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Altered Proteome Biology of Cardiac Mitochondria Under Stress Conditions

Jun Zhang^{1,2}, David A. Liem^{1,2}, Michael Mueller⁴, Yueju Wang^{1,2}, Chenggong Zong^{1,2}, Ning Deng^{1,2}, Thomas M. Vondriska^{3,2}, Paavo Korge¹, Oliver Drews^{1,2}, W. Robb MacLellan^{2,1}, Henry Honda^{2,1}, James N. Weiss^{2,1}, Rolf Apweiler⁴, and Peipei Ping^{1,2}

¹Departments of Physiology, David Geffen School of Medicine at UCLA, Los Angeles, CA

²Medicine/Division of Cardiology, David Geffen School of Medicine at UCLA, Los Angeles, CA

³Anesthesiology/Division of Molecular Medicine, David Geffen School of Medicine at UCLA, Los Angeles, CA

⁴EMBL/EBI, Hinxton, UK

Abstract

Myocardial ischemia-reperfusion induces mitochondrial dysfunction and, depending upon the degree of injury, may lead to cardiac cell death. However, our ability to understand mitochondrial dysfunction has been hindered by an absence of molecular markers defining the various degrees of injury. To address this paucity of knowledge, we sought to characterize the impact of ischemic damage on mitochondrial proteome biology. We hypothesized that ischemic injury induces differential alterations in various mitochondrial sub-compartments, that these proteomic changes are specific to the severity of injury, and that they are important to subsequent cellular adaptations to myocardial ischemic injury. Accordingly, an *in vitro* model of cardiac mitochondria injury in mice was established to examine two stress conditions: *reversible injury* (induced by mild calcium overload) and *irreversible injury* (induced by hypotonic stimuli). Both forms of injury had a drastic impact on the proteome biology of cardiac mitochondria. Altered mitochondrial function was concomitant with significant protein loss/shedding from the injured organelles. In the setting of mild calcium overload, mitochondria retained functionality despite the release of numerous proteins, and the majority of mitochondria remained intact. In contrast, hypotonic stimuli caused severe damage to mitochondrial structure and function, induced increased oxidative modification of mitochondrial proteins, and brought about detrimental changes to the sub proteomes of the inner mitochondrial membrane and matrix. Using an established *in vivo* murine model of regional myocardial ischemic injury, we validated key observations made by the *in vitro* model. This pre-clinical investigation provides function and sub-organelle location information on a repertoire of cardiac mitochondrial proteins sensitive to ischemia reperfusion stress and highlights protein clusters potentially involved in mitochondrial dysfunction in the setting of ischemic injury.

Keywords

proteome biology; ischemia injury; cardiac mitochondria; reversible injury; irreversible injury

Correspondence: Peipei Ping, PhD, Departments of Physiology and Medicine/Cardiology, David Geffen School of Medicine at UCLA, Suite 1619 MRL Building, CVRL, Los Angeles, CA 90095, Tel: 310.267.5624; Fax: 310.267.5623; pping@mednet.ucla.edu.

Supporting Information: Supplemental Material providing additional details on methods, data, and discussion is provided via the Internet at <http://pubs.acs.org>.

Introduction

Myocardial Ischemia-reperfusion injury induces necrotic and apoptotic cell death. It is well recognized that mitochondrial perturbations contribute to cardiac dysfunction during injury (1-4), in particular calcium overload and generation of reactive oxygen species (ROS) (2, 4-6). However, the manner in which these factors impact the mitochondrial proteome during ischemic injury remains largely undefined.

Mitochondria are dynamic organelles whose abundance, morphology, and function are subject to regulation under physiological and pathological conditions (7-10). Several investigations have made key contributions to our understanding of mitochondrial subproteomes (11-17), linking subproteomic changes to ischemic injury. However, how ischemic injury modifies the mitochondrial proteome (including the identities of affected proteins, their sub-organelle location and biological functions), has not been defined. In particular, it is unknown whether ischemic injury triggers differential alterations of protein clusters in various sub-mitochondrial compartments, whether changes of mitochondrial subproteomes vary with the severity of injury, and whether these proteomic changes contribute to the subsequent cellular adaptive responses to myocardial ischemic injury. Using functionally validated cardiac mitochondria, our recent investigation (18) provided a comprehensive mitochondrial proteome atlas. On the basis of this mitochondrial protein dataset, we designed a pre-clinical study to carefully address the above questions.

Depending upon the degree of injury, ischemic factors may cause the prolonged opening of mitochondrial permeability transition pore, which is accompanied by the loss of membrane potential and swelling of mitochondria (3). During lethal injury, these changes may lead to cardiac cell death (myocardial infarction). The ability to recognize differences between various degrees of injury has been hindered by an absence of a clear blue print of the molecular targets (i.e. the proteins) indicative of these distinct types of injury. Accordingly, to define changes in mitochondrial proteome biology in the setting of ischemic injury, we established an *in vitro* model to study two conditions of mitochondrial injury: a condition of reversible injury (mild calcium overload) and a condition of irreversible injury (hypotonic insult).

The purpose of the present study was two-fold: first, we sought to define mitochondrial proteome biology during ischemic injury, whereby changes of proteins are characterized according to their sub-mitochondrial compartmentalization and their function in relation to the degree of mitochondrial injury; the second objective was to identify a candidate pool of mitochondrial proteins sensitive to ischemic injury, which serves as a proteomic basis for the discovery of novel protein markers indicative of myocardial ischemic injury.

Experimental Procedures

All procedures were performed in accordance with the Animal Research Committee guidelines at UCLA and the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health. An overview of our experimental procedures is presented in Figure 1.

1. Material

Anti-VDAC1 was purchased from EMD Biosciences (San Diego, CA). Anti-catalase, creatine kinase and frataxin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cytochrome c, prohibitin, moesin, metaxin-2, flotillin-1 and TIM44 were purchased from BD Pharmingen (San Diego, CA). Anti-peroxiredoxin5 was from Abcam (Cambridge, MA). Anti-TOM40 was purchased from BioVision (Mountain View, CA). N-

dodecyl -D-maltoside (DDM) was purchased from Avanti Polar Lipids, Inc (Alabaster, AL). OxyBlot protein oxidation detection kit was from Chemicon international (Temecula, CA). ECL was from GE Healthcare (Piscataway, NJ). EGTA-free Protease inhibitors cocktail was from Roche (Indianapolis, IN). HEPES, Percoll, cytochrome c oxidase assay kit, phosphate inhibitors cocktails and all other chemicals were purchased from Sigma-Aldrich (St Louis, MO).

