Cardiac mitochondrial proteomic expression in inbred rat strains divergent in survival time after hemorrhage

Harold G. Klemcke,1 Robert M. DeKroon,2,4 Mihaela Mocanu,2,3 Jennifer B. Robinette,2,3 and Oscar Alzate2,3,4
1U.S. Army Institute of Surgical Research, Fort Sam Houston, Texas; 2Systems Proteomics Core Laboratory, University of North Carolina, Chapel Hill, North Carolina; 3Program of Molecular Biology and Biotechnology, University of North Carolina, Chapel Hill, North Carolina; and 4Department of Cell and Developmental Biology, University of North Carolina, Chapel Hill, North Carolina

Submitted 27 August 2012; accepted in final form 5 February 2013

Klemcke HG, DeKroon RM, Mocanu M, Robinette JB, Alzate O. Cardiac mitochondrial proteomic expression in inbred rat strains divergent in survival time after hemorrhage. Physiol Genomics 45: 243–255, 2013. First published February 5, 2013; doi:10.1152/physiolgenomics.00118.2012.—We have previously identified inbred rat strains differing in survival duration (StaH). In attempts to identify cellular mechanisms and ultimately genes that are important contributors to enhanced StaH, we conducted a study to characterize potential differences in cardiac mitochondrial proteins in these rats. Inbred rats from three strains [Brown Norway/Medical College of Wisconsin (BN); Dark Agouti (DA), and Fawn Hooded Hypertensive (FHH)] with different StaH (DA = FHH > BN) were assigned to one of three treatment groups (n = 4/strain): nonoperated controls, surgically catheterized rats, or rats surgically catheterized and hemorrhaged for 24 h postsurgery. Rats were euthanized 30 min after handling or 30 min after initiation of a 26 min hemorrhage. After euthanasia, hearts were removed and mitochondria isolated. Differential protein expression was determined using 2D DIGE-based Quantitative Intact Proteomics and proteins identified by MALDI/TOF mass spectrometry. Hundreds of proteins (791) differed among inbred rat strains (P < 0.038), and of these 81 were identified. Thirty-eight were unique proteins and 43 were apparent isoforms. For DA rats (longest StaH), 36 proteins increased and 30 decreased compared with BN (shortest StaH). These 81 proteins were associated with lipid (e.g., acyl CoA dehydrogenase) and carbohydrate (e.g., fumarase) metabolism, oxidative phosphorylation (e.g., ubiquinol-cytochrome C reductase), ATP synthesis (F1 ATPase), and H2S synthesis (3-mercaptopyruvate sulfurtransferase). Although we cannot make associations between these identified mitochondrial proteins and StaH, our data do provide evidence for future candidate proteins with which to consider such associations.

inbred rats; hemorrhage; proteomics; respiration; cellular pathways; mitochondrial proteomics; 2D DIGE; quantitative intact proteomics

Previous investigations in humans (114), dogs (32), and rats (125) have indicated considerable variability in their ability to survive severe hemorrhage and attendant shock. In attempts to understand the nature of this variability, we have recently initiated studies to identify genetic determinants of survival time after controlled hemorrhage (StaH) (68–69). Our initial investigations have identified several inbred rat strains sufficiently divergent in survival time to warrant their continued use for further genetic- and cellular-level investigations (68, 69). To supplement our search for genetic determinants, in the current study we have investigated mitochondrial characteristics of inbred rat strains divergent in StaH (Dark Agouti (DA) = Fawn Hooded Hypertensive (FHH) > Brown Norway/Medical College of Wisconsin (BN)).

After acute hemorrhage a complex of interacting systems-level responses occur as the body attempts to maintain homeostasis (101). As the biological pump that provides oxygen and nutrients to tissues and removes waste products, the heart must continue appropriate functioning as a necessary precondition for survival. Adversely altered cardiac function posthemorrhage has long been identified as a contributory mechanism to hemorrhagic shock in multiple species (e.g., 86, 92, 133) that ultimately can lead to death (54). Early observations indicated that decreased cardiac function resulted in part from reduced coronary arterial blood flow (21, 40) and presumed concomitant myocardial hypoxia (40, 115). Acute cardiac failure has been shown to be responsible for the transition from reversible to irreversible shock in multiple species (32, 33).

The complexity of the response at the organismal level is also evident at the cellular and molecular levels, and mitochondrial function undoubtedly exerts a major role in cellular level responses (19, 25). Hemorrhagic shock has been defined as "an abnormality of the circulatory system that results in inadequate organ perfusion and tissue oxygenation" (2a). Mitochondria provide ~95% of cellular energy requirements in the form of ATP and consume almost 90% of cellular oxygen (19, 25). Moreover, mitochondria are also important in apoptosis via a number of mitochondrial proteins (76). With reduced oxygen availability, such as that occurring during severe hemorrhage, mitochondrial function is compromised as indicated by decreased ATP generation, impaired electron transport, and increased production of reactive oxygen species (ROS) (19, 25, 112, 121).

