Biphasic modulation of the mitochondrial electron transport chain in myocardial ischemia and reperfusion

Hsin-Ling Lee,1,2 Chwen-Lih Chen,3 Steve T. Yeh,1 Jay L. Zweier,1 and Yeong-Renn Chen1,3

1Davis Heart and Lung Research Institute, College of Medicine, Ohio State University, Columbus, Ohio; 2Graduate Institute of Clinical Medicine, College of Medicine, National Cheng Kung University, Tainan, Taiwan; and 3Department of Integrative Medical Sciences, College of Medicine, Northeast Ohio Medical University, Rootstown, Ohio

Submitted 22 July 2011; accepted in final form 28 December 2011

Lee HL, Chen CL, Yeh ST, Zweier JL, Chen YR. Biphasic modulation of the mitochondrial electron transport chain in myocardial ischemia and reperfusion. Am J Physiol Heart Circ Physiol 302: H1410–H1422, 2012.—Mitochondrial electron transport chain (ETC) is the major source of reactive oxygen species during myocardial ischemia-reperfusion (I/R) injury. Ischemic defect and reperfusion-induced injury to ETC are critical in the disease pathogenesis of postischemic heart. The properties of ETC were investigated in an isolated heart model of global I/R. Rat hearts were subjected to ischemia for 30 min followed by reperfusion for 1 h. Studies of mitochondrial function indicated a biphasic modulation of electron transfer activity (ETA) and ETC protein expression during I/R. Analysis of ETAs in the isolated mitochondria indicated that complexes I, II, III, and IV activities were diminished after 30 min of ischemia but increased upon restoration of flow. Immunoblotting analysis and ultrastructural analysis with transmission electron microscopy further revealed marked downregulation of ETC in the ischemic heart and then upregulation of ETC upon reperfusion. No significant difference in the mRNA expression level of ETC was detected between ischemic and postischemic hearts. However, reperfusion-induced ETC biosynthesis in myocardium can be inhibited by cycloheximide, indicating the involvement of translational control. Immunoblotting analysis of tissue homogenates revealed a similar profile in peroxisome proliferator-activated receptor-γ coactivator-1α expression, suggesting its essential role as an upstream regulator in controlling ETC biosynthesis during I/R. Significant impairment caused by ischemic and postischemic injury was observed in the complexes I–III. Analysis of NADH ferricyanide reductase activity indicated that injury of flavoprotein subcomplex accounts for 50% decline of intact complex I activity from ischemic heart. Taken together, our findings provide a new insight into the molecular mechanism of I/R-induced mitochondrial dysfunction.

Marked hyperoxygenation induced by reperfusion in the postischemic myocardium was detected by in vivo electron paramagnetic resonance (EPR) oxymetry (50).

The proteins of ETC are the major site for energy transduction and oxygen free radicals production in mitochondria. It was reported that cardiac ischemia resulted in damage to the mitochondrial ETC and this ischemic damage to the ETC contributed to myocardial injury during reperfusion (12, 36). Furthermore, mitochondrial ultrastructural injury occurred and progressed during myocardial ischemia (34). Despite the above alteration induced by ischemia in the ETC, recovery of mitochondrial function with restoration of contractility was detected upon reperfusion. However, it was observed that ETC-mediated oxygen consumption is disproportionately high relative to reduced contractility (23, 35). Therefore, defect of mitochondrial integrity in the postischemic heart was linked to the oxidative damage caused by reactive oxygen species (ROS) overproduction. Several groups (12, 13, 36, 50) have reported that the activities of the ETC from isolated mitochondria are decreased during reperfusion. The molecular mechanisms of ischemia-induced defects and reperfusion-induced damage to the ETC are still poorly understood due to a lack of deep insights into biochemical characterization of the ETC proteins in the ischemic and postischemic hearts. Recent studies have identified that alterations of ETC in the ischemic and postischemic myocardium are involved in posttranslational modifications, including increasing protein tyrosine nitration of complex I (30) and complex II (7), decreasing protein S-glutathionylation of complex II (13), and increasing hyperphosphorylation of complex IV (39). In vitro studies (7, 8, 13, 149) have also clarified that some of the above protein modifications contributed to the decrease in the enzymatic activity of specific ETC components.

Isolated mitochondria are commonly used to evaluate bioenergetics in hearts undergoing ischemia and reflow. The present study was therefore performed to assess the biochemical properties of the mitochondria isolated from ischemic and postischemic hearts, including 1) the enzymatic activities of ETC, 2) regulation of protein biosynthesis of ETC, and more specifically 3) impairment of complex I. Our findings indicate that the mitochondrial ETC is specifically undergoing a biphasic modulation of enzymatic catalysis and protein biosynthesis during the phase of ischemia and the phase of reperfusion. The detected dynamic behavior of the ETC, at least in part, contributes to the ischemic defect of mitochondria and reperfusion-induced mitochondrial dysfunction.

MATERIALS AND METHODS

Animals, Langendorff heart preparation, and measurement of left ventricular function and coronary flow. Male Sprague-Dawley rats (3 to 4 mo, 350–400 g) were purchased from Harlan (Indianapolis,
IN). All procedures were performed with the approval (protocol no. 10-003) of the Institutional Animal Care and Use Committee at Northeast Ohio Medical University (Rootstown, OH) and conformed to the Guide for the Care and Use of Laboratory Animals. Langendorff-isolated heart was performed as described previously (51). All hearts were subjected to a 20-min baseline period under constant perfusion pressure. Hearts were randomly assigned to one of three study groups including control (perfusion for 60 min without ischemia), global ischemia (30 min), and reperfusion following global ischemia, and left ventricular (LV) developed pressure (LVDP) was continuously recorded using Powerlab 4/25 ADC (AD Instruments, Newton, NH). The following derived indexes of LV mechanical function were instantaneously recorded: peak systolic pressure (PSP), end diastolic pressure (LVEDP), developed pressure (LVDP = PSP – LVEDP), heart rate (HR), and rate pressure product (RPP = LVDP \times HR).