2. Isolation and Functional Characterization of Cardiac Mitochondria

Cardiac mitochondria were isolated from mouse hearts by differential centrifugation according to a standard procedure in our group (18-20). Briefly, cardiac mitochondria were isolated from adult (8-10 week old, ICR strain) mouse hearts in isolation buffer (250mM sucrose, 1mM EGTA, 20mM HEPES, pH7.5) with protease inhibitors cocktail and two different phosphate inhibitors cocktails. For purification, the crude mitochondrial pellet was resuspended in 19% Percoll in isolation buffer, and slowly layered on two layers of 30% and 60% Percoll (v/v) (18). After centrifugation at 10,000g for 15min, mitochondria were collected between the 30% and 60% layers. Percoll purified mitochondria were washed (3×) prior subjecting them to further analyses. Mitochondrial intactness was measured by cytochrome c oxidase activity (18). All procedures were performed at 4°C. Mitochondrial O₂ consumption and membrane potential were measured as described (20). For morphological assessment, mitochondrial suspensions were fixed in 2% glutaraldehyde and 2% formaldehyde in isolation buffer at room temperature for 2 hours. The samples were fixed, sectioned, and stained, and examined by JEOL 100CX electron microscope at 80kV (18).

3. An *in Vitro* Model of Mitochondrial Injury

An *in vitro* model of mitochondrial injury was established using either mild calcium overload or hypotonic buffer. These two challenges engender distinct levels of injury to cardiac mitochondria as determined by their functional changes post injury. For calcium overload, freshly-isolated Percoll-purified mitochondria (800µg) were resuspended in isolation buffer (without EGTA) and incubated with CaCl₂ to final concentration 100µM for 5min at room temperature. For the hypotonic challenge, mitochondria were resuspended in 5mM Tris-HCl buffer (pH7.6) for 5 min at room temperature. The degrees of mitochondrial injury by these two treatments were carefully characterized using multiple parameters including the intactness of mitochondria, the release of cytochrome c, and the responses of mitochondria to calcium induced swelling (18, 19, 21). In the case of mild calcium injury, the majority of injured mitochondria retained their morphology and function (Figs. 2A-2C); the responses of mitochondria to calcium swelling could be prevented by cyclosporin A; this injury is defined as reversible injury (**RI**). In contrast, in the case of treatment by hypotonic buffer, a significant number of mitochondria were broken concomitant with release of cytochrome c (Figs. 2A-2C); in addition, the loss of function that could not be protected by cyclosporin A; this is defined as irreversible injury (**II**).

4. Experimental Strategies for Defining Proteomic Changes of Injured Mitochondria

A major challenge in characterizing ischemic injury-induced proteomic changes in cardiac mitochondria is the high abundance of specific proteins in mitochondria, which obscure the mass spectrometry-based detection of less abundant proteins in the same sample constituting the mitochondrial proteome; furthermore, it is even more difficult to detect changes in this pool of less abundant proteins. To circumvent this technical issue, we designed an experimental strategy to detect changes in the mitochondrial proteome from the proteins released into the milieu surrounding mitochondria, i.e., the changes of proteins in the extra-mitochondrial environment. In the control group, mitochondria were placed in a

physiological milieu (in its isotonic sucrose buffer, no injury). Thus, when mitochondria were placed in calcium buffer or hypotonic buffer, the proteins released from the mitochondria and were detected by the LC/MS/MS would be specific due to injury (a change of their surrounding milieu).

5. Liquid Chromatography and Tandem Mass Spectrometry Analyses

Protein samples, i.e., proteins released from the injured mitochondria, were collected from the *in vitro* model of mitochondrial injury. Briefly, the samples were subjected to centrifugation at 8,000g for 10min at 4°C and analyzed by one dimensional SDS/PAGE and LC/MS/MS. Mitochondria resuspended in isolation buffer without any treatment were used as control (18, 22). A total of three independent experimental runs were performed for both conditions (reversible injury by mild calcium overload, **RI**; and irreversible injury by hypotonic buffer, **II**). All three sets of protein samples were subjected to mass spectrometry analyses.

All MS/MS spectra were searched against the IPI mouse database (version 3.12) using the SEQUEST algorithm incorporated into the Bioworks software package (Thermo, Version 3.2) (23). Partially tryptic peptides meeting the following criteria were accepted: Xcorr 2.00 for charge state 1+; Xcorr was 4.3 with charge state 2+; Xcorr was 4.7 with charge state 3+, DeltaCN 0.1 and Rsp=1. For the protein level, the consensus score was >5.0 and all proteins were identified on the basis of 2 or more unique peptides (24). The searching parameter for the peptide tolerance is 2Da. Additionally, proteins with less than three peptide hits were manually evaluated to confirm the identification based on the following criteria: all the MS/MS spectra must have good quality fragment ions with acceptable signal to noise ratio (> 5); if more than one spectrum was assigned to a peptide (each with acceptable scores), then the spectrum with the highest score was used for manual analysis; the fragment ion series must consist of at least three consecutive y or b ions; and some of the intense fragment ions must correspond to either singly or doubly charged fragment ions, or their dehydrated or deammoniated counterpart ions.

6. Semi-Quantitative Analysis of Proteins Using Spectra Abundance Factors (SAF)

Relative quantitative analysis of mitochondrial proteins was performed using Spectra Abundance Factors (SAF) as described (25, 26). First, the stringent data filtering criteria detailed above were employed for all peptides/proteins assessed to ensure inclusion of only high quality spectra for quantitation. The overlapping proteins identified in the conditions of calcium challenge and of hypotonic injury were compared. Those proteins that were shared by two out of three independent runs in both experimental conditions were chosen to collect spectra counts. Peptide spectra counts were summed for the given protein and then divided by protein length (Spectral Abundance Factor, SAF), which was further normalized against the sum of all SAFs for a particular run. Statistical analyses were further applied.

7. Analyses of Post-translational Modifications

Oxidation modification of proteins was detected by immunoblotting using OxyBlot protein oxidation detection kit (Chemicon, Temecula, CA).

