.8% to 3% O2) is similar to the phenomenon observed in hibernating regions of myocardium, in that contractile activity is reduced and recovers with reoxygenation. We have previously shown that isolated noncontracting cardiomyocytes are also capable of reversibly downregulating metabolic activity during hypoxia (<5 μmol/L, <0.5% O2).8

In the present study, we have assessed the effect of hypoxia on cellular energy consumption not associated with contraction by measuring the rates of protein synthesis, RNA synthesis, Na+,K+-ATPase activity, and proton leak. Whereas hibernation was previously thought to be due only to reduced rates of contractile activity,4–7 in the present study, we show that rates of protein synthesis, RNA synthesis, and proton leak are also downregulated by noncontracting cardiomyocytes exposed to reduced oxygen availability.

Materials and Methods

Isolation of Cardiomyocytes

Cardiomyocytes from 1-day-old neonatal Sprague-Dawley rats (Animal Resource Centre, Murdoch, Western Australia) were isolated as described previously.9

Estimation of Oxygen Consumption by Protein Synthesis, RNA Synthesis, and Na+,K+-ATPase Activity

Rates of oxygen consumption were measured in a closed-cell chamber as previously described by Guppy et al.10 Cells were resuspended in medium 199 for experiments associated with protein and RNA synthesis measurement or in a HEPES-buffered Krebs-Henseleit solution (137 mmol/L NaCl, 5.4 mmol/L KCl, 1.0 mmol/L Na2HPO4, 0.8 mmol/L MgSO4, 25 mmol/L HEPES, and 5.5 mmol/L glucose; pH 7.3 at 37°C) for Na+,K+-ATPase activity experiments.

Inhibitors of protein synthesis (cycloheximide, 1.5 mmol/L), RNA synthesis (actinomycin D, 100 nmol/L), Na+,K+-ATPase activity (ouabain, 1.5 mmol/L), Na+-Ca2+ exchange activity (2-[2-(4-(4-nitrobenzoyloxy)phenyl)ethyl]sulfiniouracil methanesulfonate, 10 μmol/L), sarcolemmal Ca2+ channels (verapamil, 10 μmol/L; NiCl2, 50 μmol/L), and mitochondrial oxygen consumption (myxothiazol, 4 μmol/L) were added. Rates of oxygen consumption were measured 5 to 10 minutes after the addition of each inhibitor. It was necessary to prevent the entry of extracellular Ca2+ through
Calculation of Energy Production

Energy production has been calculated in terms of ATP equivalents because this concept is familiar to most investigators. The calculation of energy production incorporates energy production for proton leak. Estimates of energy wastage by this process are described in Figure 2.

Rates of energy production were calculated by determining the yield of ATP from the rates of oxidative phosphorylation (assuming a P/O ratio of 2.58),15 anaerobic glycolysis (assuming that 1 mol ATP is produced per mole of lactate), and ATP and phosphocreatine (PCr) depletion.

Protein and RNA Synthesis Measurements

Experiments were performed at 37°C in an orbital shaker. Cardiomyocytes (2×10^6 cells/mL) were resuspended in conical flasks containing a final volume of 3 mL. Rates of protein and RNA synthesis were measured under three experimental conditions, moderate hypoxia (5 μmol/L, 0.5% O_2), severe hypoxia (<0.05 μmol/L, <0.5% O_2), and normoxia (203 μmol/L, 21% O_2), in the presence of myoxygen. Each experiment was matched to a control group in which rates were measured under normoxic conditions. In all experiments, both flasks of cells were initially maintained under normoxia for 30 minutes before the appropriate gas mixture was applied or myoxygen was added. Cells were then incubated for a further 45 minutes to ensure that the correct oxygen concentration had been attained. [3H]Phenylalanine (10 μCi/μmol) or [3H]uridine (30 μCi/μmol) were added at 135 minutes. Rates of protein synthesis were measured using a method described previously.16 In brief, cell samples were immediately centrifuged (10 000 g, 5 minutes) and the supernatant was retained for spectrophotometric determination of lactate concentration. The cell pellets were washed using a method described previously.16 In brief, cell samples were immediately centrifuged (10 000 g, 5 minutes), and the supernatant was retained for spectrophotometric determination of lactate concentration. The cell pellets were washed four times in ice-cold MgCl_2 (0.1 mol/L), resuspended in 5 mmol/L CsCl (1 mL), and sonicated for 30 minutes before the supernatant was removed and frozen, and a aliquot was retained for determination of protein concentration by the Bio-Rad DC Protein Assay Kit. The samples were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes, which were blocked for 1 hour in 5% nonfat milk in TBST (20 mmol/L Tris-HCl, pH 7.6, 137 mmol/L NaCl, and 0.1% Tween 20) before incubation for 2 hours with a 1:5000 dilution of primary antibody (rabbit anti-eIF2α phosphorylation, Research Genetics) in 1% nonfat milk in TBST. The membranes were then washed in TBST and incubated for 1 hour with a 1:10 000 dilution of secondary antibody in 5% nonfat milk in TBST (sheep anti-rabbit IgG conjugated to horseradish peroxidase, Sile-nus). Protein immuno-captures were performed at room temperature. Immunoreactive bands were detected by enhanced chemiluminescence (Supersignal West Fento Maximum Sensitivity Substrate, Pierce), followed by exposure to autoradiography film (Hyperfilm MP, Amersham). Densitometry was performed on scanned films by using NIH Image 1.62. Immunoreactive bands were quantified by comparing their density with the density of known quantities of standard eIF2α phosphorylation. The standard was made by using recombinant rat eIF2α and heme-controlled repressor kinase, which were both gifts from Dr Scot Kimball (Penn State College of Medicine, Hershey, PA). Exposure times to the autoradiographic film were adjusted to ensure that the density of the bands was linear with quantity.