Mitochondrial DNA codes for 13 mitochondrial proteins (MP) (3), and nuclear DNA is responsible for the additional MP that not only vary in number among species (44, 99, 117), but also among tissues within species (44). Such MP may be divided into categories of known function that include: 1) oxidative phosphorylation-associated proteins; 2) signaling proteins; 3) transporter and channel-associated proteins; 4) RNA, DNA, and protein synthesis-related proteins; 5) metabolic proteins; 6) cell death and defense proteins; 7) redox proteins; 8) proteases and protein...
targeting; and 9) unknown or unclassified proteins (123). From this listing it is quite obvious that the mitochondria fulfill a multitude of interacting functions in addition to the Krebs cycle and oxidative phosphorylation. Hence, there is substantial opportunity for existence of variability in the genetic code that in turn might lead to modified MP affecting cellular and organismal function during response to severe hemorrhage. Objectives of our current research are to determine if there are inbred rat strain-related differences in mitochondrial proteins that might 1) predispose a given strain to better survive hemorrhagic shock, 2) demonstrate strain-related differences in response to the stress of surgery, and 3) demonstrate strain-related differences in response to severe hemorrhage that might assist in the compensatory responses to that hemorrhage.

MATERIALS AND METHODS

Animals. All rats were maintained in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International. These studies were approved by the Institutional Animal Care and Use Committee of the US Army Institute of Surgical Research, Fort Sam Houston, TX. Furthermore, “This study has been conducted in compliance with the Animal Welfare Act, the implementing Animal Welfare Regulations, and the principles of the Guide for the Care and Use of Laboratory Animals.”

Inbred rat strains were obtained from the following vendors: BN from Charles River Laboratories (Wilmington, MA); FHH from Physigene-nix (Wauwatosa, WI); and DA/OlaHsd were from Harlan (Indianapolis, IN). All rats were males that were sacrificed at ~10 wk of age and were held for an 18–24 day acclimation period in our facility before experimentation. Rats were maintained individually in plastic cages at 22°C. Rats were provided food and water constantly available. Animals were housed on a 12:12 light:dark cycle. Ambient temperature was maintained at 22°C. Rats were randomly assigned treatment, day of surgery, order of surgery on each day, and order of hemorrhage on each day.

Experimental design and generation of the experimental groups. Four rats from each of the above three inbred rat strains were randomly assigned to one of the following treatment groups: 1) Normal, noncatheterized rats without any treatment (this set generated the control groups, i.e., BN-Ctrl, DA-Ctrl, and FHH-Ctrl); 2) Catheterized rats, ~24 h postsurgery and without hemorrhage (three experimental groups: BN-Cath, DA-Cath, and FHH-Cath); and 3) Catheterized rats, ~24 h postsurgery and 30 min after initiation of a conscious hemorrhage that occurred during a 26 min period (generating three more experimental groups: BN-CathBLD, DA-CathBLD, and FHH-CathBLD). Hence, a total of 36 rats were used in the study. Control rats were simply removed from their cages and euthanized as described below. Twenty-four hours after surgery, catheterized, but nonhemorrhaged rats were restrained, a catheter extension was attached, and rats were then placed in a cage for ~30 min prior to euthanasia. For this study it was assumed that all rats had a normalized blood volume of 5.83 ml/100 g body wt as previously noted (68). However, further investigation subsequent to the study reported herein has shown that blood volumes in these three inbred rat strains are different (69). Hence based on these later-obtained data, in the current study equal hemorrhages were not achieved. Rather, we calculated that the percentage of the total blood volume that was removed was 41.4 ± 0.14% for BN, 50.1 ± 0.15% for DA, and 47.0 ± 0.28% for FHH rats. The time period of 30 min after initiation of hemorrhage was chosen as we anticipated from previous work (68) that some rats of each strain might die soon after the hemorrhage. As we wanted to ensure that tissues were taken at the same time after initiation of hemorrhage for all rats, a fairly short time interval was used. Such a design was used to remove any possible confounding effects of taking tissues at different times after hemorrhage. It should be noted that survival times after a comparable 47% hemorrhage for the different inbred rat strains were such that both DA and FHH survived longer (190 ± 29 min vs. 113 ± 24 min, respectively) than did BN (52 ± 5 min) but did not differ from each other (69).