8. Murine Model of Regional Myocardial Ischemic Injury

Myocardial infarction was induced in the anesthetized mouse using an established protocol of regional ischemic injury (19, 27). Briefly, male ICR mice were subjected to a 30-min left anterior descending coronary artery occlusion followed by 4h of reperfusion. The region of the left ventricle rendered ischemic was visually identified during the surgery by myocardial cyanosis. The infarcted region was determined post-mortem by perfusion with a 1% solution

of 2,3,5-triphenyltetrazolium chloride in phosphate buffer (pH 7.4, 37°C). To delineate the risk region, the coronary artery was tied at the site of the previous occlusion, and the aortic root was perfused with a 1% solution of Evans blue dye. The risk zone and non-risk zone were collected as previously described. Animals underwent a sham protocol, i.e., subjected to open chest surgery without coronary occlusion, were used as control. The mitochondria and cytosol were separated by differential centrifugation (4,000g and 100,000g, respectively).

9. Statistical Analysis

For swelling assays and spectra analyses using SAF, data are reported as MEAN±SEM. Differences among the experimental groups were analyzed using one-way ANOVA. If the ANOVA showed an overall significance, *post hoc* contrasts were performed with Student *t* test (28).

Results

1. Examination of Mitochondria Function Under Stress Conditions

Figure 1 shows the schematic diagram of the experimental strategies: purified and functionally validated murine cardiac mitochondria were divided into three experimental groups: Group 1 served as the control group with no treatment; in Group 2, mitochondria were challenged with 100μM calcium (reversible injury); and in Group 3, mitochondria underwent the treatment of hypotonic buffer (irreversible injury). Mitochondria following reversible injury and irreversible injury were examined by assessment of their integrity, function, and morphology.

The integrity of mitochondria was assessed by cytochrome c oxidase activity (Fig. 2A). Compared with control group (> 95% of the mitochondria remained intact), after reversible injury, the majority of mitochondria remained intact (>85%) with excellent function (Figs. 2B and 2C). In contrast, the irreversible injury protocol caused more than 80% of mitochondria to burst (Fig. 2A), concomitant with significant alterations in function (Figs. 2B and 2C). As expected, greater cytochrome c release was induced during irreversible injury (Fig. 2B).

The function of mitochondria was evaluated using swelling assay; compared with the control group, mitochondria from the reversible injury group underwent moderate swelling that was prevented by the administration of cyclosporine A, indicating that these mitochondria retained excellent function; cyclosporine A by itself had no effect on mitochondrial swelling in the control group. In contrast, the irreversible injury group developed significant mitochondrial swelling that was not rescued by cyclosporine A, indicating that these mitochondria were severely damaged (Fig. 2C).

Furthermore, we evaluated mitochondrial morphology by electron microscopy (Fig. 2D). The left panel shows that control group was homogeneous with intact membranes and orthodox cristae structure. The middle panel shows a similar picture of mitochondrial morphology during reversible injury, with few ruptured mitochondria and only moderately perturbed cristae structure. In contrast, the right panel shows a rupture of the majority of mitochondria with a marked absence of intact structure post irreversible injury.

Finally, because mitochondria are a major source of cellular ROS generated by electron transport reactions (29), we also investigated oxidation modifications of mitochondrial proteins (by derivatizing the modified carbonyl groups to DNP-hydrazone and immunoblotting with anti-DNP). Compared with the reversible injury protocol, the irreversible injury protocol led to much stronger total DNP signals (Fig. 2E), suggesting that

irreversible injury (and the concomitant organelle killing effects) produced more ROS which subsequently oxidized more proteins. Among these, VDAC1 (an outer membrane protein) was chosen as an example to test the oxidative modification state. Supernatants (released proteins) from mitochondria having undergone either reversible injury or irreversible injury were subjected to VDAC1 immunoprecipitation, followed by DNP blotting. In agreement with the total profiles of oxidative modified proteins, irreversible injury induced much greater release of VDAC1 with an increased oxidative modification of VDAC1.

These evaluations of mitochondrial phenotypes provide functional basis for subsequent proteomic comparisons of altered mitochondrial protein repertoires under stress conditions.

2. Characterization of Mitochondrial Released Proteins Under Stress Conditions

Protein samples collected from all three experimental groups of cardiac mitochondria were subjected to an initial analysis using one dimensional SDS-PAGE (*Supplemental Material*, Fig. S2) with LC/MS/MS. This initial analysis shows that (i) the proteins released following the two types of injuries are different; (ii) more proteins are released with the irreversible injury protocol; and (iii) no detectable proteins are released in the control group without treatment.

For mitochondria underwent injury, LC/MS/MS analysis identified 230 proteins from the mitochondrial milieu of the reversible injury group and 363 proteins from the mitochondria of the irreversible injury group; among these two sets of proteins, 188 proteins were common to both conditions (*Supplemental Material*, Tables S1 and S2). The physicochemical proprieties of these proteins are shown in Figure S3 (*Supplemental Material*).

2.a. Function and Location of Identified Proteins—To gain biological insights into stress-modified mitochondrial proteomes, we annotated the spatial distributions and function of these released proteins using the Gene Ontology Annotation database (GOA) and Swiss-Prot protein knowledgebase (30).

The organellar locations of these proteins are classified into outer mitochondrial membrane (OMM), inter-membrane space (IMS), inner mitochondrial membrane (IMM), and mitochondria matrix (Fig. 3A). Those proteins for which the sub-organelle compartmentalization could not be determined were labeled “mitochondrion”. In addition, some identified proteins have unknown locations; some proteins were found to reside primarily in other cellular locations and were not previously annotated as mitochondrial proteins. The explanation for their presence in this organelle remains to be determined. During the irreversible injury protocol, a large number of proteins were lost from the inner mitochondrial membrane or the matrix (Fig. 3A and Table S4 in *Supplemental Material*), suggesting that irreversible injury disrupts the integrity of both mitochondrial membranes. In contrast, the reversible injury protocol induced release of fewer proteins from the inner membrane and matrix, whereas the number of identified proteins originating from the outer membrane and intermembrane space of mitochondria was similar following both insults.

Analysis of biological functions demonstrates that both type of injuries impacted multiple functional clusters (Fig. 3B and Table S5 in *Supplemental Material*). In particular, reversible injury was accompanied by loss of mitochondrial proteins in the functional clusters of apoptotic proteins, transporters, redox-related proteins, and proteases. The irreversible injury protocol resulted in changes in proteins involved in metabolism and oxidative phosphorylation (with most of the proteins residing in mitochondrial inner membrane and matrix).