Intracellular concentrations of eIF2α phosphorylation were normalized against normoxic concentrations because of the variation between different cell preparations. Normoxic concentrations of eIF2α phosphorylation from all experiments averaged 37±0.3 ng/10^6 cells (n=6).

K⁺ Influx Measurements and Intracellular Na⁺ and K⁺ Measurements

Rates of Na⁺.K⁺-ATPase activity were determined under normoxic, moderately hypoxic, and severely hypoxic conditions in the presence or absence of myoxygen (4 μmol/L) by measuring the rate of Rb⁺ uptake with the use of methods described previously.16,19 This technique has been shown to be as accurate as the use of 86 Rb and has the advantage of making the experiments safer.18 Cells were resuspended in a modified HEPES-buffered Krebs-Henseleit solution containing 1.8 mmol/L CaCl₂ and 1 mmol/L instead of 5.4 mmol/L KCl. Flasks were preincubated under normoxia for 30 minutes before the appropriate gas mixture was applied or myoxygen was added. After a further 45 minutes, RbCl (4.4 mmol/L) was added with or without ouabain (1.5 mmol/L). Samples (250 μL) were removed every 10 minutes between 75 and 115 minutes, placed directly into ouabain (1.5 mmol/L) to inhibit any further Rb⁺ uptake, and immediately centrifuged (5000g, 3 minutes). Cell pellets were washed four times in ice-cold MgCl₂ (0.1 mol/L), resuspended in 5 mmol/L CsCl (1 mL), and sonicated for 30 seconds (3×10 seconds). After centrifugation (10 000g, 10 minutes), cell pellets were resuspended in 0.3 mol/L NaOH (250 μL). Protein content in the pellet was determined as described previously, whereas the Bio-Rad protein assay kit was used to measure the protein concentration in the supernatant. The Rb⁺ content in the supernatant was measured by emission flame photometry (Varian).
Rates of Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity were normalized against a matched control in each experiment. Normoxic rates of Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity from all experiments were 5.3±0.8 nmoL/min per milligram protein (n=11).

Intracellular Na\textsuperscript{+} and K\textsuperscript{+} concentrations were measured under normoxia and severe hypoxia by using a modified protocol. In brief, cells were resuspended in HEPES-buffered Krebs-Henseleit solution, and all measurements were made in the absence of ouabain. Samples (1 mL) were removed at 75 and 115 minutes and immediately washed by vacuum filtration with 35 mL MgCl\textsubscript{2} (0.1 mol/L). The filter paper was resuspended in 5 mmol/L CsCl (1.4 mL) and sonicated. Na\textsuperscript{+} and K\textsuperscript{+} concentrations in the supernatant were measured by emission flame photometry at wavelengths of 589 and 766 nm, respectively, with a slit width of 0.2 nm.