Preparation of the mitochondria from the rat hearts. Mitochondria were prepared by differential centrifugation (47). To increase recovery of the mitochondria from both viable and nonviable cardiomyocytes, Polytron/nagarase homogenate was subjected to centrifugation at 20,000 × g for 10 min and resuspended in the buffer M containing 230 mM mannitol, 70 mM sucrose, 1 mM EDTA, 5 mM Trizma/HC1 buffer (pH 7.4), and protease/phosphatase inhibitors (1 tablet Complete and 1 tablet phosphoSTOP in 10 ml; Roche Applied Science, Indianapolis, IN) before oxygen consumption measurement. The mitochondria as prepared contain 410.2 nmol heme b/mg protein and 470.4 nmol aas/mg protein and no detectable contamination of nuclear and endoplasmic reticulum. The recovery of mitochondria was determined by measuring percent recovery of citrate synthase activity in isolated mitochondria and tissue homogenate according to published methods (5, 16).

Assay of enzymatic activities of the mitochondrial ETC. Mitochondrial preparations were subjected to analysis of electron transfer activities (ETAs) using a U/VIS spectrophotometer. The ETA of complex I was determined by following the rotenone-sensitive ubiquinone-2 (Q2H2)-stimulated dichlorophenyl indophenol reduction (13). The ETA of complex III was assayed by thenoyltrifluoroacetone-sensitive ubiquinone-2 (Q2)–stimulated cytochrome c reduction and verified by inhibition with antimycin A (13). The ETA of complex IV was assayed by measuring ferrocytochrome c oxidation, and further confirmed by inhibition with potassium cyanide (15).

Immunoblotting analysis. Western blotting with mitochondrial preparations was performed as described previously (13). Immunoblotting was carried out with anti-51-kDa antibody [against the flavin mononucleotide (FMN)-binding subunit of complex I, generated in house], or anti-70-kDa antibody (against the FAD-binding complex I), or anti-ND1 (hydrophobic protein of complex I), generated in house], or anti-75-kDa antibody (against 75-kDa subunit of complex III) was prepared from submitochondrial particles according to published methods (26). Mitochondrial size was calculated based on micrograph at ×18,500, and volumetric density of mitochondria was computed based on micrograph at ×6,800.

Preparations of isolated NADH-cytochrome c reductase supercomplex from bovine heart. Bovine heart mitochondrial NADH-cytochrome c reductase (NCR; supercomplex hosting complex I and complex III) was prepared from submitochondrial particles according to the published method (22). The NCR preparation contains 2.2 nmol heme b/mg protein with an electron transfer activity of 111.8 nmol cyt c reduced·min⁻¹·nmol heme b⁻¹ in the assay mixture containing 20 mM potassium phosphate buffer, 150 μM NADH, 50 μM ferricytochrome c, 180 μg/ml azolectin, 1 mM EDTA, and 2 mM NaN3 (22).

EPR spin-trapping experiment. EPR spin trapping experiment with 5-diethoxyphosphoryl-5-methyl-1-pyrrole N-oxide (DEPMPO) was used to detect O2•−, which was performed on a Bruker EMX MICRO spectrometer (in Rootstown campus of NEOMED) operating at 9.86 GHz with 100 kHz of modulation frequency at room temperature. The reaction mixture was transferred to a 50-μl capillary, which was then positioned in the high-sensitivity cavity. The instrumental setting and the parameters of computer simulation was followed according to published method (14).

Statistical analysis. All data were reported as group averages ± SE. Statistical analyses of LV function were performed at the end of the baseline period and at the end of 15, 30, or 60 min of reperfusion. Comparison between two groups was assessed by independent t-test and among multiple groups was assessed by one-way ANOVA followed by the least significant difference, Tukey’s honestly significant difference or Games-Howell post hoc tests. Differences between two groups were determined with Student’s t-test. A value of P < 0.05 was considered statistically significant.

RESULTS

Myocardial functional recovery in the postischemic heart. As compared with control preischemic baseline level, impairment of LV function was detected at the end of 15, 30, and 60 min of reperfusion. The functional recovery of LVDP was 20.0 ± 4.3% (15-min reperfusion, n = 6), 24.9 ± 4.9% (30-min reperfusion; n = 6), and 26.2 ± 3.8% (60-min reperfusion, n = 6). The RPP recovered to 18.3 ± 4.4% (15-min reperfusion), 22.2 ± 4.9% (30-min reperfusion), and 26.1 ± 5.8% (60-min reperfusion) compared with isolated hearts subjected to equal duration of reperfusion without ischemia. The parameters of hemodynamic performance are shown in the Table 1.