Taking the function and localization data into consideration (Fig. 3C), this analysis presents a comprehensive picture of mitochondrial proteome biology during stress conditions. It shows that a large number of proteins classified as matrix and inner mitochondrial membrane (which may be involved in metabolism and electron transport,) were affected by irreversible injury, in agreement with the mitochondrial integrity data in Figure 2 which demonstrate rupture of both membranes following irreversible injury.

2.b. Differential Release of Proteins Under Two Stress Conditions—Using normalized spectral abundance factors (NSAF), we evaluated differential release of proteins following either the reversible or the irreversible injury protocols (Figs. 4A-4D and Table S6 in *Supplemental Material*), a list containing 83 proteins. Within a given run, spectrum counts for the same protein were divided by protein length (Spectral abundance factor, SAF), and then normalized against the sum of all SAFs from the same run. Each protein considered for this analysis had been identified in at least two of three independent experiments. The data indicate that proteins released during reversible injury (related to apoptosis/signal transduction, transport, and proteolysis) showed similar or higher abundance as compared to that found in irreversible injury. Because the absolute amount of proteins released during irreversible injury was much greater than reversible injury, this observation implies that this cluster of proteins may play a very important role in reversible injury. In addition, the release of superoxide dismutase (IPI00109109), an important antioxidant defense enzyme, occurred to a greater degree following irreversible injury, potentially supporting a greater role for ROS induced damage with irreversible injury. Similarly, isoforms of ANT, the major transporters residing in the inner membrane of mitochondria and catalyzing the exchange of ATP/ADP cross membrane, were found in greater abundance following irreversible injury, again supportive of the destruction of both inner and outer mitochondrial membranes. In contrast, another family of major transporters residing in the outer membrane of mitochondria, VDAC, showed similar release during both types of injury. Moreover, following irreversible injury, the released proteins were enriched in molecules residing in the mitochondrial inner membrane or matrix involving in metabolism and oxidative phosphorylation. These data suggested that the alternations of mitochondrial subproteome are dependent on the type and severity of the injury.

3. Target Validation of Released Proteins

The mitochondrial proteins lost into their surrounding milieu (i.e., cytosol) during different insults were further validated by two approaches. First, using isolated mitochondria (in vitro model), immunoblotting demonstrated changes of a number of key proteins were validated using isolated mitochondria (Fig. 5). Note that creatine kinase and SOD were released after both reversible injury and irreversible injury (Fig. 5, left panel), whereas moesin, TOM40, and frataxin appeared dramatically in reversible injury (Fig. 5, left panel). In contrast, the inner mitochondrial membrane proteins TIM44 and prohibitin, metaxin-2, fatty acid binding protein, and flotillin-1 were released to a much greater degree following irreversible injury (Fig. 5, right panel).

Finally, to determine whether our *in vitro* model of mitochondrial injury recapitulates what occurs *in vivo*, we performed target validation studies using an established murine model of regional myocardial ischemic injury. Following 30 min of ischemia and 4h of reperfusion (Fig. S5 in *Supplemental Material*), cardiac tissues from either the risk zone or the non-risk zone were collected (Fig. 6A); cytosol and mitochondria were separated by differential centrifugation. In this setting, proteins released from mitochondria *in vivo* were captured in the cytosolic fractions (the surrounding milieu of mitochondria). Immunoblotting (Fig. 6B) detected increased amount of peroxiredoxin 5, TIM44, and catalase in the cytosol of the risk zone from hearts that underwent regional ischemic injury as compared to sham operated

control, indicating these proteins were released from mitochondria during the severe ischemic injury in the intact animal model and confirming our observations from the isolated mitochondria setting.

Discussion

This investigation defined how mitochondrial injury impacts proteome biology of this organelle by systematically identifying proteins affected during the injury, by functional annotation of their sub-organellar compartmentalization, and by characterization of the protein functional clusters affected by the injuries. The data presented here reports a large repertoire of proteins sensitive to injury and detail their respective release from mitochondria following different degrees of injury. This study confirms the utility of previously established markers (e.g., cytochrome *c* and catalase) as well as offers supporting evidence of new proteins that maybe considered as novel markers indicative of mitochondrial injury.

1. Mitochondrial Proteome Biology during Reversible and Irreversible Injuries

1.a. The In Vitro Model of Mitochondrial Injury—Mitochondrial dysfunction is a key causative event in cardiac dysfunction that is characterized by loss of metabolic capacity (decrease of cellular oxygen concentration and ATP production) and increased production of toxic products (ROS and calcium) (31, 32). It is established that ischemic injury to the myocardium induces functional changes including mitochondrial permeability transition (MPT), a loss of mitochondrial membrane potential and disruption of the outer membrane that leads to cardiac cell death. The severity of injury also determines whether mitochondrial function is retained; in the case of a lethal injury, complete loss of mitochondrial function leads to cell death by necrosis and/or apoptosis (3, 4, 6, 29). Thus, a clear measure of mitochondrial dysfunction that discriminates degrees of injury will be essential to delineate the pathogenesis of myocardial infarction at the subcellular level. Accordingly, we defined mitochondrial injuries as “irreversible” (those that cause irreversible damage to mitochondria and cardiomyocytes) versus those that are “reversible” (regulation of mitochondrial volume and cell fate in response to transient sub-lethal stresses).

Examinations of mitochondrial integrity, function, and morphology, confirmed the feasibility of this *in vitro* model of mitochondrial injury. Our data showed that in the setting of calcium induced reversible injury, mitochondria retained functionality and the majority of mitochondria remained intact. In contrast, irreversible hypotonic injury caused severe damage in mitochondrial structure and function. These results validated our experimental model for further proteomic study and provided the biological bases for proteomic comparisons of the proteins released under reversible and irreversible insults.

1.b. Defining Stress-Induced Alterations in Mitochondrial Proteome Biology—

Using the above models we characterized changes in mitochondrial proteome biology during injury. This is achieved by identifying mitochondrial proteins lost during the injury and by defining their sub-organellar location, the relevant mitochondrial functions in which they participate, and their relative importance during the two degrees of injury.

Both types of injury significantly modified mitochondrial proteome, affecting mitochondrial proteins of 11 different functional clusters, including apoptosis/signal transduction, metabolism and oxidative phosphorylation. Furthermore, our data suggests that the cardiac injury triggers differential alterations of protein clusters in various sub-mitochondrial compartments. Among the results, the more interesting findings are the pool of proteins identified unique to either reversible injury or to irreversible injury; the latter were highly enriched in proteins involved in metabolism and oxidative phosphorylation (most of the

proteins residing in mitochondrial inner membrane and matrix). However, the majority of the unique proteins identified following reversible injury were low abundance and regulatory proteins, such as apoptotic proteins, transporters and proteases.