Surgical and hemorrhage procedures. Appropriate rats were surgically catheterized in the carotid artery by sterile procedures as described previously (68). Briefly, rats were anesthetized with 2–5% isoflurane (Foran; Baxter Healthcare, Deerfield, IL) in 100% oxygen. A catheter was inserted into the left common carotid artery and exteriorized in the dorsal neck region and sealed. Rats were injected with buprenorphine (2.5 μg/100 g body wt sc) and with 10 ml of 0.9% saline (sc)400 g body wt to provide analgesia and hydration, respectively, during recovery. Approximately 24 h after surgery, conscious, unrestrained rats were weighed and then bled as described previously (68) or, depending on assigned treatment, restrained and observed. To avoid potential influences of endogenous circadian rhythms, all rats were hemorrhaged between 07:00 and 12:00. Air temperature was maintained at ~25°C throughout the hemorrhage, and the subsequent observational period with a lamp. At the appropriate time, rats were deeply anesthetized with 5% isoflurane, the thoracic cavity opened, and each rat exsanguinated via removal of the heart. Each heart was placed in ice-cold phosphate-buffered saline (0.9 M NaCl) that was placed on ice. A portion of the apex of the heart (~300–450 mg) that consisted of both left and right ventricles was removed for isolation of mitochondria.

Mitochondria isolation. Isolation of mitochondria made use of an isolation kit and essentially used procedures detailed with this kit (cat. #MITOISO1; Sigma, St Louis, MO). Briefly, each recently obtained, not-previously-frozen heart sample was minced, subjected to trypsin digestion, homogenized, and centrifuged at 660 gmax for 10 min at 4°C. The supernatant from this centrifugation was recentrifuged at 25,000 gmax for 10 min at 4°C. The supernatant was removed, and the pellet was resuspended in 3 ml of 15% Percoll (Sigma). This suspension was then gently layered over a discontinuous Percoll density gradient consisting of 3.5 ml 23% Percoll over 3.5 ml 40% Percoll. This gradient was then centrifuged at 30,750 gmax for 10 min at 4°C (116). For our preparation, four bands of cellular material were formed within the Percoll gradient. Measurement of mitochondrial inner membrane-associated cytochrome C oxidase activity (see below) indicated that band 4 (greatest density) had the most intact mitochondria, with the highest specific activity (units of product/mg protein). Hence this band was used for all subsequent proteomics procedures. Bands 3 and 4 (~1.5–2.5 ml) were routinely collected and added to 8 ml of ice-cold extraction buffer A (10 mM HEPES, 0.2 M mannitol, 20 mM sucrose, 1 mM EGTA; pH 7.5: Sigma #E2778). After gentle mixing, these suspensions were centrifuged at 25,200 gmax for 10 min at 4°C. The lower 1 ml of this centrifuged suspension that contained a loose pellet was removed, transferred to a 1.5 ml conical centrifuge tube, and recentrifuged at 11,000 gmax for 10 min at 4°C. The supernatant was removed, and the pellet was resuspended in 500 μl of a second HEPES buffer (10 mM HEPES, 0.25 mM sucrose, 1 mM ATP, 0.08 mM ADP, 5 mM sodium succinate, 2 mM K2HPO4, 1 mM dithiothreitol, pH 7.5). The pellet was resuspended by careful trituration using a Gilson P1000 Pipetman (Gilson, Middleton, WI) and stored on ice until used in the enzymatic assay. These procedures allowed for an approximately sixfold increase in cytochrome C oxidase (COX) specific activity (units/mg protein) in band 4 compared with a crude homogenate, and an approximately twofold increase in specific activity when compared with a crude mitochondrial preparation. After the COX assay, mitochondria were rapidly frozen in liquid nitrogen and stored at −80°C until proteomic procedures were performed.

COX assay. This assay was conducted according to instructions detailed in a Sigma technical bulletin using their COX (EC 1.9.3.1) assay kit (Sigma #CYTOCOX1). An important feature of this procedure is that it provides the ability to distinguish intact from damaged mitochondria via use of the detergent n-dodecyl β-D-maltoside. This detergent solubilizes the mitochondrial membranes sufficiently to
allow cytochrome C substrate to access the inner mitochondrial membrane and COX. Briefly, in this assay reduced ferrocytochrome C is added to the above-noted mitochondrial preparation in the presence (total COX activity) or absence (COX activity in damaged mitochondria) of detergent. The oxidation of cytochrome C by COX is measured at 550 nm for 60 s at 10 s intervals at 25°C, and the change in absorbance is calculated. The percentage of intact mitochondria is calculated as: \( I_m = \left( \frac{A_0 - A_t}{A_0} \right) \times 100 \), where \( I_m \) is percentage of intact mitochondria, \( A_0 \) = activity in the presence of detergent, and \( A_t \) = activity in the absence of detergent.