State 3 ADP-stimulated respiration and mitochondrial integrity in the ischemic and postischemic heart. Overproduction of oxygen free radicals during myocardial ischemia/reperfusion can depolarize mitochondrial membrane potential and uncouple mitochondrial respiration (19, 20). Therefore, respiratory control ratio (RCR; defined as the ratio of state 3 to state 4) of mitochondrial preparation from hearts was evaluated at 30°C. As indicated in the Fig. 1, A and B, NADH-linked and ADP-stimulated respiration (state 3; driven by 5 mM malate/140 mM glutamate) of the mitochondria from ischemic hearts was decreased from 190.7 ± 18.0 to 73.6 ± 4.8 nmol O2·min⁻¹·mg protein⁻¹ (n = 4; P < 0.05), FCCP-induced uncoupling respiration was decreased from 219.9 ± 21.9 to 99.6 ± 6.6, and the RCR was decreased from 4.1 ± 0.4 to 1.4 ± 0.1 (n = 4; P < 0.05). In the postischemic heart, NADH-linked and ADP-stimulated/uncoupling respiration was decreased to 117.2 ± 9.1/160.3 ± 13.3 nmol O2·min⁻¹·mg protein⁻¹ (n = 4; P < 0.05), and the RCR was decreased to 2.6 ± 0.3 (n = 4; P < 0.05). These results indicate a significant defect in respiration and mitochondrial integrity in the ischemic and postischemic hearts. However, the functional recovery of LVDP...
during reperfusion was supported by partial restoration of mitochondrial RCR, ADP-stimulated, and uncoupling respirations. The results support the data directly showing recovery of high-energy phosphates including tissue ATP and phosphocreatine in the heart subjected to a similar protocol of ischemia and reperfusion as previously reported by Ambrosio et al. (1, 2).

Mitochondrial ETC activities and protein and mRNA expressions in the ischemic and postischemic hearts. The recovery of mitochondria from myocardial tissue is 33.6 ± 6.5% (control heart; n = 5), 29.5 ± 5.2% (ischemic heart; n = 5), and 35.2 ± 5.2% (postischemic heart; n = 5) based on citrate synthase assay. There is no statistical difference in the mitochondrial

Table 1. Hemodynamic values from isolated rat hearts subjected to ischemia and reperfusion and the effect of cycloheximide

<table>
<thead>
<tr>
<th>Measure</th>
<th>Equilibrium Baseline (20 min)</th>
<th>Ischemia (0 min) + RP (60 min)</th>
<th>End of Ischemia (30 min)</th>
<th>RP (15 min)</th>
<th>RP (30 min)</th>
<th>RP (60 min)</th>
<th>CHX + RP (30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, beats/s</td>
<td>285 ± 15</td>
<td>293 ± 12</td>
<td>ND</td>
<td>262 ± 16</td>
<td>254 ± 11</td>
<td>269 ± 21</td>
<td>243 ± 19</td>
</tr>
<tr>
<td>LVDP, mmHg</td>
<td>96.7 ± 5.7</td>
<td>94.3 ± 3.6</td>
<td>ND</td>
<td>19.3 ± 1.8</td>
<td>24.1 ± 3.1</td>
<td>26.7 ± 4.1</td>
<td>10.1 ± 1.7</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>4.7 ± 1.3</td>
<td>5.1 ± 1.7</td>
<td>31.1 ± 4.3</td>
<td>71.8 ± 9.4</td>
<td>58.6 ± 9.5</td>
<td>41.2 ± 6.0</td>
<td>69.0 ± 8.2</td>
</tr>
</tbody>
</table>

Values are means ± SE. RP, reperfusion; LVDP, left ventricular developed pressure; LVEDP, left-ventricular end-diastolic pressure; CHX, cycloheximide; ND, not determined.

Fig. 1. A and B: state 3 and state 4 respiratory rates of mitochondrial preparations from control, ischemic, and postischemic hearts. Control hearts were obtained from 60-min reperfusion without ischemia. Ischemic and postischemic hearts were obtained through the conditions of global ischemia (30 min) and global ischemia (30 min)/reperfusion (60 min). Mitochondria were prepared and resuspended in the buffer M. Mitochondrial respirations were measured by the polarographic method at 30°C (7). Oxygen consumption by mitochondria (0.5 mg/ml) was induced by malate (5 mM)/glutamate (140 mM). State 3, oxygen consumption stimulated by ADP (0.5 mM); state 4, oxygen consumption after the addition of oligomycin (6 μg/mg mitochondria) followed by ADP addition. RCR is the ratio of state 3 and state 4 oxygen consumption rates; uncoupling respiration is oxygen consumption after the addition of FCCP (2.5 μM). *P < 0.05 vs. baseline control (N). §P < 0.05, ischemia (I) vs. ischemia/reperfusion (IR). C and D: enzymatic activity of electron transport chain (ETC) in the mitochondria of ischemic and postischemic hearts (n = 5). ETA; electron transfer activity; NFR, NADH ferricyanide reductase.
recovery among control, ischemic, and postischemic hearts. Even though the amount of mitochondria recovered was roughly similar in the three conditions, the quality was not. It should be noted that the isolation procedures employed for “healthy” mitochondria may work differently for ischemic mitochondria, which could be ending up “enriching” selected populations of mitochondria, not necessarily representative of the whole mitochondrial population. Thus, the use of centrifugation at 20,000 g (rather than 5,000 g) should boost the representative of the whole mitochondrial population. Furthermore, two populations (subsarcolemmal mitochondria and interfibrillar mitochondria) of mitochondria with differential susceptibility to damage in ischemia should be mentioned as a potential limitation of current study.

Mitochondria were then subjected to analysis of ETC enzymatic activities. The specific activities of complex I, complex II, complex III, and complex IV in the mitochondria of normal heart were 244.2 nmol NADH oxidized·min⁻¹·mg protein⁻¹, 386.5 nmol dichlorophenyl indophenol reduced·min⁻¹·mg protein⁻¹, 2255.6 nmol ferricytochrome c reduced·min⁻¹·mg protein⁻¹, and 2,008.0 nmol ferricytochrome c oxidized·min⁻¹·mg protein⁻¹, respectively.