Among the pool of proteins affected by both types of injuries, NSAF analyses provided an indication of the relative abundances. These analyses suggest that mitochondrial proteins which were involved in apoptosis/signal transduction or proteolysis showed similar or higher abundance following reversible, as compared to following irreversible, injury. However, the absolute amount of proteins released after irreversible injury was much greater, implying that this cluster of proteins may play a very important role in reversible injury. For example, twice the amount of adenylate kinase isoenzyme 2 (IPI00269076), which is a small ubiquitous enzyme essential for maintenance and cell growth residing in the intermembrane space of mitochondria, was released following reversible injury compared with irreversible injury (Fig. 4A). Programmed cell death protein 8 (also called apoptosis-inducing factor 1, IPI00129577), which translocates to the nucleus upon induction of apoptosis and induces nuclear chromatin condensation and large scale DNA fragmentation (33), was released to a similar level following both reversible and irreversible injuries. In contrast, a greater amount of proteins involved in metabolic and oxidative phosphorylation was released following irreversible injury; e.g. the release of mitochondria matrix protein dihydrolipoyl dehydrogenase (IPI00115569), one of the subunits of pyruvate dehydrogenase (PDH) complex, into cytosol from mitochondria was 3 times more during II compared with RI. To this end, several proteins involved in transport were affected differently during the two degrees of injuries (Fig. 4A), e.g. ANT1, indicating that protein transport systems may be disrupted at different levels during different injuries. In addition, mitochondrial chaperone heat shock protein 60, a protein involved in protein folding was significantly affected in the irreversible injury. Proteins released from mitochondria into cytosol during injury may serve as trafficking signals bridging biological processes between different compartments (the cytosol versus mitochondria). For example, both cytochrome c and apoptosis-inducing factor 1, two key regulators of apoptotic pathways (17, 34, 35), were released after both types of injury. The functional impacts of these proteins in these settings afford exciting opportunities for future investigations.

2. Role of Oxidation Modifications during Reversible and Irreversible Injury

ROS are generated as a byproduct of mitochondrial oxidative phosphorylation and, when in excess, can modify cellular components (including proteins) to elicit damage. A slight increase of ROS may serve as a second messenger and activate regulatory pathways; however, excessive ROS generation may have deleterious effects and lead to cell death. Our data showed that proteins released from mitochondria during either reversible or irreversible injury had oxidative modifications, consistent with previous reports that many mitochondrial proteins are the targets of ROS. For example, several subunits of mitochondrial respiratory chains have been shown to be regulated by ROS and exhibit diminished activity (2, 36). As one example of ROS-induced damage leading to altered mitochondrial biology, Kroemer's group reported that oxidation of a thiol residue of the protein ANT induces permeability transition pore opening and cell death (37). VDAC, a main entrance for metabolites across the outer membrane mitochondrial, has been reported not only to be a target of ROS, but also to serve as a channel to control the release of the ROS from mitochondria to cytosol (38, 39).

3. Target Validation by an *In Vivo* Model of Regional Myocardial Ischemic Injury

To evaluate our *in vitro* model of mitochondrial injury, we used a well-established murine model of regional ischemia to induce myocardial infarction in mice (27; also see Fig. S5 in *Supplemental Material*). This model closely reproduces physiological and pathological

changes of myocardial infarction in human (19, 27). Corroborating the findings from the *in vitro* model of mitochondrial injury, our data demonstrate increased protein expression of peroxiredoxin 5, TIM44, and catalase in the cytosol of risk zone following ischemic injury, confirming that these proteins were released from mitochondria during injury.

One important finding of this investigation is the evidence presented regarding molecular markers of mitochondrial injury (Fig. 5, and Tables S1 & S2 in *Supplemental Material*). These molecular markers include peroxiredoxin 5, TIM44, moesin, cytochrome *c*, and catalase; they are either unique to reversible injury, irreversible injury, or showed a graded response to both types of injuries. Some of these proteins (e.g., cytochrome *c*, catalase) are well documented to be released during ischemic injury; others are novel markers not previously characterized. The later group includes peroxiredoxin and TIM 44. Both of these proteins contribute to the preservation of mitochondrial function and integrity, known to be critical for cell survival following ischemia/reperfusion. Studies from Boengler's group (42) indicated that the protein expression level of TOM20, one of the subunits of TOM/TIM transporter complexes, was reduced in the injured cardiac mitochondria in mammals. Furthermore, they reported that the mitochondrial TOM20 content was preserved in ischemic preconditioned hearts. Their findings suggested that the release of the mitochondrial TOM/TIM complex proteins were good indicators of mitochondrial dysfunction after ischemic injury and that preconditioning protected the myocardial dysfunction, which was concomitant with a restored expression of this protein marker. Peroxiredoxins are antioxidants that play an important role in redox regulation underlying ischemic injury. Nagy's study (43) showed that mice null for isoform 6 of peroxiredoxins were more susceptible to ischemic injury; here we present the first data linking peroxiredoxins 5 with this same form of myocardial injury.

In addition to the above postulated role as markers indicative of stress conditions, many other functional roles of the mitochondrial releasing proteins remain to be elucidated. There exist possibilities how these proteins may function in response to myocardial stress. Several hypothesized mechanisms would include, but not limited to, the following (Fig. S6 in *Supplemental Material*): (i) some of these mitochondrial released proteins may serve to transduce mitochondrial stress signals to other organelles, or to mediate mitochondrial interactions with other stress-sensing organelles (e.g. ER); (ii) some of these mitochondrial released proteins may serve to transduce mitochondrial stress signals to their neighboring mitochondria and thus to coordinate or to synchronize mitochondrial function in the myocardium; (iii) some of these mitochondrial released proteins may function to direct signals to nucleus, promoting transcriptional based adaptation/remodeling during myocardial ischemic injury; and finally (iv) some of these mitochondrial released proteins may circulate to plasma and thus are candidates as biomarkers to indicate the level of myocardial ischemia injury.