COX activity was calculated as designated (Sigma #CYTOCOX1) and expressed as mg of per mg of total protein. Each mitochondrial preparation was assayed in duplicate in both the presence and absence of detergent. The average coefficient of variability (CV) for duplicate measures of COX in mitochondria of band 4 across 36 samples was 5.9%. For the amount of substrate (10 mM) and mitochondrial protein used per assay, there was always a linear increase of product formation during the 60 s incubation period, indicating that measurements were taken during the time period of initial velocity. The total protein present in each mitochondrial preparation was measured by a modified Lowry protein assay (80) validated for use with membrane preparations in our lab.

Quantitative intact proteomic analysis by two-dimensional difference gel electrophoresis. We treated 200 µl from each mitochondrial sample with two-dimensional (2D) clean-up kit (GE Healthcare, Piscataway, NJ) following the manufacturer’s instructions. Each resulting pellet was resuspended in 100 µl of lysis buffer (8 M urea, 2 M thiourea, 20 mM Tris·HCl, pH 8.5, 4% CHAPS), and the concentration was determined with a 2D Quant kit (GE Healthcare) as instructed by the manufacturer. From each sample 100 µg was used for pair-wise comparisons, and 50 µg was used to create the internal control (IC). The 2D difference gel electrophoresis (DIGE) experimental design followed the standard IC methodology (2, 37). Therefore, BN-Ctrl was compared with BN-Cath and to BN-CathBLD, and BN-Cath was compared with BN-CathBLD. The same comparisons were also performed with the DA and the FHH samples. The IC was created by pooling 50 µg from each sample, which was labeled with NHS-Cy2 (GE Healthcare); this IC was loaded in all the gels and used for normalization and statistical analysis as explained below. This experimental design allows us to compare all the mitochondrial proteomes among all the samples in a single group and between groups.

Proteins were covalently labeled with NHS-Cy3 and NHS-Cy5 (GE Healthcare; 2, 37). Labeled proteins were separated in the first dimension using isoelectric focusing with Immobilized pH Gradient (IPG) strips (GE Healthcare) in an IPG-Phor (GE Healthcare; 36). Protein separation in the second dimension was performed using 24 cm gels (Sigma) fixed to low-fluorescent glass plates with bind-silane (GE Healthcare). After 2D protein separation, proteins were visualized with a Typhoon Trio + scanner (GE Healthcare; 36). Differential protein expression was analyzed with the 2D DeCyder 7.0 computer program (GE Healthcare) using \( P < 0.05 \) as threshold to determine statistically significant changes based on t-test and ANOVA calculations (36, 47). The DeCyder algorithm was used to remove as many artifacts as possible before matching the gels. Once the t-test was calculated, each spot with a significant \( P \) value was manually inspected to confirm it as an unambiguous real protein. Ambiguous spots included: 1) proteins with dust peaks or other artifacts on it, 2) spots at the edge of the gel (the urea front) such that they may represent an overlap of several proteins, 3) spots that were part of a protein streak and therefore not well resolved, and 4) sometimes a protein shoulder or valley as it would not be very informative for mass spectrometry. Such ambiguous spots were excluded from consideration as being real proteins.

Protein identification by mass spectrometry. Protein spots displaying statistically significant expression changes were removed from the 2D gels using an Ettan Spot Picker (GE Healthcare) and submitted to the UNC mass spectrometry facility for protein identification by matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry (103).

Statistical and analytical procedures. COX data were analyzed with a two-way analysis of variance (PROC GLM) using the Statistical Analysis System (SAS, Cary, NC). All data were tested for homogeneity of variance (Levene’s test) and normality of distribution (PROC Univariate Normal with associated Kolmogorov-Smirnov test). Gels were analyzed using DeCyder 2D v. 7.0 software (GE Healthcare) as described previously (36). In this procedure, the IC present on each gel allows for the generation of a ratio for each protein peak detected between that samples’ protein peak, and the identical protein peak in the IC. Hence, use of such an IC assures both accuracy and specificity and adjusts for between-gel variability (2). Spot maps for each gel image (samples and internal control) were matched, and a statistical comparison (t-test) performed between groups (e.g., BN control vs. DA control vs. FHH control). For mitochondrial protein spots, probability levels reported are the t-test values that correspond to \( q \) values of \( \leq 0.05\). The latter values represent the acceptable degree of “false discovery” (the probability that a comparison found to be significant is actually nonsignificant) and allows adjustment for the many multiple comparisons using the false discovery rate (14, 64).

Protein network analysis with Ingenuity. To determine functions for the identified proteins we used each protein’s RefSeq identifier (obtained from http://www.uniprot.org/). Protein networks were analyzed with Ingenuity Pathway Analysis (IPA, version 8.8-3204, http://www.ingenuity.com/). As IPA considered the various putative posttranslationally modified proteins as duplicates, it automatically chose that replicate with the greatest fold-change.

RESULTS

The intactness of band 4 mitochondria was 93.4 ± 0.6% when averaged across all strains and treatments and did not differ among strains or treatments. COX activity also did not differ among treatments or strains (Table 1).