As indicated in Fig. 1C, a significant decrease of ETA was detected in the ischemic heart compared with control, including complex I (52.1 ± 4.6% of ETA remained; n = 5; P < 0.05), complex II (70.1 ± 5.1% remained; n = 5; P < 0.05), complex III (60.7 ± 3.9% remained; n = 5; P < 0.05), and complex IV (75.4 ± 5.8% remained; n = 5; P < 0.05). The concentrations of heme b and aa₃ remained to 350.9 and 409.6 nmol/mg, respectively. These results clearly demonstrated an ischemia-induced defect of the mitochondrial ETC, and the defect was correlated to the impairments of ADP-stimulated respiration and mitochondrial integrity (Fig. 1B).

We then assessed the ETAs of 60 min postischemic heart. A significant decrease in the complex I activity (72.3 ± 3.3% remained; n = 5; P < 0.05) and complex III activity (80.9 ± 2.0% remained; n = 5; P < 0.05) was observed, suggesting the involvement of postischemic injury. However, we did not detect a significant impairment of absolute ETAs in complex II, complex IV (n = 5, not statistically significant), and the concentrations of heme b and aa₃.

Protein expression in mitochondria was probed by Western blot with equal amounts (120 μg) of protein loading, and the SOD-2 was used as housekeeping protein or protein loading control. As shown in the Fig. 2, a significant reduction of ETC protein expression was detected in the mitochondria isolated from ischemic heart, but no reduction was observed in those isolated from the postischemic heart. Based on the densitometrical analysis of blots using ImageJ Software, protein expression in ischemic heart was decreased by 47.4 ± 7.8% for complex I (probed with Ab against a 51-kDa subunit; n = 5; P < 0.05), by 33.7 ± 8.9% for complex II (probed with Ab against a 70-kDa subunit; n = 5; P < 0.05), by 34.8 ± 7.1% for complex III (probed with Ab against RISP; n = 5; P < 0.05), by 23.1 ± 3.3% for complex IV (probed with Ab against subunit I; n = 5; P < 0.05). Additional subunits of ETC proteins were probed by Western blotting (indicated by Fig. 2), including 75-kDa subunit of complex I (decreasing by 15.6 ± 1.6%; n = 5; P < 0.05), ND1 subunit of complex I (decreasing by 37.2 ± 2.2%; n = 5; P < 0.05), iron sulfur protein of complex II (decreasing by 18.3 ± 3.6%; n = 5; P < 0.05), and subunit Vb of complex IV (decreasing by 24.5 ± 4.5%; n = 5; P < 0.05). These data suggested ischemic degradation of mitochondrial ETC and marginal elevation of ETC biosynthesis during reperfusion following ischemia.

The mRNA level of ETC in the myocardium was measured by real-time PCR using 18S rRNA as a loading control. As shown in the Fig. 3, it was decreased by 18–29% (n = 5; P < 0.05) after 30 min of ischemia, likely due to a global increase of nuclease activity induced by ischemia. There was no signif-
Ultrastructural evidence of decreased mitochondrial ETC in the ischemic hearts. The ultrastructure of normal or control myocardium from LV muscle is shown in Fig. 4, A and B. The mitochondria in normal or control myocytes were characterized by having electron dense matrices and tightly packed cristae, i.e., high density of cristae in the mitochondria (34).

Ultrastructural changes were occurred during progressive ischemia in the myocardium. After 30 min of ischemia, myofibrils were loosely packed, indicating severe swelling (34). Virtually all mitochondria were swollen (Fig. 4C), matrices were electron lucent, and cristae were degraded, severely disorganized, and dramatically decreased in density. The number of mitochondria in myocardium was not found appreciably reduced. This result was supported from the results from immunoblotting analysis of mitochondria isolated from ischemic heart (Fig. 2), confirming a dynamic process of ischemic degradation and subsequent reduction of ETC protein in the mitochondria.

The ultrastructure of myocardium after 60 min of postischemic reperfusion indicated increasing density of cristae in mitochondria, suggesting reduction of mitochondrial swelling. Electron dense matrices and highly packed cristae in most mitochondria were reverted. However, most mitochondria exhibited morphological changes with irregular shapes although they were clearly marginalized (Fig. 4D).

Morphometric analysis indicated mitochondrial sizes from control myocardium and 1 h postischemic heart are 3.7 ± 0.2 μm (n = 25) and 4.9 ± 0.1 μm (n = 25; P < 0.001 vs. control) indicated in the Fig. 4E. Owing to severe swelling, rupture, and vague border of mitochondria from ischemic myocardium, calculation of the mitochondrial size is not feasible. Mitochondrial fractional area was calculated and computed as the percentage of total myocardial fiber area, indicating 43.6 ± 2.8% for control myocardium, 69.9 ± 1.4% for ischemic heart, and 53.6 ± 6.7% for 1 h postischemic heart (Fig. 4F).

Involvement of peroxisome proliferator-activated receptor-γ coactivator-1α in the postischemic biosynthesis of the mitochondrial ETC. Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) is a coactivator of the PPARγ (40). PGC-1α functions to coactivate several downstream transcriptional factors and plays a central integrative role in mitochondrial biogenesis (48). To provide further evidence regarding the role of PGC-1α in the ischemic deficiency and postischemic biosynthesis of the mitochondrial ETC, we analyzed the protein expression level of PGC-1α in myocardium by immunoblotting, and GAPDH was used as a protein loading control. We observed a dramatic decrease (by 46.9 ± 6.1%; P < 0.05; n = 6) in cytosolic PGC-1α expression after 30 min ischemia and a progressive increase during reperfusion at 15, 30, and 60 min (Fig. 5A). A significant change of PGC-1α expression due to myocardial ischemia and reperfusion was not observed in the nuclear fraction (Fig. 5A). Analysis of total PGC-1α expression combining the cytosolic and nuclear fractions indicated a 28.0 ± 3.3% (n = 6; P < 0.05) reduction after 30-min ischemia (Fig. 5B). Therefore, ETC protein expression in mitochondria was correlated with PGC-1α expression in the cytosol during ischemia and reperfusion.

