Delayed Ischemic Preconditioning Activates Nuclear-Encoded Electron-Transfer-Chain Gene Expression in Parallel With Enhanced Postanoxic Mitochondrial Respiratory Recovery

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Background—Delayed ischemic preconditioning promotes cardioprotection via genomic reprogramming. We hypothesize that molecular regulation of mitochondrial energetics is integral to this cardioprotective program.

Methods and Results—Preconditioning was induced by use of 3 episodes of 3-minute coronary artery occlusion separated by 5 minutes of reperfusion. Twenty-four hours later, infarct size was reduced by 58% after preconditioning compared with sham-operated controls (P<0.001). Cardiac mitochondria were isolated from sham and preconditioned rat hearts. Mitochondrial respiration and ATP production were similar between the groups; however, preconditioned mitochondria exhibit modest hyperpolarization of the inner mitochondrial membrane potential (≥22% versus control, P<0.001). After 35-minute anoxia and reoxygenation, preconditioned mitochondria demonstrated a 191±006% improvement in ADP-sensitive respiration (P=0.002) with preservation of electron-transfer-chain (ETC) activity versus controls. This augmented mitochondrial recovery was eradicated when preconditioning was abolished by the antioxidant 2-mercaptopropionyl glycine (2-MPG). These biochemical modulations appear to be regulated at the genomic level in that the expression of genes encoding rate-controlling complexes in the ETC was significantly upregulated in preconditioned myocardium, with a concordant induction of steady-state protein levels of cytochrome oxidase, cytochrome c, and adenine nucleotide translocase-1. 2-MPG abolished preconditioning induction of these transcripts. Moreover, transcripts of nuclear regulatory peptides known to orchestrate mitochondrial biogenesis, nuclear respiratory factor-1 and peroxisome-proliferator–activated receptor gamma coactivator 1α, were significantly induced in preconditioned myocardium.

Conclusions—Delayed preconditioned mitochondria display increased tolerance against anoxia-reoxygenation in association with modifications in mitochondrial bioenergetics, with concordant genomic induction of a mitochondrial energetic gene regulatory program. This program appears to be mediated by reactive oxygen species signaling. (Circulation. 2004;110:534-539.)

Key Words: ischemia • preconditioning • mitochondria • metabolism

Delayed preconditioning is an adaptive program of the myocardium to a preceding nonlethal transient ischemia resulting in augmented resistance to subsequent ischemia-reperfusion injury.1,2 This ischemia-tolerant phenotype is thought to be regulated predominantly at the transcriptional level and manifests 12 to 24 hours after the preconditioning trigger.3

An intuitive biological program to orchestrate tolerance against ischemia would be the maintenance of cellular energetics, to resist ischemia and reperfusion damage.3,4 The evaluation of the role of the maintenance or restoration of ATP production in the context of delayed preconditioning has not been comprehensively investigated. In contrast, during the short-lived tolerance associated with classical preconditioning a uniform bioenergetic consequence of preconditioning (PC) is an enhanced capacity to restore mitochondrial ATP synthesis (reviewed by Opie and Sack).5 These data imply modification of mitochondrial bioenergetic regulation as a component of preconditioning-induced myocardial protection. We reasoned that delayed preconditioning, with its regulatory control at the transcriptional level, would be the ideal biological program to investigate the biochemical and gene-regulatory modulation of mitochondrial bioenergetics in promoting ischemic tolerance.
Our objective was to delineate the mitochondrial phenotype of delayed-preconditioned mitochondria under basal conditions and after anoxia-reoxygenation. Furthermore, the mitochondrial response after the inhibition of ischemic preconditioning by use of 2-mercaptopyrropropionyl glycine (2-MPG) was evaluated.6,7 Basal mitochondrial respiratory function was assessed by inner mitochondrial membrane potential (ΔΨm), state-3 respiration, cytochrome c oxidase activity, and ATP levels comparing PC, PC in the presence of 2-MPG (PC+MPG), and sham-operated rat cardiac mitochondria. Furthermore, the capacity to recover respiratory function was evaluated in isolated mitochondria after anoxia-reoxygenation. The nuclear-encoded genomic control of mitochondrial respiration was evaluated by determining expression levels of genes encoding rate-controlling proteins in the electron transfer chain (ETC) and their putative cognate nuclear regulatory proteins.