Proton Leak Measurement
Rates of oxygen consumption and mitochondrial membrane potential (MMP) were measured simultaneously in the closed cell chamber (1.5 mL) and spectrophotometer (2 mL), respectively. Spectrofluorometric measurements took place at 37°C within a stirred water-jacketed cuvette located on the floor of the spectrophotometer. Oxygen concentrations within the cuvette were maintained either under normoxia (150 μmol/L, 15% O\textsubscript{2}) by leaving the cuvette open to air or under moderate hypoxia (5 to 10 μmol/L, 0.5% to 1% O\textsubscript{2}) by gassing with a humidified gas mixture of 1.5% O\textsubscript{2}/balance N\textsubscript{2} (~15 μmol/L). MMP was measured by using 5,5',6,6'-tetrachlorotetraethylbenzimidazolocarbocyanine iodide (JC-1, 200 nmol/L), a cationic lipophilic carbocyanine dye whose mitochondrial uptake follows Nernst’s equation. JC-1 is mainly present in a monomeric form at low MMP emitting a green fluorescence, whereas at high MMP, JC-1 tends to form aggregates which are associated with orange-red fluorescence. In agreement with the findings of others,\textsuperscript{20} we found that MMP was best described by using the ratio of the fluorescent emission at 590 nm (monomeric form)/535 nm (J-aggregate emission) after excitation at 514 nm.

Analytical Measurements
Rates of lactate accumulation were measured spectrophotometrically by using a method described previously.\textsuperscript{31} Intracellular ATP and PCr were extracted from samples (100 μL) by the addition of 6.6 mol/L perchloric acid (10 μL) before being centrifuged (10 000g, 10 minutes). The supernatant was removed and neutralized by the addition of 2.4 mol/L KOH and 3 mol/L KCl. Samples were centrifuged again, and the supernatant was stored at −80°C. ATP and PCr concentrations were measured by using a bioluminescence method described previously.\textsuperscript{31}

Statistical Analysis
Results are expressed as mean±SEM. Data were analyzed by the Student t test or repeated-measures ANOVA, with statistical differences determined by least significant difference post hoc tests. Data were considered significantly different at P<0.05.

Results
Rates of ATP Consumption/Wastage Under Normoxic Conditions
We estimate that at least 88% of the resting respiration rate of isolated neonatal cardiomyocytes under normoxic conditions is used to fuel the major ATP-consuming (protein synthesis, RNA synthesis, and Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity) and ATP-wasting (proton leak) processes (Figure 1). Rates of Ca\textsuperscript{2+}-ATPase activity were assumed to be negligible and not measured, because no significant difference existed between the normoxic rates of oxygen consumption measured in the presence (1.48±0.15 nmoL/min per 10\textsuperscript{6} cells) and absence (1.44±0.07 nmoL/min per 10\textsuperscript{6} cells) of sarcolemmal Ca\textsuperscript{2+} channel inhibitors (n=3). This result also confirms that cardiomyocytes were not undergoing contraction, because rates of oxygen consumption by contracting cardiomyocytes have been shown to be at least three times higher than rates by noncontracting cardiomyocytes.\textsuperscript{7,22}

Effect of Moderate Hypoxia
As shown in Figure 2, cardiomyocytes responded to moderate hypoxia by reducing their rates of oxygen consumption to 78±3% of normoxic rates (P<0.05). This reduction in aerobic metabolic activity was not associated with a compensatory switch to glycolysis, as indicated by the finding that glycolytic rates of ATP production accounted for only 5±1% of the total rates of ATP production (Figure 2). In addition, we found that the rates of energy production were decreased by 21±3% under these conditions (P<0.05; Figure 2).

The energetic status of cells maintained under moderate hypoxia for 1 hour was monitored by measuring intracellular ATP and PCr concentrations. PCr, in particular, is a sensitive indicator of energetic stress (a situation that occurs when the rates of energy consumption exceed the rates of energy produc-
Cardiomyocytes maintained under severe hypoxia were energetically stressed because intracellular ATP and PCr concentrations were decreased to 65±5% and 50±8% of normoxic concentrations (P<0.05; Figure 3B). Yet despite this severely compromised energetic state, cardiomyocytes remained viable and were capable of reversing energetic stress with reoxygenation, because intracellular ATP and PCr concentrations recovered to within 93±12% and 90±6% of normoxic concentrations, respectively (n=6).

In contrast to the relationship seen during moderate hypoxia, reduced rates of energy production under severe hypoxia were associated with decreased rates of protein and RNA synthesis (P<0.05; Figure 4). This downregulation was reversible with reoxygenation, because the rates of protein and RNA synthesis recovered to within 85±9% and 83±5% of normoxic rates, respectively (n=3). Rates of Na⁺,K⁺-ATPase activity under severe hypoxia were not significantly less than the rates measured under normoxia (Figure 4). This finding was supported by the measurements of intracellular [Na⁺] and [K⁺], inasmuch as the ratio of intracellular [Na⁺]/[K⁺] under severe hypoxia was not significantly different from the ratio measured under normoxic conditions (4.3±0.6 versus 4.9±0.3, respectively; n=3).