Proteomics analysis. Among all treatments and strains, a total of 1,958 spots were detected by the DeCyder 2D software. Of these spots, 948 were confirmed via direct observations as true proteins and not simply artifacts from dust particles or impurities in the glass plates (Fig. 1). Of the protein spots, only two showed significant effects of hemorrhage within an inbred rat strain: #387, FHH Cath-Bld 1.13-fold < Cath; #407, FHH Cath-Bld 1.08-fold < Cath. These two spots were not subsequently identified. Because of very few treatment effects, all treatment groups were statistically combined within inbred rat strain groups for the remaining spots and compared among strains. Multiple protein spots differed among strains (DA vs. BN, \( n = 485 \) proteins differed; DA vs. FHH, \( n = 314 \); FHH vs. BN, \( n = 430 \); \( P \leq 0.038\); \( q \leq 0.05\)). Of all protein spots, 141 were isolated and subjected to MALDI-TOF mass spectrometry for identification. We successfully identified 81 of the spots. Of these, 38 nonredundant/unique proteins were present (Supplemental Tables S1 and S2), and 43 identified proteins presumably represent multiple posttranslational modifications (PTM) (i.e., isoforms) of 24 of these nonredundant/unique proteins.1

Although it is appropriate to statistically combine treatments within strain, differences did exist among the strains for each treatment group. Control rats represent the most normal baseline condition, catheterized rats represent the status from which hemorrhage occurred, and hemorrhaged rats represent the sta-

1The online version of this article contains supplemental material.
tus 30 min after hemorrhage initiation. Hence, mitochondrial protein differences that are in common for all these treatments and for strains with better StaH (DA and FHH) compared with that strain having a poorer StaH (BN) could be informative. Of all protein spots detected, 20 met the criteria of being common to both DA and FHH rats and were different from BN rats in all three groups. Of these 20 protein spots, 15 were identified (Table 2).

Strain comparisons of all identified MP indicate that each strain had both nonredundant/unique and putative PTM isoforms that were expressed to a greater and to a lesser extent than other strains (Table 2, Supplemental Table S2). In general, differences in identified MP for DA vs. FHH were ~50% less than differences noted between either DA vs. BN or FHH vs. BN. For comparisons of the longer-lived DA and FHH inbred strains vs. the shorter-lived BN, 63% of these fold-changes were in the same direction (increase or decrease). For example, both isoforms of fumarase (catalyzes the stereospecific interconversion of fumarate and L-malate; MP spots 804, 813) were decreased in both DA and FHH vs. BN. Further comparisons of both DA vs. FHH and BN strains revealed a 50% difference between the hemorrhaged BN vs. those rats that were not hemorrhaged. See Table 2.

Table 1. Percentage of isolated mitochondria that were intact and the COX activity associated with those mitochondria

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Intact</th>
<th>COX Activity</th>
<th>% Intact</th>
<th>COX Activity</th>
<th>% Intact</th>
<th>COX Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>93.1 ± 2.3</td>
<td>1,901 ± 564</td>
<td>92.8 ± 0.6</td>
<td>2,554 ± 92</td>
<td>92.0 ± 3.0</td>
<td>2,068 ± 481</td>
</tr>
<tr>
<td>Surgery</td>
<td>96.6 ± 1.3</td>
<td>2,490 ± 370</td>
<td>93.9 ± 1.3</td>
<td>1,868 ± 467</td>
<td>92.8 ± 2.1</td>
<td>2,403 ± 140</td>
</tr>
<tr>
<td>Surgery + hemorrhage</td>
<td>95.8 ± 1.1</td>
<td>2,174 ± 372</td>
<td>90.3 ± 2.4</td>
<td>1,549 ± 379</td>
<td>93.7 ± 1.2</td>
<td>2,383 ± 383</td>
</tr>
</tbody>
</table>

Cytochrome C oxidase (COX) activity is expressed in milliUnits (mU)/mg protein. 1 mU, the oxidation of 1 moles of cytochrome C/min at 25°C and pH 7.0. Absorbance of ferrocyanochrome C was measured at 550 nm. The percentage of intact mitochondria was calculated as: Im = [(A1 - A0)/A0]×100, where Im is percentage of intact mitochondria, A0 = activity in the presence of detergent, and A1 = activity in the absence of detergent. There were no significant differences among treatments or inbred rat strains. % intact: Treatment, P = 0.49; strain, P = 0.16, treatment * strain, P = 0.58. Activity: treatment, P = 0.79; strain, P = 0.65, treatment × strain, P = 0.35. n = 4 rats per treatment × strain combination.