The protein expression of cytosolic PGC-1α from preconditioned heart (2 episodes of 5-min ischemia separated by one 10-min and two 5-min reperfusion) was found significantly increased by 49.2 ± 8.8% (n = 3; P < 0.05) while compared

Fig. 3. Primer sequences (forward/reverse) for real-time PCR were chosen from the Genbank mRNA sequence of rat using Primer Express software (Applied Biosystems, Foster City, CA) were 51 kDa of complex I: 5'-GAAGACTT-CATTGGCTGTCG-3'/5'-GCAGCATCTTTTGCCTTG-3'; 70 kDa of complex II (structural gene, SdhA): 5'-AGCCGCAAGTACATGG TG-3'/5'-CGACTCTTCCTCCAGATGGTC-3'; cytochrome b of complex III: 5'- CCTATATTATGCTATCCCCAT-3'/5'-GTTATGATCTTGTTTGCTGG-3'; RISP of complex III: 5'-GTGAAGACCCCTTCTTGTTG-3'/5'-CGGCCACCTTGAGTTCGTTG-3'; COX I of complex IV-AGTATTCGCAATCATCATACGGCTGCT-3'/5'-GCTTTTGCTCATGTGTCATTTAGG-3'; COX II of complex IV-GCTTTTGCTCATGTGTCATTTAGG-3'/5'-GCTGGAATTACCGCGTGCT-3'; COX III of complex IV-CoX N-AGTATTCGCAATCATCATACGGCTGCT-3'/5'-GCTTTTGCTCATGTGTCATTTAGG-3'; COX IV of complex IV-GCTTTTGCTCATGTGTCATTTAGG-3'/5'-GCTGGAATTACCGCGTGCT-3'; COX V of complex IV-GCTTTTGCTCATGTGTCATTTAGG-3'/5'-GCTGGAATTACCGCGTGCT-3'.
with the level of ischemic heart (Fig. 5C), thus confirming the role of PGC-1α in cardioprotection (33). We further detected precondition-mediated upregulation of ETC activities and protein expression as shown in Fig. 5D and E.

**Inhibition of postischemic protein translation of mitochondrial ETC with cycloheximide.** Cycloheximide is an inhibitor of protein biosynthesis in eukaryotic organisms. Cycloheximide exerts its effect by interfering with the translocation step in protein synthesis, thus blocking translational elongation. To test whether translational control was involved, we treated rat heart with cycloheximide (1 mM) before 30 min of reperfusion. Tissue homogenates of cycloheximide-treated hearts were subjected to analysis. The protein expression (Fig. 6A) and ETA (Fig. 6B) in cycloheximide-treated heart were depressed to the level of ischemic heart. The expression of ETC proteins after cycloheximide treatment was decreased to 73.3 ± 6.3% for complex I (P < 0.05; n = 5), 75.7 ± 5.1% for complex II (P < 0.05; n = 5), 75.0 ± 6.0% for complex III (P < 0.05; n = 5), and 71.1 ± 6.5% for complex IV (P < 0.05; n = 5; Fig. 6A). The activities of ETC after cycloheximide treatment were decreased to 74.1 ± 5.2% for complex I (P < 0.05; n = 5), 78.7 ± 6.6 for complex II (P < 0.05; n = 5), 73.6 ± 5.9% for complex III (P < 0.05; n = 5), and 75.2 ± 7.8% for complex IV (P < 0.05, n = 5; Fig. 6B). Furthermore, we have detected that cycloheximide significantly inhibited reperfusion-mediated functional recoveries of LVDP (28.3 ± 5.9 vs. 9.0 ± 0.8%), RPP (23.9 ± 4.3 vs. 7.1 ± 1.1%), and RCR (2.7 ± 0.3 vs. 1.9 ± 0.5) as indicated in the Fig. 6C and D, and Table 1. These results thus supported involvement of translational control in the rapid biosynthesis of mitochondrial ETC proteins during reperfusion. Note that cycloheximide (1 mM) does not affect the respiration and the ETA of isolated mitochondria. Also, the ETA and ETC protein expression in mitochondria was not significantly affected in the normal heart perfused with Kreb buffer containing cycloheximide (data not shown).

**Mitochondrial NCR activity in the ischemic and postischemic hearts.** NCR is the supercomplex hosting complex I and complex III and mediates electron transport from NADH...
to ferricytochrome c. The NCR activity in the ischemic and postischemic hearts was decreased by 53.1 ± 4.8 and 29.1 ± 3.0% when assayed by NADH oxidation in the presence of ferricytochrome c (Fig. 7A). The NCR activity also can be assayed by measuring ferricytochrome c reduction (absorbance increase at 550 nm) in the presence of NADH; a decrease of 42.0 ± 8.6 and 13.0 ± 3.7% was observed (Fig. 7A). These data further supported that injury of ETC during reperfusion was specifically occurred in complex I and complex III (Fig. 1C). Presumably complex I and complex III are the major source of $O_2^{•−}$ production and sensitive to the oxidant stress induced by postischemic reperfusion. To provide direct evidence in supporting of complex I/complex III-mediated $O_2^{•−}$ generation, supercomplex of NCR was isolated from the mitochondria of bovine heart (22). Mediation of $O_2^{•−}$ by the complex I component from NCR supercomplex under the conditions of enzyme turnover was assessed with EPR spin trapping with DEPMPO (Fig. 7B). The detected DEPMPO/•OOH adduct could be inhibited by SOD (Fig. 7C). No detectable DEPMPO/•OOH adduct was mediated by the NCR or the complex III component from NCR in the presence of NADH and cyt c (Fig. 7D) or ubiquinol-2 and cyt c (Fig. 7E), suggesting that $O_2^{•−}$ mediated by NCR or its complex III component was minimized in the supercomplex under the minimized conditions of enzyme turnover. In contrast, when the complex III was isolated or detached from NCR supercomplex, significant $O_2^{•−}$ production can be mediated by complex III under the conditions of enzyme turnover (Fig. 7F).