In the present study, we present data illustrating mitochondrial bioenergetic function and gene-regulatory control during delayed preconditioning. This regulatory program appears to prevent futile ATP production and adenosine nucleotide pool depletion under normal conditions but is associated with a nascent capacity to augment ATP production after anoxia and reoxygenation. Gene expression levels demonstrate a coordinated upregulation of nuclear-encoded mitochondrial respiratory chain enzymes in response to ischemic preconditioning. Furthermore, a putative role of reactive oxygen species signaling in mediating this respiratory and genomic control is implied.

Methods

Animal Studies

One hundred seventy-two adult male Wistar rats (Charles River Laboratories, Wilmington, Mass) were used for the preconditioning and sham-control protocols. The surgical process of left coronary artery occlusion has been described previously.6 Transient regional ischemia was induced by tightening a ligature around the left coronary artery, and the supplied territory was subjected to 3 episodes of 3 minutes of ischemia coupled to 5 minutes of reperfusion. Sham operations involved encircling the left coronary artery without tightening the suture. PC+MPG (0.42 mg · kg⁻¹ · min⁻¹ 2-MPG infusion)6,7 was used as an additional control to assess the effect of reactive oxygen species signaling on the mitochondrial and genomic phenotype. After chest closure, animals were allowed to recover. Hearts were then extracted at 2 and 4 hours for RNA extraction and at 24 hours after the procedures for (1) Langendorff perfusion and ischemia/reperfusion studies as previously described, (2) mitochondrial isolation10 and analysis, and (3) Western blot analysis. A schematic of study protocols is shown in Figure 1. All experiments were performed in accordance with institutional guidelines.

Biochemical Analysis

Mitochondria were extracted from heart tissue 24 hours after preconditioning or sham-control procedures as described previously.10 Baseline respiration was measured polarographically at 25°C in response to ADP and glutamate10 and mitochondria were then exposed to a 25-minute anoxic insult in the oxygraph chamber, followed by reoxygenation.11 Recovery of respiratory function was evaluated by expressing the state 3 respiration after anoxia over the state 3 respiration before anoxia.

The activity of cytochrome oxidase (complex IV) was measured polarographically by a Clark-type electrode in the presence of 50 mmol/L potassium phosphate (pH 7.4), 40 μmol/L cytochrome c, 12.5 mmol/L ascorbate, 0.63 mmol/L N,N,N′,N′-tetramethyl-p-phenylenediamine, and 0.03% Triton X-100.12 Inner mitochondrial membrane potential determination was performed on isolated mitochondria by use of potentiometric dyes (3,3′,3′-diethyloxacarbocyanine iodide, Sigma11 and JC-1 [5,5′,6′,6′-tetrachloro-1,1,3,3′-tetrathylbenzimidazolocarbocyanine iodide, 1 μg/mL, Molecular Probes]) and FACSCalibur (Becton Dickinson) analysis as described previously.13 Oligomycin and CCCP were used as controls to confirm that fluorescence correlated with changes in inner mitochondrial membrane potential.

Tissue ATP concentrations were assessed by use of luciferin-luciferase luminescence on left ventricular whole-cell preparations as described previously.15 Protein concentrations were determined by the Lowry method.

Gene Expression and Protein Analysis

mRNA isolation and Northern blot analysis were performed as described previously.16 cDNA probes were generated by use of reverse transcription–polymerase chain reaction (RT-PCR) from rat heart tissue with oligonucleotide primers designed from GenBank to encode cDNAs for succinate dehydrogenase (flavoprotein subunit, complex II), cytochrome c, subunit (complex III), cytochrome c oxidase subunit 4 (complex IV), cytochrome c, and adenine nucleotide translocase 1.

The nuclear regulatory factor gene transcripts encoding for nuclear respiratory factor 1 (NRF-1) and peroxisome-proliferator–activated receptor gamma coactivator 1-α (PGC-1α) were quantified by use of LUX (Invitrogen) FAM-labeled primers on the MJ Research Opticon II real-time PCR thermocycler and multiplexed with JOE-labeled TATA-binding protein as internal controls. Real-time PCR was also used to evaluate the expression patterns of the nuclear-encoded ETC genes in response to preconditioning in the presence and absence of 2-MPG versus control.