DISCUSSION

To our knowledge this work represents the first determination and documentation of differences in the mitochondrial proteome among these three inbred rat strains. As above-noted, these three rat strains have different survival times after hemorrhagic shock (StaH) (69). In our model we have chosen to focus initially only on hemorrhage and early survival time to somewhat simplify our search for genes. In current combat, hemorrhage accounts for ~80% of potentially survivable deaths (38), and the vast majority of combat deaths occur early during a prehospital setting (12, 13, 22). For severe uncontrolled hemorrhage in a combat prehospital setting, a minimal amount of fluid for hypotensive resuscitation should be used (85). Therefore, major tissue trauma, resuscitation with attendant reperfusion, and delayed multiple organ failure, all of which would entail additional genetic components, are not part of the current model. There were only two proteins affected by treatments, hence our discussion will focus on inbred rat strain-related effects. Moreover, the reader is reminded that in this study the hemorrhages were not equal (see MATERIALS AND METHODS). Hence, some strain-related differences among hemorrhaged rats might in part reflect these different percent hemorrhages. However, such effects are undoubtedly minimal as hemorrhage only affected expression of two proteins. Absence of treatment effects may reflect 1) a true absence of effects of these stressors (surgery and hemorrhage) at the applied intensities on these proteins or 2) an apparent absence of effects due to the time periods at which measures were taken. Postsurgery, it is possible that some alterations in proteins occurred but by 24 h had returned to basal values. It is not unusual for proteins to demonstrate a time-dependent increase and decrease in expression (e.g., 16, 128).
Posthemorrhage, changes may not have been observed due to the short time interval between hemorrhage and tissue collection that was dictated by the need to collect tissues at the same time in all rats. Such a time interval allowed for observation of treatment-associated changes in mRNA expression (unpublished data) but may have been insufficient to observe significant changes in protein expression. However, it should be noted that for some proteins 30 min is sufficient for increased expression after an appropriate stimulus (e.g., 31, 65, 88), and the onset of measurable protein accumulation may be stimulus specific (e.g., 52). Moreover, changes in phosphorylation of proteins can occur within 5 min (97). Indeed, after 30 min of in vitro ischemia, there were significant alterations in specific rat cardiac protein levels, three of which were unaffected in the current study (43). Furthermore, chronic intermittent hypoxia (6 h daily for 42 days) did alter cardiomyocyte expression of seven mitochondrial proteins unaffected by treatment in our study (140). Hence, our current interpretation is that the strength of the ischemic challenge was insufficient to stimulate measurable protein changes at the time interval measured.

In understanding the biological relevance of these strain-related differences in mitochondrial protein expression under...
**Table 2. Mitochondrial proteins from 3 inbred rat strains that were removed from 2D DIGE gels and identified via MALDI/TOF mass spectrometry**