Effect of Myxothiazol

It was not clear why the rates of protein and RNA synthesis were reduced when cells were exposed to severe hypoxia and not when they were exposed to moderate hypoxia. Because the cells were energetically stressed during severe hypoxia but not during moderate hypoxia, we added myxothiazol, an inhibitor of electron flow through complex III in the mitochondria, to cause energetic stress under normoxic conditions. Our results show that nonmitochondrial rates of oxygen consumption accounted for 3±1% of total rates of oxygen consumption (Figure 2). Intracellular ATP and PCr concentrations were decreased by 50±4% and 69±5%, respectively, after 1 hour of exposure to myxothiazol (P<0.05; Figure 3C). Rates of ATP turnover were reduced by 80±3% and were associated with decreased rates of protein synthesis, RNA synthesis, and Na⁺,K⁺-ATPase activity (P<0.05; Figure 4). These results are consistent with the concept that nonmitochondrial oxygen consumption is associated with cellular energetic stress.
that energetic stress was involved in signaling metabolic downregulation.

Effect of Hypoxia and Myxothiazol on elf2α Phosphorylation
A rate-limiting step in the initiation of protein synthesis is the formation of the 43S initiation complex, which is catalyzed by elf2. The activity of elf2 is downregulated by phosphorylation of its α subunit. We measured the phosphorylation status of elf2α under severe and moderate hypoxia to establish whether decreased rates of protein synthesis were associated with an inhibition of protein initiation. Our results show that the concentration of elf2α phosphorylation was unchanged at moderate hypoxia when intracellular concentrations of ATP and PCr were maintained and increased by 46±7% after exposure to severe hypoxia for 30 minutes (P<0.05; Figure 5A), when cells were energetically stressed. Phosphorylation was reversible with reoxygenation, inasmuch as the concentration of phosphorylated elf2α decreased to 104±11% (n=3) of original normoxic concentrations (Figure 5A). Again, elf2α phosphorylation seemed to be related to the cellular energy state because intracellular PCr concentrations recovered to 95±12% of original normoxic concentrations with reoxygenation.

To further explore the link between energetic stress and downregulated rates of protein synthesis, we added myxothiazol under normoxic conditions and measured intracellular concentrations of phosphorylated elf2α. After 45 minutes of exposure to myxothiazol, phosphorylated elf2α concentrations were increased by 115±15% (P<0.05; Figure 5B), and intracellular ATP and PCr concentrations were decreased by 59±5% and 42±12%, respectively.

Effect of Moderate Hypoxia on Rates of Proton Leak
The proportion of mitochondrial oxygen consumption used to drive proton leak under normoxia and moderate hypoxia was determined by abolishing all oxygen consumption coupled to ATP synthesis with the ATP synthase inhibitor oligomycin (300 nmol/L). The resulting decreased rate of oxygen consumption, which represents proton leak, was associated with an increase in MMP (MMP 137% and 127% after 25 minutes under normoxia [Figure 6A] and moderate hypoxia [Figure 6B], respectively; P<0.05). It was necessary to titrate the MMP back its resting value (100%; Figure 6A and 6B) with myxothiazol (210 nmol/L) before rates of oxygen consumption could be used to estimate rates of proton leak, because rates of proton leak are highly dependent on MMP.

Our results show that rates of proton leak under moderate hypoxia were reduced to 60±9% of normoxic rates (P<0.05; Figure 6C). Rates of proton leak were not measured under severe hypoxia because of lack of a suitable method of measurement under these conditions.

Discussion
In addition to having identified protein synthesis, RNA synthesis, Na⁺,K⁺-ATPase activity, and proton leak as the major energy-consuming processes in resting isolated neonatal cardiomyocytes, the present study, to the best of our knowledge, is the first to have identified particular energy-consuming processes other than contraction downregulated during hypoxia. These energy-using processes have been estimated to consume ≈15% of total rates of oxygen consumption in working rat hearts. Reduced energy use through the inhibition of contractile and noncontractile energy-using processes would be expected to further prolong cell survival during ischemia.
We found that cardiomyocytes responded to reduced oxygen concentrations in either of two distinctly different ways that were dependent on the severity of hypoxia.