In summary, the studies presented here are the first, to our knowledge, to examine responses of mitochondrial proteome during varied degrees of injury. The mitochondrial proteome is affected by loss of proteins into the surrounding milieu (cytosol) during organelle rupture and death (following irreversible injury); furthermore, the proteome is also substantially altered following a milder reversible injury. Given that ischemic damage to heart tissue involves these specific varied levels of mitochondrial injury, the blueprint of mitochondrial proteins affected by injuries provided in this study is an important first step in advancing our mechanistic understanding of cell death and in our search for novel biomarkers of injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Lesnefsky EJ, Moghaddas S, Tandler B, Kerner J, Hoppel CL. Mitochondrial dysfunction in cardiac disease ischemia-reperfusion aging and heart failure. *J Mol Cell Cardiol.* 2001; 33:1065–1089. [PubMed: 11444914]
2. Varadarajan SG, An J, Novalija E, Smart SC, Stowe DF. Changes in $[Na^{+}](i)$ compartmental $[Ca^{2+}]$ NADH with dysfunction after global ischemia in intact hearts. *Am J Physiol.* 2001; 280:H280–293.
3. Weiss JN, Korge P, Honda HM, Ping P. Role of the mitochondrial permeability transition in myocardial disease. *Circ Res.* 2003; 93:292–301. [PubMed: 12933700]
4. Honda HM, Korge P, Weiss JN. Mitochondria and ischemia/reperfusion injury. *Ann N Y Acad Sci.* 2005; 1047:248–258. [PubMed: 16093501]
5. Halestrap A, Calcium P. mitochondria and reperfusion injury: a pore way to die. *Biochemical Society Transactions.* 2006; 34:232–237. [PubMed: 16545083]
6. Griffiths EJ, Halestrap AP. Mitochondria non-specific pores remain closed during cardiac ischemia but open upon reperfusion. *Biochem J.* 1995; 307:93–98. [PubMed: 7717999]
7. Shaw JM, Nummari J. Mitochondrial dynamics division in budding yeast. *Trends Cell Biolo.* 2002; 12:178–184.
8. Sickmann A, Reinders J, Wagner Y, Joppich C, Zahedi R, Meyer HE, Schonfisch B, Perschil I, Chacinska A, Guiard B, Rehling P, Pfanner N, Meisinger C. The proteome of *Saccharomyces cerevisiae* mitochondria. *Proc Natl Acad Sci.* 2003; 100:13207–13212. [PubMed: 14576278]
9. Reinders J, Zahedi RP, Pfanner N, Meisinger C, Sickmann A. Toward the complete yeast mitochondrial proteome: multidimensional separation techniques for mitochondrial proteomics. *J Proteome Res.* 2006; 5:1543–1554. [PubMed: 16823961]
10. McDonald TG, Van Eyk JE. Mitochondrial proteomics. Undercover in the lipid bilayer. *Basic Res Cardiol.* 2003; 98:219–227. [PubMed: 12835951]
11. Arrell DK, Elliott ST, Kane LA, Guo Y, Ko YH, Pedersen PL, Robinson J, Murata M, Murphy AM, Marban E, Van Eyk JE. Proteomic analysis of pharmacological preconditioning: novel protein targets converge to mitochondrial metabolism pathways. *Circ Res.* 2006; 99:706–714. [PubMed: 16946135]
12. White MY, Cordwell SJ, McCarron HC, Prasan AM, Craft G, Hambly BD, Jeremy RW. Proteomics of ischemia/reperfusion injury in rabbit myocardium reveals alterations to proteins of essential functional systems. *Proteomics.* 2005; 5:1395–1410. [PubMed: 15800873]
13. Yan L, Ge H, Li H, Lieber SC, Natividad F, Resuello RR, Kim SJ, Akeju S, Sun A, Loo K, Peppas AP, Rossi F, Lewandowski ED, Thomas AP, Vatner SF, Vatner DE. Gender-specific proteomic alterations in glycolytic and mitochondrial pathways in aging monkey hearts. *J Mol Cell Cardiol.* 2004; 37:921–929. [PubMed: 15522269]
14. Hopper RK, Carroll S, Aponte AM, Johnson DT, French S, Shen RF, Witzmann FA, Harris RA, Balaban RS. Mitochondrial matrix phosphoproteome: effect of extra mitochondrial calcium. *Biochemistry.* 2006; 45:2524–2536. [PubMed: 16489745]
15. Van Loo G, Demol H, van Gurp M, Hoorelbeke B, Schotte P, Beyaert R, Zhivotovsky B, Gevaert K, Declercq W, Vandekerckhove J, Vandenabeele P. A matrix-assisted laser desorption/ionization post-source decay (MALDI-PSD) analysis of proteins released from isolated liver mitochondria treated with recombinant truncated Bid. *Cell Death Differ.* 2002; 9:301–308. [PubMed: 11859412]
16. Teilum M, Hansson MJ, Dainiak MB, Mansson R, Surve S, Elmer E, Onnerfjord P, Mattiasson G. Binding mitochondria to cryogel monoliths allows detection of proteins specifically released following permeability transition. *Anal Biochem.* 2006; 348:209–221. [PubMed: 16310157]