<table>
<thead>
<tr>
<th>DA vs. BN</th>
<th>FHH vs. BN</th>
<th>DA vs. FHH</th>
<th>Protein</th>
<th>Spot #</th>
<th>Abbreviation</th>
<th>UniprotKB Accession</th>
<th>Coding Chromosome</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA&lt;BN (-3.74)</td>
<td>FHH&lt;BN (-2.5)</td>
<td>DA&lt;FHH (-1.49)</td>
<td>acyl-coenzyme A dehydrogenase, very long chain</td>
<td>775</td>
<td>ACADVL</td>
<td>Q5M9H2</td>
<td>10q24</td>
</tr>
<tr>
<td>DA&lt;BN (-1.84)</td>
<td>FHH&lt;BN (-1.33)</td>
<td>DA&lt;FHH (-1.38)</td>
<td>mitochondrial aldehyde dehydrogenase precursor chain B, Rat Liver F1-Atpase</td>
<td>694</td>
<td>ALDH2-3</td>
<td>P11884</td>
<td>12q16</td>
</tr>
<tr>
<td>DA&lt;BN (+2.25)</td>
<td>FHH&lt;BN (+2.62)</td>
<td>N/S</td>
<td>carnitine palmitoyltransferase 2 (Rattus norvegicus)</td>
<td>737</td>
<td>ATP5B-2</td>
<td>P10719</td>
<td>7q11</td>
</tr>
<tr>
<td>DA&lt;BN (+1.75)</td>
<td>FHH&lt;BN (+2.37)</td>
<td>DA&lt;FHH (-1.36)</td>
<td>carnitine palmitoyltransferase 2 (Rattus norvegicus)</td>
<td>420</td>
<td>CPT2-1</td>
<td>P18886</td>
<td>5q35</td>
</tr>
<tr>
<td>DA&lt;BN (+1.89)</td>
<td>FHH&lt;BN (+2.02)</td>
<td>N/S</td>
<td>carnitine palmitoyltransferase 2 (Rattus norvegicus)</td>
<td>433</td>
<td>CPT2-2</td>
<td>P18886</td>
<td>5q35</td>
</tr>
<tr>
<td>DA&lt;BN (-3.66)</td>
<td>FHH&lt;BN (-3.66)</td>
<td>N/S</td>
<td>carnitine palmitoyltransferase 2 (Rattus norvegicus)</td>
<td>445</td>
<td>CPT2-3</td>
<td>P18886</td>
<td>5q35</td>
</tr>
<tr>
<td>DA&lt;BN (-2.4)</td>
<td>FHH&lt;BN (-2.46)</td>
<td>N/S</td>
<td>mitochondrial short-chain enoyl-coenzyme A hydratase 1 precursor</td>
<td>450</td>
<td>CPT2-6</td>
<td>P18886</td>
<td>5q35</td>
</tr>
<tr>
<td>DA&lt;BN (+2.94)</td>
<td>FHH&lt;BN (+3.01)</td>
<td>N/S</td>
<td>mitochondrial short-chain enoyl-coenzyme A hydratase 1 precursor</td>
<td>1588</td>
<td>ECHS1-3</td>
<td>P14604</td>
<td>1q41</td>
</tr>
<tr>
<td>DA&lt;BN (-3.31)</td>
<td>FHH&lt;BN (-1.47)</td>
<td>DA&lt;FHH (-1.43)</td>
<td>fumarase</td>
<td>804</td>
<td>FH-1</td>
<td>P14408</td>
<td>13q25</td>
</tr>
<tr>
<td>DA&lt;BN (-2.01)</td>
<td>FHH&lt;BN (-1.69)</td>
<td>N/S</td>
<td>fumarase</td>
<td>813</td>
<td>FH-2</td>
<td>P14408</td>
<td>13q25</td>
</tr>
<tr>
<td>DA&lt;BN (+1.66)</td>
<td>FHH&lt;BN (+1.83)</td>
<td>N/S</td>
<td>isovaleryl coenzyme A dehydrogenase precursor (Rattus norvegicus)</td>
<td>925</td>
<td>IVD</td>
<td>P12007</td>
<td>3q35</td>
</tr>
<tr>
<td>DA&lt;BN (+1.38)</td>
<td>FHH&lt;BN (+1.33)</td>
<td>N/S</td>
<td>3-mercaptopyruvate sulfurransferase</td>
<td>1386</td>
<td>MPST</td>
<td>P97532</td>
<td>7q34</td>
</tr>
<tr>
<td>DA&lt;BN (+2.23)</td>
<td>FHH&lt;BN (+2.0)</td>
<td>N/S</td>
<td>propionyl-coenzyme A carboxylase, alpha polypeptide</td>
<td>394</td>
<td>PCCA</td>
<td>P14882</td>
<td>15q25</td>
</tr>
<tr>
<td>DA&lt;BN (+1.44)</td>
<td>FHH&lt;BN (+1.42)</td>
<td>N/S</td>
<td>propionyl coenzyme A carboxylase, β-polypeptide precursor (Rattus norvegicus)</td>
<td>551</td>
<td>PCCB</td>
<td>Q68FZ8</td>
<td>8q31</td>
</tr>
<tr>
<td>DA&lt;BN (-4.56)</td>
<td>FHH&lt;BN (-3.52)</td>
<td>N/S</td>
<td>ubiquinol-cytochrome c reductase, Rieske iron-sulfur</td>
<td>1618</td>
<td>UQCRSF1-2</td>
<td>149045287*</td>
<td>17p12</td>
</tr>
</tbody>
</table>

Statistical comparisons between strains were conducted using the DeCyder 2D version 7.0 software. All comparisons noted as > or < are different at a P ≤ 0.038 and a false discovery rate of q ≤ 0.05. N/S, the MP spots were not different. UniprotKB are accession numbers associated with the Protein Information Resource (PIR), a UniProt Consortium member (http://www.uniprot.org/). Increased (+) or decreased (−) fold changes are presented in parentheses. Inbred rat strains are: Dark Agouti (DA), Fawn Hooded Hypertensive (FHH), and Brown Norway/Medical College of Wisconsin (BN). Abbreviations present in column 6 represent those symbols used by Ingenuity Pathways to designate each specific protein. Numerical suffixes (e.g., CPT2-1 and CPT2-2) have been added to designate different isoforms. The spot numbers are as shown in Fig. 1. There were 4 rats per inbred rat strain in each treatment group for a total of 36 rats.