**Effect of Hypoxia on Cellular Energy State and Rates of Various Energy-Consuming Pathways**

Rates of energy production were decreased when cardiomyocytes were exposed to both moderate and severe forms of hypoxia. However, rates of energy consumption by cellular protein and RNA synthesis were downregulated only under severe hypoxia, when oxygen concentrations were low enough to induce energetic stress. Interestingly, hepatocytes isolated from the western painted turtle (Chrysemys picta bellii) have previously been shown to suppress energy usage by as much as 94% during anoxia.26–29 Additionally, it has been demonstrated that under these conditions, reduced rates of ATP turnover are associated with a suppressed rate of protein synthesis, protein degradation, Na⁺/K⁺-ATPase activity, urea synthesis, and gluconeogenesis.26–28

Rates of Na⁺/K⁺-ATPase activity were not downregulated during either moderate or severe hypoxia. This result compares well with the findings of others. Buttgereit and Brand30 have suggested that there is a hierarchy of ATP-consuming processes in mammalian cells, with protein and RNA synthesis being more sensitive to energy supply than Na⁺/K⁺-ATPase activity. In addition, Buck and Hochachka26 have demonstrated that Na⁺/K⁺-ATPase activity requires up to 75% of the total ATP turnover in hepatocytes from the western painted turtle under anoxia. They have suggested that this finding demonstrates the importance of ion pumping and the maintenance of ion homoeostasis during hypoxia. Finally, Pesquero et al31 have demonstrated that the Na⁺/K⁺-ATPase of trout erythrocytes is unaffected by exposure to anoxia. Because it is widely accepted that Na⁺/K⁺-ATPase preferentially uses glycolytically produced ATP,32,33 it has been suggested that the pump is protected under anoxic conditions, when the rates of aerobic ATP production are decreased.

**Are Energy-Consuming Pathways Downregulated in Response to Energetic Stress?**

Energetic stress may be the initial signal responsible for downregulating protein and RNA synthesis during severe hypoxia. This suggestion is in agreement with results obtained by Lefebvre et al34 who found that rates of protein and RNA synthesis were reduced when isolated preparations of hepatocytes were exposed to reduced oxygen concentrations. However, this group overlooked the link between metabolic downregulation and energetic stress, despite showing that ATP concentrations were decreased and ADP concentrations were increased under these conditions. It was surprising to note that in the study by Lefebvre et al,34 hepatocytes exhibited decreased rates of energy consumption and signs of energetic stress at oxygen concentrations as high as 100 μmol/L, inasmuch as ATP concentrations usually fall only when isolated cardiomyocytes are exposed to near-anoxic oxygen concentrations.22,35 We believe that despite being exposed to a gas mixture containing 100 μmol/L O₂, the oxygen concentration at the cell level was probably <0.5 μmol/L because of the presence of oxygen diffusion gradients. This suggestion is supported by the findings of Wolff et al,36 who reported that stationary culture conditions lead to cellular anoxia. This explanation would account for the discrepancy between our data and those of Lefebvre et al.34

Interestingly, the use of chemical hypoxia to model severe hypoxia had an unexpected effect on Na⁺/K⁺-ATPase activity, with rates of the pump being more depressed when cells were incubated under normoxic conditions in the presence of myxothiazol than when cells were incubated under severe hypoxia. Additionally, intracellular ATP concentrations were more depressed under these conditions. These findings support the suggestion that energy stress may involved in signaling metabolic downregulation.

**Effect of Hypoxia and Myxothiazol on Phosphorylation of eIF2α**

We are the first to show that downregulated rates of protein synthesis by isolated cardiomyocytes during severe hypoxia are associated with an inhibition of protein initiation. This finding is consistent with results obtained by Munoz et al,37 who demonstrated an increase in eIF2α phosphorylation during anoxia in PC12 cells. In agreement with our suggestion that energy consumption may be downregulated in response to energy stress, we found that eIF2α phosphorylation was increased when cardiomyocytes were energetically stressed and that phosphorylation was reversible when the energetic status of the cell recovered.
Effect of Moderate Hypoxia on Proton Leak
We have previously shown that isolated preparations of cardiomyocytes exhibit decreased rates of oxygen consumption when they are exposed to reduced oxygen concentrations.9 This response, termed oxygen conformance, is not associated with energetic stress and has also been demonstrated in isolated preparations of adult cardiomyocytes,22 hepatocytes,38 and embryonic chick cardiomyocytes.35 In the present study, we demonstrate that oxygen conformance is caused, at least in part, by decreased rates of the component of oxygen consumption not coupled to ATP synthesis, which is most likely proton leak. Our results compare well with the findings of Gnaiger et al39 and St-Pierre et al,40 who showed that rates of proton leak were decreased in isolated mitochondria exposed to reduced oxygen concentrations.

Implications of the Study
The results of the present study are the first to suggest that rates of energy consumption by protein and RNA synthesis may be downregulated in response to energetic stress. Downregulating rates of protein and RNA synthesis when oxygen availability is limited may provide a short-term means of conserving energy and prolonging cell survival. However, the long-term implications of such a response for the survival of the heart are unclear.

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