**DISCUSSION**

**Main findings.** The main findings of this study elucidate biphasic change in the protein level of mitochondria during ischemia and reperfusion, which appears to be correlated to dynamic biosynthesis of PGC-1α and regulated by gene transcription and translational control. Furthermore, we have characterized the complex I of ischemic/postischemic hearts at the levels of Fp subcomplex and NCR supercomplex (Fig. 7).

**Ischemic defects of mitochondrial ETC.** This study has demonstrated that a biphasic process occurred in the protein biosynthesis and enzymatic activity of the mitochondrial ETC during the transition from ischemia to reperfusion. The transition was likely due to the physiological alteration in which $P_{O_2}$ in the myocardium was overshooting upon flow restoration (50). We detected a marginal reduction of ETC protein expression in the mitochondria of ischemic heart, which should contribute to the ischemic defect. Ischemic defects in the respiration and ETC activity have been reported in the isolated heart mitochondria (12). It has been suggested that oxidative damage caused by ROS production has been attributed to ischemic defects due to higher ability to generate $H_2O_2$ by the mitochondria isolated from ischemic heart, which subsequently leads to the injury of ETC.

Two major molecular mechanisms are proposed to explain ischemia-induced downregulation of mitochondrial ETC protein. The first mechanism is likely caused by a downregulation of the mRNA level in the ischemic heart ($−20−30$% downregulation compared with the normal control; Fig. 3). The second mechanism is related to reduction in the transcriptional factor PGC-1α in the ischemic heart (Fig. 5). PGC-1α deficiency in the ischemic hearts was presumably due to protein degradation triggered by low $P_{O_2}$, and then it downregulated ETC biosynthesis, augmenting the ischemic defect and impairment of the ETC activity. The additional mechanism involved was most likely regulated by the myocardial proteasome or increasing autophagy during ischemia. Powell et al. (38) has reported that myocardial 20S-proteasome activity was modestly enhanced during the early phase of global ischemia, which would actively remove ETC proteins during ischemia. Furthermore, in response to the physiological conditions of $O_2$, and ATP depletion, autophagy can be induced to remove redundant ETC proteins during myocardial ischemia (32). Although ischemia-induced autophagy is driven by AMPK activation (32), which seems to contradict PGC-1α downregulation, AMPK activation mediated by ischemia likely sets a tone for the following upregulation of PGC-1α during early phase of reperfusion.

**Biosynthesis of ETC during reperfusion.** Noteworthy up-regulation (vs. ischemic defect) of mitochondrial ETC biosynthesis and enzymatic activity was detected during reperfusion. Discordance was observed between recovery of ETA and protein levels vs. poor recovery of cardiac contractile function. However, recovery of respiratory control index (or RCR in the Fig. 1A) was measured $−35$%, which is close to recovery of contractility. Therefore, modest injury of complexes I, II, and III during reperfusion should impair the protein-protein interaction of ETC (Fig. 1B), leading to poor recovery of RCR and contractility. The mechanisms contributing to reperfusion-induced ETC biosynthesis are discussed below:

1) We suggest that ETC protein expression is triggered in response to $P_{O_2}$ elevation, and the molecular mechanism that controls ETC biosynthesis likely serves as a sensor of $P_{O_2}$. Upregulation of cytosol PGC-1α and its mRNA level was consistently detected in the postischemic heart (Fig. 5A) and correlated with measures of ETC biosynthesis during reperfusion (Fig. 2). The above mechanism is further supported by the evidence showing that PGC-1 gene was activated during reperfusion.

---

**Fig. 5.** Cardiac peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) expression in the ischemic and postischemic hearts ($n = 6$). Myocardial tissues were homogenized in 3 mM HEPES, 250 mM sucrose, 0.1% Triton X-100, and 2 mM DTT. Supernatant (cytosolic fraction) of tissue homogenate was collected by centrifugation at 8,000 g for 20 min. Pellet was resuspended in 25 mM Tris–Cl pH 7.6 containing 150 mM NaCl, 1% NP-40, 1% deoxycholate, 0.1% SDS, 2 mM EDTA, 5 mM DTT, and protease/phosphatase inhibitors cocktail (1×). Supernatant was collected by centrifugation at 16,000 g for 5 min (nuclear fraction) and subjected to analysis of PGC-1α expression in the nuclear fraction. A: cytosolic PGC-1α of tissue homogenates (190 μg) and nuclear PGC-1α of pellets (190 μg) were immunoblotted with a polyclonal antibody against PGC-1α. Protein expression level of GAPDH in tissue homogenates was used as a loading control. B: immunoblotting analysis using the sample mixture combined from cytosol and nuclear fractions. *P < 0.05 vs. baseline control, IR-15 min, IR-30 min, IR-1 h. §P < 0.05 vs. baseline control. C: immunoblotting analysis using the cytosolic fractions from ischemic heart (30-min I) and ischemic heart (30-min I) after precondition (IPC) using 2 episodes of 5-min ischemia separated by one 10-min and two 5-min reperfusion. Tissue homogenates were prepared for biochemical analysis after 30-min ischemia. D: same as C, except that enzymatic activities of mitochondrial ETC were measured. E: same as C, except that protein expression of ETC was probed with immunoblotting analysis.
Fig. 6. Cycloheximide (CHX) partially blocks reperfusion-induced ETC protein increase \((n = 5)\). Hearts were subjected to a 5-min baseline period under constant perfusion pressure. Hearts were randomly assigned to 1 of 2 study groups including 1 control and 1 CHX treatment group. Immediately 15 min before global ischemia, with the perfusion rate set at \(0.25 \text{ ml/min}\), these groups received oxygenated Krebs buffer only (control group) or Krebs buffer containing CHX (1 mM). After 30 min of global ischemia, hearts were reperfused with oxygenated Krebs buffer (control) or Krebs buffer with CHX (1 mM) for additional 30 min. Tissue homogenates of left ventricles were subjected to immunoblotting analysis (A), ETA (B) including complexes I-IV, and functional recovery of left ventricular developed pressure and rate pressure product (C) and RCR (D). #P < 0.01 vs. IR-30 min control, *P < 0.05 vs. IR-30 min control. Note, 100% refers to the basal level or basal activity of IR-30 min control. E: cardiac eIF2α (eukaryotic initiation factor) expression in the ischemic and postischemic hearts \((n = 3)\). Cytosolic fraction of tissue homogenates was immunoprecipitated with the polyclonal Ab against eIF2α, and then probed with the Ab against phosphorylated (Ser51) eIF2α. Note that blotting of control sample (lane 1 from left) was inserted due to a noncontiguous sample loading from ischemia and IR samples (lanes 2–4 from left).