17. Kim N, Lee Y, Kim H, Joo H, Youm JB, Park W, Warda M, Cuong DV, Han J. Potential biomarkers for ischemic heart damage identified in mitochondrial proteins by comparative proteomics. *Proteomics*. 2006; 6:1237–1249. [PubMed: 16402359]
18. Zhang J, Li XH, Mueller M, Wang Y, Liem DA, Young J, Korge P, Weiss JN, Apweiler R, Ping P. Systematic characterization of the murine mitochondrial proteome using functionally validated cardiac mitochondria. *Proteomics*. (in press).
19. Wang G, Liem DA, Vondriska TM, Honda HM, Korge P, Pantaleon DM, Qiao X, Wang Y, Weiss JN, Ping P. Nitric oxide donors protect murine myocardium against infarction via modulation of mitochondrial permeability transition. *Am J Physiol*. 2005; 288:H1290–1295.
20. Korge P, Honda HM, Weiss JN. K⁺-dependent regulation of matrix volume improves mitochondrial function under conditions mimicking ischemia-reperfusion. *Am J Physiol*. 2005; 289:H66–77.
21. Baines CP, Song CX, Zheng YT, Wang GW, Zhang J, Wang OL, Guo Y, Bolli R, Cardwell EM, Ping P. Protein kinase Cepsilon interacts with and inhibits the permeability transition pore in cardiac mitochondria. *Circ Res*. 2003; 92:873–880. [PubMed: 12663490]
22. Edmondson RD, Vondriska TM, Biederman KJ, Zhang J, Jones RC, Zheng Y, Allen DL, Xiu JX, Cardwell EM, Pisano MR, Ping P. Protein kinase C epsilon signaling complexes include metabolism-transcription/translation-related proteins: complementary separation techniques with LC/MS/MS. *Mol Cell Proteomics*. 2002; 1:421–433. [PubMed: 12169683]
23. Eng JK, McCormack AL, Yates JR 3rd. An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J Am Soc Mass Spectrom*. 1994; 5:976–989.
24. Qian WJ, Liu T, Monroe ME, Strittmatter EF, Jacobs JM, Kangas LJ, Petritis K, Camp DG 2nd, Smith RD. Probability-based evaluation of peptide protein identifications from tandem mass spectrometry and SEQUEST analysis: the human proteome. *J Proteome Res*. 2005; 4:53–62. [PubMed: 15707357]
25. Florens L, Carozza MJ, Swanson SK, Fournier M, Coleman MK, Workman JL, Washburn MP. Analyzing chromatin remodeling complexes using shotgun proteomics and normalized spectral abundance factors. *Methods*. 2006; 40:303–311. [PubMed: 17101441]
26. Paoletti AC, Parmely TJ, Tomomori-Sato C, Sato S, Zhu D, Conaway RC, Conaway JW, Florens L, Washburn MP. Quantitative proteomic analysis of distinct mammalian Mediator complexes using normalized spectral abundance factors. *Proc Natl Acad Sci*. 2006; 103:18928–18933. [PubMed: 17138671]
27. Ping P, Song C, Zhang J, Guo Y, Cao X, Li RC, Wu W, Vondriska TM, Pass JM, Tang XL, Pierce WM, Bolli R. Formation of protein kinase C(epsilon)-Lck signaling modules confers cardioprotection. *J Clin Invest*. 2002; 109:499–507. [PubMed: 11854322]
28. Wallenstein S, Zucker CL, Fleiss JL. Some statistical methods useful in circulation research. *Circ Res*. 1998; 47:1–9. [PubMed: 7379260]
29. Le Bras M, Clement MV, Pervaiz S, Brenner C. Reactive oxygen species and the mitochondrial signaling pathway of cell death. *Histol Histopathol*. 2005; 20:205–219. [PubMed: 15578439]
30. Mueller M, Martens L, Reidegeld KA, Hamacher M, Stephan C, Bluggel M, Korting G, Chamrad D, Scheer C, Marcus K, Meyer HE, Apweiler R. Functional annotation of proteins identified in human brain during the HUPO brain proteome project pilot study. *Proteomics*. 2006; 6:5059–5075. [PubMed: 16912974]
31. Chen Q, Camara AK, Stowe DF, Hoppel CL, Lensmeyer EJ. Modulation of electron transport protects cardiac mitochondria and decreases myocardial injury during ischemia and reperfusion. *Am J Physiol*. 2007; 292:C137–147.
32. Kim J, Hem L, Lemasters JJ. Mitochondrial permeability transition: a common pathway to necrosis/apoptosis. *Biochem Biophys Res Commun*. 2003; 304:463–470. [PubMed: 12729580]
33. Jacotot E, Ferri KF, El Hamel C, Brenner C, Druillennec S, Hoebeke J, Rustin P, Metivier D, Lenoir C, Geuskens M, Vieira HL, Loeffler M, Belzacq AS, Briand JP, Zamzami N, Edelman L, Xie JH, Reed JC, Roques BP, Kroemer G. Control of mitochondrial membrane permeabilization by adenine nucleotide translocator interacting with HIV-1 viral protein R and Bcl-2. *J Exp Med*. 2001; 193:509–520. [PubMed: 11181702]

34. Munoz-Pinedo C, Guio-Carrion A, Goldstein JC, Fitzgerald P, Newmeyer DD, Green DR. Different mitochondrial intermembrane space proteins are released during apoptosis in a manner that is coordinately initiated but can vary in duration. *Proc Natl Acad Sci.* 2006; 103:11573–11578. [PubMed: 16864784]
35. Patterson SD, Spahr CS, Daugas E, Susin SA, Irinopoulou T, Koehler C, Kroemer G. Mass spectrometric identification of proteins released from mitochondria undergoing permeability transition. *Cell Death Differ.* 2000; 7:137–144. [PubMed: 10713728]
36. Sadek HA, Szweda PA, Szweda LI. Modulation of mitochondrial complex I activity by reversible Ca^{2+} NADH mediated superoxide anion dependent inhibition. *Biochemistry.* 2004; 43:8494–8502. [PubMed: 15222760]
37. Costantini P, Belzacq AS, Vieira HL, Larochette N, de Pablo MA, Zamzami N, Susin SA, Brenner C, Kroemer G. Oxidation of a critical thiol residue of the adenine nucleotide translocator enforces Bcl-2-independent permeability transition pore opening and apoptosis. *Oncogene.* 2000; 19:307–314. [PubMed: 10645010]
38. Han D, Antunes F, Canali R, Rettori D, Cadenas E. Voltage-dependent anion channels control the release of the superoxide anion from mitochondria to cytosol. *J Biol Chem.* 2003; 278:5557–5563. [PubMed: 12482755]
39. Perez Velazquez JL, Frantseva MV, Huzar DV, Carlen PL. Mitochondrial porin required for ischemia-induced mitochondrial dysfunction neuronal damage. *Neuroscience.* 2000; 97:363–369. [PubMed: 10799768]
40. Linn TC, Pettit FH, Reed LJ. Alpha-keto acid dehydrogenase complexes. X. Regulation of the activity of the pyruvate dehydrogenase complex from beef kidney mitochondria by phosphorylation dephosphorylation. *Proc Natl Acad Sci.* 1969; 62:234–241. [PubMed: 4306045]
41. Azarashvili TS, Tynnela J, Odinkova IV, Grigorjev PA, Baumann M, Evtodienko YV, Saris NE. Phosphorylation of a peptide related to subunit c of the F₀F₁-ATPase/ATP synthase and relationship to permeability transition pore opening in mitochondria. *J Bioenerg Biomembr.* 2002; 34:279–284. [PubMed: 12392191]
42. Boengler K, Gres P, Cabestrero A, Ruiz-Meana M, Garcia-Dorado D, Heusch G, Schulz R. Prevention of the ischemia-induced decrease in mitochondrial Tom20 content by ischemic preconditioning. *J Mol Cell Cardiol.* 2006; 41:426–430. [PubMed: 16828795]
43. Nagy N, Malik G, Fisher AB, Das DK. Targeted disruption of peroxiredoxin 6 gene renders the heart vulnerable to ischemia-reperfusion injury. *Am J Physiol.* 2006; 291:H2636–2640.
44. Liu H, Sadygov RG, Yates JR III. A model for random sampling estimation of relative protein abundance in shotgun proteomics. *Anal Chem.* 2004; 76:4193–4201. [PubMed: 15253663]