Western blot analysis was performed on heart tissue at the 24-hour time point after the preconditioning and sham-controlled procedures as described previously.16 Antibodies (cytochrome c, adenine nucleotide translocase-1 [ANT-1], and β-actin) were obtained from Santa Cruz Biotechnology, and cyclooxygenase IV was obtained as a control. The secondary antibodies were conjugated with horseradish peroxidase, and the protein was detected by use of enhanced chemiluminescence (Amersham). The immunoblots were probed with β-actin to confirm equal loading.

Statistical Analysis

All results are expressed as mean±SEM. Student’s t-test was used for 2-group comparisons. Sequential time points of gene expression were analyzed by 1-way ANOVA followed by the Student-Newman-Keuls post hoc test. A value of P<0.05 was considered significant.

Results

To validate the biological system, we confirmed delayed preconditioning cardioprotection in the rat heart. Infarct size was reduced from 63.1±11.2% to 26.5±9.1% (P<0.001) of...
the risk zone after delayed preconditioning versus sham controls, with no change in infarct size between sham-operated and nonoperated controls (data not shown).

Delayed Preconditioning Modulates Mitochondrial Respiration and Augments Energetic Capacity After Anoxia

The initial objective was to evaluate the mitochondrial respiratory phenotype in response to delayed preconditioning. Twenty-four hours after sham or ischemic preconditioning surgery, the cardiac levels of ATP as assessed by luciferase activity were similar (0.19±0.04 versus 0.18±0.02 nmol/L ATP/μg protein, respectively; data not shown). In parallel, mitochondrial energy-production, reflected by the rate of oxygen consumption (in the presence of ADP and glutamate-state 3 respiration) was not different between the 2 groups (Figure 2). In contrast, the preconditioned mitochondria exhibited moderate but significantly higher inner mitochondrial membrane potential (hyperpolarization) as quantified by FACS analysis by use of JC-1 and 3,3′-dihexyloxacarbocyanine iodide potentiometric dyes (≥11% increase in fluorescence; data not shown). Because mitochondrial hyperpolarization implies an enhanced capacity to generate ATP, we evaluated the respiratory recovery of these mitochondria after anoxia-reoxygenation. After 25 minutes of anoxia, preconditioned mitochondria exhibited a 191.4±12.4% greater capacity to restore state 3 respiration compared with control mitochondria (P=0.002; Figure 3). To evaluate whether ETC enzymatic activity corresponds to these modulations in respiratory capacity, cytochrome oxidase activity (complex IV of the ETC) was compared between groups at baseline and in response to anoxia-reoxygenation. In parallel with state 3 respiration, cytochrome oxidase activity was similar in both groups at baseline but exhibited a 2-fold greater capacity to maintain enzyme activity after anoxia in the preconditioned mitochondria versus control mitochondria (P=0.001 versus control, Figure 3).

Genomic Induction of the ETC in Response to Delayed Preconditioning

Delayed preconditioning is a genomic reprogramming of the heart that is characterized by transcriptional induction of putative cytoprotective peptides.3 The temporal delineation of regulatory control of cytoprotective proteins demonstrates that genes encoding cytoprotective peptides are induced within hours after the preconditioning trigger, with a subsequent induction of the cognate encoded proteins coinciding with ischemia tolerance.17,18 After the biochemical observations identified in our study, we reasoned that the nuclear genes encoding mitochondrial ETC proteins may be induced by delayed preconditioning. As shown in Figure 4, genes encoding regulatory peptides in complexes II (succinate dehydrogenase), III (cytochrome c1), IV (cytochrome oxidase subunit IV), cytochrome c, and ANT-1 are all significantly upregulated in excess of 60% to 130% above the expression levels in sham-operated control heart tissue at 4 hours after the preconditioning trigger. A modest upregulation of these nuclear-encoded mitochondrial inner membrane protein-encoding genes was observed 2 hours after ischemic preconditioning (data not shown). To establish whether these modulations in gene expression translate into upregulation of steady-state protein levels concordant with ischemic tolerance, Western blot analysis was performed by use of antibodies directed against cytochrome c, cytochrome c oxidase, and ANT-1. These proteins were concordantly upregulated by ≥115% compared with sham-operated controls 24 hours after the operative procedures (data not shown).