Erusion via marginal increase of its mRNA level by \(~25.7\%\) (data not shown). Cardiac PGC-1α functions to coactivate several downstream transcriptional factors thus likely plays the role to coordinate rapid protein translation of ETC during reperfusion. It should be noted that experiments here were performed in crystalloid-perfused hearts, which are exposed to oxygen tension much higher than in vivo. Thus it is conceivable that the crucial role of oxygen tension seen in this study under review may be less important under different conditions.

McLeod et al. (33) have previously noted a significant elevation of PGC-1α gene transcription induced in the preconditioned myocardium (vs. ischemia/reperfusion myocardium). In agreement of this result, PGC-1α protein expression from preconditioned heart significantly enhanced after ischemia (Fig. 5), thus supporting the role of PGC-1α in augmented resistance to ischemic injury.

2) Modest suppression of ETC translation and enzymatic activities by cycloheximide (CHX in Fig. 6) has significance since this observation implicated the involvement of translational control. The impact of translational control was detected on both nuclear \((51 \text{ kDa}, 70 \text{ kDa}, \text{RISP})\) and mitochondria (the subunit of complex IV)-encoded subunits of ETC (Fig. 6A). The magnitude of cycloheximide effect to inhibit protein biosynthesis was well correlated to the level of ETC recovery upon reperfusion (Fig. 2), but actinomycin D only executed a minor effect. Further evidence was provided by detecting the status of eIF2α phosphorylation showing marked upregulation during ischemia and modest downregulation during perfusion (Fig. 6E). However, a significant change of eIF4E phosphorylation was not observed, which was in agreement with the reports in the literatures (17, 18). It is likely that mitochondrial ribosome was similarly regulated to affect the translation of mitochondria and nuclear encoded subunits in concert.

Interestingly, real-time PCR analyses failed to show an increase of ETC mRNA level during reperfusion when compared with the mRNA level of ischemic heart (Fig. 3). Thus a lack of correlation was identified between ETC mRNA and protein levels. The findings supported that protein biosynthesis can occur within a short experimental duration (within 60 min) via translational control via internal ribosomal entry site (IRES) during reperfusion. Recent studies by Holcik and Sonenberg (24) indicate that some genes containing an IRES in the 5′-untranslated region of mRNA can escape the general translational control and undergo protein translation induced by special physiological conditions (24). These special physiological conditions include oxidant stress and apoptosis (24, 41). Specific protein translation mediated by IRES depends on specific physiological conditions. A well-documented example of IRES-mediated translational control is stress-induced Nrf-2 activation via enhancing internal initiation (29, 41). Therefore, rapid ETC translation may be mediated by a similar mecha-
nism under the conditions of reperfusion-induced oxidant stress.

In addition, the role of myocardial proteosome in the postischemic injury is worth noting. Significant impairment of 20S/26S-proteosomal activities has been detected in the 60 min of postischemic rat heart (38). Bulteau et al. (6) have reported a similar result in an in vivo rat heart model of coronary occlusion/reperfusion. Therefore, diminished activity for protein degradation can facilitate the accumulation of excess ETC proteins and dysfunctional mitochondria in the postischemic heart.