Abbreviations

DDM	N-dodecyl -D-maltoside
COX	cytochrome <i>c</i> oxidase
CsA	cyclosporin A
NSAF	normalized spectral abundance factors
DNP-hydrazone	2,4-dinitrophenylhydrazone
OMM	outer mitochondrial membrane
IMS	inter-membrane space
IMM	inner mitochondrial membrane
<i>pI</i>	isoelectric point
GRAVY	grand average hydrophobicity

Figure 1. Schematic Diagram of Experimental Design Using the *in vitro* Model of Cardiac Mitochondrial Injury

Figure 2. Examination of Mitochondrial Function Under Stress Conditions. 2A

Index of mitochondrial integrity was assessed after reversible injury (**RI**) or irreversible injury (**II**) by measuring cytochrome c oxidase activity. Activity measured in the presence of DDM which destroys mitochondrial outer membranes, was taken as total cytochrome c oxidase activity as detailed in *Experimental Procedures*. Compared with control group, reversible calcium treatment (**RI**) induced injury of about 15% of mitochondria. In contrast, hypotonic treatment (**II**) caused more than 80% of mitochondria to burst. **2B**. Measurement of cytochrome c release by immunoblotting. Irreversible injury (**II**) induced more cytochrome c release from mitochondria compared with reversible injury (**RI**). **2C**.

Determination of mitochondrial swelling. Mitochondrial swelling was determined in control group, where CsA did not have an effect; in the group of reversible injury (**RI**), where CsA prevented calcium-induced swelling; and in the group of irreversible injury (**II**), where CsA did not prevent calcium-induced swelling. **2D**. The morphology of the isolated mitochondria as determined by electron microscopy. Left panel shows that control, non-treated mitochondria were a homogeneous population with intact membranes and orthodox cristae structure. The middle panel shows a similar picture of mitochondria after reversible injury (**RI**) with few injured/swollen mitochondria and moderately perturbed cristae structure. In contrast, irreversible injury (**II**) induced rupture of the majority of mitochondria with a marked absence of intact structure (right panel) (magnification 19,000 $\times$). **2E**. Detection of oxidation as posttranslational modification of identified proteins by immunoblotting. Upper panel shows a stronger signal in irreversible injury, suggesting that irreversible injury was associated with the release of a greater level of ROS-modified proteins. Middle panel showed VDAC1 was modified by ROS in irreversible injury. Lower panel showed total VDAC1 protein expression as internal control.

Figure 3. Proteome Biology of Identified Proteins

Each protein was assigned to its respective functional cluster(s) and spatial location(s) according to the Gene Ontology Annotations database (GOA) and Swiss-Prot protein knowledgebase (<http://www.expasy.org/>). **3A.** Sub-organellar location of identified proteins. Mitochondrial released proteins were previously annotated to be localized in the OMM, IMS, IMM, Matrix, or mitochondria in general. In addition, a subpopulation of mitochondrial released proteins was not previously annotated as mitochondrial proteins; their primarily annotated subcellular locations are shown in the lower panel. **3B.** Biological function of identified proteins; **3C.** Combined analyses of location and function. The *x* axis represents the subcellular compartment(s) for protein annotation according to current databases, whereas the *y* axis indicates the functional cluster the identified proteins belong to. The detailed information regarding the characterization of these proteins by mass spectrometry, functional and subcellular annotation (Swiss-Prot, GOA, and our own Annotations), the spectra counts data and probability scores for each peptide were included in Tables S1 and S2 in the *Supplemental Material*.

Figure 4. Semi Quantitative Analysis of Identified Proteins Using Spectra Abundance Factors (SAF)

We established an index called normalized SAF, which is defined as peptide spectra counts of individual proteins divided by total peptide spectra counts. Protein IDs with their IPI numbers (shown in all x axis of this figure) can be located in Tables S1 and S2 in *Supplemental Material*. Protein names corresponding to IPI numbers in this figure were listed Table S4 in *Supplemental Material*. **4A.** Protein samples from both experimental groups were analyzed according to their functional clusters, e.g., redox. Three proteins (sulfite oxidase, [IPI00153144], superoxide dismutase [IPI00109109], and peroxiredoxin 5, [IPI00129517]) in the redox group were compared. The release of superoxide dismutase was found to be in a significant high amount in the experimental group of irreversible injury. **4B.** Protein samples from both experimental groups were analyzed on the oxidative phosphorylation cluster. The majority of released proteins in this category were found to be in a greater level following irreversible injury, e.g., the release of ATP synthase alpha chain (IPI00130280) was found to be 7 times greater in the experimental group of irreversible group. **4C and 4D.** Protein samples from both experimental groups were analyzed on metabolic cluster according to their submitochondrial localization. The significant amount of released proteins in this category was found to be associated with mitochondrial matrix; e.g., dihydrolipoyl dehydrogenase (IPI00115569).

Figure 5. Target Validation of Identified Proteins

Immunoblotting based verification of identified proteins. Creatine kinase and SOD were detected in both experimental groups of reversible injury and irreversible injury (upper left panel); whereas moesin, TOM40, and frataxin appeared dramatically more abundant in the experimental group of reversible injury (lower left panel). In contrast, the inner mitochondrial membrane proteins TIM44 and prohibitin, metaxin-2, fatty acid binding protein, and flotillin-1 were found to be much greater in the experimental group of irreversible injury (right panel).

Figure 6. The *in vivo* murine model of regional myocardial ischemia injury. 6A

The protocol to produce a myocardial infarction and the assessment of the risk zone were illustrated as shown. The resultant infarct size was determined by postmortem tetrazolium staining. **6B.** Validation of identified proteins in the experimental groups of irreversible injury using the *in vivo* model of regional myocardial ischemia/reperfusion injury. Risk zone and non-risk zone were collected and separated into cytosol and mitochondria by differential centrifugation. Increased presence of peroxiredoxin 5, TIM44, and catalase was observed in the cytosol of the risk zone as compared that observed in the sham control hearts, suggesting that these proteins were released from the mitochondria *in vivo* during injury (left panel); whereas no significant changes were detected in the mitochondrial fraction. Ponceau S image showed equal loading of proteins (lower panel).