Recently, the nuclear regulatory proteins that induce the ETC proteins have been identified.19–21 We evaluated the gene expression levels of 2 prime candidates, ie, NRF-1 and PGC-1α, in response to the sham and preconditioning procedures in the rat heart tissue. Interestingly, the mRNA levels of NRF-1 and PGC-1α were induced 2.3- and 2.5-fold, respectively, at 2 hours after preconditioning (P=0.04 versus sham-operated control), with a return toward basal levels at the 4-hour time after the surgical procedures (Figure 5).
An Antioxidant Intervention During the Preconditioning Trigger Abolishes the Respiratory Gene Expression and Mitochondrial Respiratory Perturbations Associated With Delayed Preconditioning

Delayed preconditioning is inhibited by the administration of antioxidant agents in concert with the preconditioning trigger. To determine whether the mitochondrial and genomic phenotype described above could be abolished by inhibiting preconditioning, we administered 2-MPG during the preconditioning trigger. Interestingly, the capacity to restore post-anoxic state-3 respiration and the expression levels of nuclear genes encoding mitochondrial respiratory chain proteins were nullified by the infusion of 2-MPG during preconditioning (Figure 6).

Discussion

The major findings in this study demonstrate that the cardioprotective program induced by delayed preconditioning includes alterations in mitochondrial respiratory function that appear to be regulated by coordinated genomic and biochemical perturbations. These preconditioning-activated regulatory events are associated with a mitochondrial bioenergetic phenotype displaying an enhanced capacity to maintain energetic balance after bioenergetic stress coupled to efficient oxygen consumption during basal respiration. The complex repertoire of mitochondrial regulatory phenotypic changes observed in our study are directly concordant with the paradigm of maintaining cellular ATP levels to delay myocyte death in ischemia/reperfusion injury and may be a central component of the delayed preconditioning–induced cardioprotective program.

Our initial findings demonstrated that delayed preconditioning promotes mitochondrial hyperpolarization (increased inner mitochondrial membrane potential) and an enhanced capacity to generate ATP in response to anoxia. Inner mitochondrial membrane potential represents the summation of the generators of the electrochemical gradient (substrate transport and metabolism in the mitochondria, with subsequent respiratory chain transfer) balanced against proton leak/slip and the reaction of ATP synthesis, transport, and turnover. Interestingly, a physiologically hyperpolarized mitochondrion is considered to be an energized mitochondrion that hypothetically has an enhanced capacity to produce ATP in response to an acute stress. Our data support this concept in that the preconditioned mitochondria demonstrated a greater capacity to restore respiration and ATP-generating capacity after anoxia-reoxygenation compared with the sham-control mitochondria. Also, this augmented respiratory capacity in response to anoxia has been shown to be present in diazoxide-induced classic preconditioning.
At baseline, the similar oxygen consumption in the control and preconditioned mitochondria is compatible with the similar energy demand of these hearts, as evidenced by similar ex vivo rate-pressure products and rates of ATP production (data not shown). The phenotype of hyperpolarization in the absence of increased oxygen consumption is compatible with the “first mechanism of respiratory control.” Here, the differential electrochemical gradient across the inner mitochondrial membrane is usually in excess of 150 mV and results in the subsequent inhibition of proton pump activity. The respiratory profile observed in this study suggests a hypothesis that delayed preconditioning results in divergent perturbations in the mitochondrial respiratory architecture to concurrently prevent futile depletion of the vital adenine nucleotide pool under basal conditions, coupled to an increased capacity to generate ATP in response to anoxic stress.