Mitochondrial complex I in the ischemic and postischemic hearts. Mitochondrial dysfunction following ischemia is mainly associated with impaired ETA activities. More severe impairment of ETA in mitochondria was observed in complex I in which 48% of intact complex I activity was reduced with 24% loss of NFR (Fp) activity after 30 min of ischemia. In addition, immunoblotting analysis indicated a 32% downregulation of the 51-kDa (FMN-binding subunit of complex I) expression in mitochondria (Fig. 2), matching the impairment of NFR activity. Since the injury of Fp represented by NFR activity accounts for 50% of the decline of intact complex I activity, there must be other factors contributing to the reduction of complex I activity during ischemia. These factors may include the loss of FMN (43), tissue acidosis and ATP depletion (42), ROS damage (36), and conformational change from active form to a ‘de-active’ form (31). It was reported that Fp can be dissociated from intact complex I in vitro under an acidic environment (14). Therefore, ischemic acidosis might facilitate Fp dissociation, which, in turn, hinders electron transfer and impairs complex I activity. Damage to the iron sulfur protein subcomplex (Ip) and/or hydrophobic protein subcomplex (Hp) could be another factor associated with the decline of intact complex I activity during ischemia. In the postischemic heart, 28% of intact complex I activity was decreased with no significant loss of NFR activity (Fig. 1D), suggesting the restoration of the Fp function. The functional recovery of Fp thus may play a role in the superoxide generation during reperfusion since a functional Fp is one of the sources for complex I-mediated $O_2^{•−}$. In vitro S-glutathionylation of complex I has been reported to marginally enhance its ETA (8). Thereby, elevation in posttranslational modification with $S$-glutathionylation of complex I in the postischemic heart may also contribute to the functional recovery of mitochondria during reperfusion (9). No significant change compared with the control heart in the protein expression of complex I (probing of 51-kDa, 75-kDa, and ND1 subunits) was detected after 60 min of reperfusion (Fig. 2). These results indicated the
occurrence of bioenergetic recovery due to reperfusion as seen in the parameters of hemodynamics and RCR (Fig. 1B). Furthermore, this physiological recovery was also supported by full or partial restoration of ETAs and protein expression in the complexes II, III, and IV (Fig. 2).

Mitochondrial complex III in the ischemic and postischemic hearts. Thirty-nine and nineteen percent of complex III activity was decreased in the ischemic and postischemic hearts, respectively. However, a 26% downregulation of RISP expression was detected in the mitochondria of ischemic heart, indicating involvement of other factors in the ischemic defect of complex III. Ischemic damage to complex III, including to the iron-sulfur protein, has been previously reported (28). There is no alteration in the amount of RISP present in the mitochondria of postischemic heart, implicating oxidative injury of complex III due to reperfusion. Lipid peroxidation of cardiolipin in vivo may contribute to the above complex III defect (37).

Oxygen free radical production in the postischemic heart. It was observed that reperfusion induced a marginal ETC biosynthesis that failed to trigger complete recovery of mitochondrial integrity based on analysis of the RCR (Fig. 1B) and transmission electron microscopy analysis of ultrastructure (Fig. 4). The detected mitochondrial defect was due to overproduction of oxygen free radicals during reperfusion, leading to oxidative damage of membrane integrity and impairment of ETC (complex I and complex III in this study). In support of the hypothesis of oxygen free radical-induced damage, preservation of cardiac function through SOD (52) or glutathione peroxidase mimetics (4, 25) during reperfusion has been established and well documented.

It should be acknowledged that most damage to the ETC occurs during ischemia, rather than reperfusion as previously reported by Hoppel and colleagues (10, 11, 27), the concept of which is substantiated in the present study. Despite increasing ETC activities caused by marginal ETC biosynthesis during reperfusion, relatively dramatic impairment in mitochondrial integrated respiration (state 3 O2 consumption in the Fig. 1A) persists. The present study substantiates the previous findings (12) in which dominant damage to ETC during ischemia sets the stage for an increase in ROS as a mechanism of persistent reperfusion injury. Equally viable based on the data in the current study is persistence of previous ischemic injury.

Ischemia/reperfusion-induced impairments of complex I and complex III in the isolated mitochondria were significant in this study (Figs. 1C and 7A). It has been documented that complexes I and III are the major sites for mitochondrial O2•− production (45, 46). In vitro analysis of NCR supercomplex with EPR spin-trapping indicated that complex I has much higher catalytic activity to generate O2•− (Fig. 7, B vs. E). No detectable O2•− adduct of DEPMPO can be mediated by the intact NCR or its complex III component. However, O2•− generation mediated by complex III was greatly enhanced once...
complex III was detached from the supercomplex (Figs. 7, E vs. F), presumably due to lack of protein-protein interaction between complex I and complex III. With the damage to mitochondrial integrity under high PO$_2$ oxygen consumption by the ETC was not effectively coupled with oxidative phosphorylation during reperfusion. This resulted in electron leakage and subsequent O$_2$$^{-•}$ production by the ETC. Complexes I likely contributed to major oxygen free radical production in the early phase of reperfusion. However, the injury of complex I during ischemia/reperfusion can impair its protein-protein interaction with complex III, thus potentially enhancing complex III-mediated O$_2$$^{-•}$ production and augmenting overall oxidant stress in mitochondria during ischemia/reperfusion.

Conclusion. The proposed diagram in Fig. 8 illustrates a biphasic modulation of ETC activity in mitochondria during myocardial ischemia/reperfusion. Physiological conditions of hypoxia during ischemia trigger an upregulation of the proteolytic degradation, downregulating mRNA level in myocardium and PGC-1α expression, and subsequently decreasing the amount of ETC in the mitochondria, which results in impairment of mitochondrial integrity (due to decreasing oxidative phosphorylation and RCR). Ischemia further facilitates autophagy that is driven by AMPK activation (32). Overshooting PO$_2$ upon reperfusion renders the cardiomyocytes under the conditions of hyperoxygenation, which triggers progressive upregulation of PGC-1α. Together with proteasome activity downregulated and translational control, increasing PGC-1α expression progressively enhances ETC biosynthesis in mitochondria during reperfusion. Failure to completely restore the mitochondrial integrity during reperfusion results in overproduction of O$_2$$^{-•}$ under the conditions of hyperoxygenation and low oxidative phosphorylation. This augments the oxidant stress in mitochondria and the consequent impairment of ETC activity. Specifically, reperfusion-induced injuries to complex I and complex III are detected in this study. The biological significance of this study was to elucidate the unique and dynamic properties of the mitochondrial ETC in response to the physiological changes of myocardial ischemia/reperfusion. Defining this molecular mechanism advances our understanding of the disease process of postischemic infarction.

REFERENCES