To modulate inner membrane mitochondrial membrane potential, protons are translocated outwardly across the inner membrane by the proton pumps in complexes I, III, and IV in accordance with the chemiosmotic theory. The proton motive force generated then allows the phosphorylation of ADP via the F$_1$F$_0$ ATPase. Because delayed preconditioning is mediated via the transcriptional induction of cytoprotective peptides, we reasoned that key proteins in these ETC complexes may be upregulated in parallel with the mitochondrial hyperpolarization evident after delayed preconditioning. Our data reveal an increase in the transcript and protein levels for several proteins in the proton-pumping complexes of the ETC, and notably the putative rate-limiting components of electron transfer, cytochrome c and cytochrome c oxidase. The fact, that the preconditioning “antagonist” 2-MPG abolishes the upregulation of these ETC protein encoding genes is further supportive evidence of the involvement of these gene-regulatory events in delayed preconditioning cardioprotection. Here, 2-MPG is postulated to attenuate the delayed preconditioning program by inhibiting the ischemic preconditioning trigger-induced reactive oxygen species signaling.

The transcriptional regulatory program controlling nuclear and mitochondrial-encoded electron chain peptides has begun to be investigated. Because NRF-1 and PGC-1α have been demonstrated to modulate energy supply and mitochondrial biogenesis control, we evaluated the steady-state transcript levels of these 2 regulatory proteins in delayed preconditioning. Interestingly, both of these inducers of mitochondrial respiratory function were found to be transiently upregulated before the upregulation of the mRNA levels of genes encoding nuclear-encoded respiratory chain proteins. In the heart, the persistent induction of PGC-1α in a cardiac-restricted pattern has been shown to be detrimental. As our PGC-1α gene expression implies, a modest and transient induction of this peptide may be cardioprotective, yet the functional evaluation of these putative divergent actions will need to be explored.

The composite data from this study generate a novel hypothesis implying nuclear regulatory control of mitochondrial respiration as an integral component of the delayed preconditioning cardioprotective program. We would propose that delayed preconditioning results in the transcriptional induction of nuclear-encoded mitochondrial ETC protein encoding genes. The subsequent upregulation of steady-state protein levels augments proton pumping across the inner mitochondrial membrane. This, in turn, evokes the first mechanism of respiratory control via the Mitchell hypothesis, resulting in the feedback inhibition of respiration (reviewed by Kadenbach). Concurrently, mitochondrial inner membrane hyperpolarization allows enhanced recovery of respiration after oxygen depletion through an as yet undefined mechanism.

Numerous limitations of this study need to be discussed. In the first instance, the complexity of the biochemical profile of the mitochondria in response to the delayed preconditioning needs further characterization. However, our data and the studies performed in classic preconditioning collectively suggest that energetic homeostasis controlled by the mitochondrion should be considered central in orchestrating the cardioprotective programs induced by preconditioning. In addition, the nuclear regulatory program identified in this study, i.e., upregulation of NRF-1 and PGC-1α, could be compatible with either the upregulation of the ETC machinery in individual mitochondria or the initiation of mitochondrial biogenesis. However, because the mitochondrial membrane potential was measured by use of a fixed number of mitochondria (FACS analysis) and the polarographic studies were corrected for total mitochondrial protein, our data would support modulations in the existing population of cardiac
mitochondria. In addition, the induction of cytochrome c may induce the mitochondrial-protective phenotype via alternative mechanisms, including an antioxidant effect. Last, nitric oxide (NO) has been very well established as a key signaling molecule in orchestrating the delayed-preconditioning cell-survival program. Interestingly, NO itself is known to modulate mitochondrial respiration by reversibly inhibiting complex IV of the ETC. Whether this function of NO prevents futile ETC cycling in preconditioned, nonstressed mitochondria is an intriguing proposal that requires evaluation.

In conclusion, these data demonstrate that mitochondrial respiratory function and capacity are modulated in response to delayed preconditioning, enabling improved energy production in response to anoxia. These modulations appear to be regulated in part via the transcriptional induction of multiple proteins in the electron transport chain. The composite mitochondrial phenotype induced by the delayed preconditioning program results in an enhanced capacity to generate ATP subsequent to the ATP-depleting effects of anoxia coupled to efficient oxygen consumption during basal myocardial metabolic demand. Moreover, we propose that the findings in this study have identified a robust biological phenomenon (delayed preconditioning) that can be exploited to advance our understanding of the role of intergenomic control of mitochondrial biology in generating this cardioprotective phenotype.

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