“basal” conditions (control, catheterized, and, absent treatment effects, hemorrhaged rats), one should remember that these protein expression levels reflect the initial ability/capacity to respond to any challenge. Such baseline levels represent but one of a multitude of biological basal levels/activities that define cellular, organ, and organismal functioning and from which activation and deactivation occur (49, 53, 58). Basal levels of a biological variable or molecule can 1) affect direction and magnitude of a subsequent response to a stimulus (50), 2) be predictive of disease (107) and treatment outcome (7, 57, 90) and serve as biomarkers for therapeutic intervention (100), 3) be predictive of susceptibility to aversive environments (45, 106), 4) reflect the functioning of an organ (127), and 5) regulate cellular proliferation and transformation and define cellular responses (102). Hence, although none of the identified proteins showed altered expression subsequent to hemorrhage, the strain differences in mitochondrial protein expression existing at the time of hemorrhage should provide information concerning a differential mitochondrial capacity to respond. Moreover, it is recognized that differences in mitochondrial protein expression could also influence cardiac responses to many other challenges such as atrial fibrillation (51), heart failure (27), exercise-associated cardiac preconditioning (46), and cardiac aging (35). Indeed, many of these proteins also have a known involvement in various pathological states for multiple organs, some of which are noted (Table 3).

Mitochondria are the primary source of energy for the body in the form of ATP and consume the majority of cellular oxygen. Cellular hypoxia associated with the global ischemia of severe hemorrhage results in mitochondrial malfunction (e.g., 24, 112). Hence, it was hypothesized that differences in mitochondrial proteins might reflect differences in mitochondrial ability to withstand such hemorrhage and associated hypoxia. Therefore, we will speculate about the possible involvement of these mitochondrial protein differences in differential StaH. Strain-related differences in these proteins may indeed suggest their influence on StaH. However, it should be emphasized that until further experimental evidence is obtained, identified associations are purely conjectural. Nonetheless, such conjectures are not without merit as differences in the mitochondrial proteome have been associated with cardioprotection after preconditioning (8) cardioplegia (84) and cardiac adaptations to hibernation (5). Additionally, <10% of the
protein spots that were different between DA or FHH and BN were identified. Hence, the available assessment of proteomic differences among these inbred rat strains represents only a partial understanding of the total mitochondrial proteome differences that could potentially influence survival after severe hemorrhage or other phenotypes. Based on the intactness of our mitochondrial preparations and the 100% mitochondrial localization of all identified proteins, we are confident that the majority of remaining unidentified proteins are associated with the mitochondria and at most represent but minor numbers of proteins from other cellular structures. Finally, although various fold increases or decreases were measured among the inbred rat strains for these proteins, it is difficult to extrapolate the effects of these fold-differences to overall effects on enzymatic products. The flow rates of products through the mitochondrial metabolic pathways depend not only on the expression levels of each enzyme, but also on their activity and substrate/product relationships (120).

Mindful of these caveats, it is nonetheless of interest to note that the two strains whose survival times after severe hemorrhage did not differ (DA vs. FHH), had fewer protein differences than did strains that differed in survival time (DA and FHH vs. BN). Most identified MP are enzymes or peptides associated with large enzymatic complexes involved in one of the three stages associated with eukaryotic cellular respiration: 1) metabolism of carbohydrates, fatty acids, and amino acids into moieties that can enter the Krebs cycle (usually acetyl CoA); 2) the Krebs cycle itself that oxidizes these molecules and transfers electrons and hydrogen ions (H+3) to oxidizing agents (coenzymes) NAD+ and FAD+; and 3) transfer of electrons and H+ to protein complexes of the respiratory chain wherein the energy released during transfer of electrons is conserved in the form of ATP (93). Because of space constraints not all identified proteins will be discussed. Rather, we will focus primarily on those proteins that were in common for DA and FHH and were different than BN for all three experimental groups. At the same time we will attempt to represent constraints not all identified proteins will be discussed. Rather, we will focus primarily on those proteins that were identified for common for DA and FHH and were different than BN for all three experimental groups. At the same time we will attempt to represent

### Table 3. Biological function and some known pathologies and ongoing translational studies associated with proteins identified in Table 2

<table>
<thead>
<tr>
<th>Protein Abbreviation</th>
<th>Biological Function</th>
<th>Pathophysiological or Translational Implication</th>
<th>Ref. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACADVL</td>
<td>oxidation and beta-oxidation of fatty acids</td>
<td>deficiency leads to lethargy, hypotonia, rhabdomyolysis liver abnormalities, cardiomyopathys, hypoketotic hypoglycemia</td>
<td>4, 75, 81</td>
</tr>
<tr>
<td>ALDH2</td>
<td>aldehyde catabolism</td>
<td>low activity associated with greater susceptibility to alcohol-induced organ damage; certain alleles associated with high sensitivity to alcohol consumption and low levels of HDL cholesterol; cardioprotective in rodent studies</td>
<td>17, 18, 26, 56, 119, 122, 137</td>
</tr>
<tr>
<td>ATP5B-2</td>
<td>ATP synthesis</td>
<td>protein expression useful for cancer diagnosis and prognosis</td>
<td>34, 61, 77</td>
</tr>
<tr>
<td>CPT2</td>
<td>lipid metabolism/transport</td>
<td>deficiency most common defect of mitochondrial beta-oxidation; form of gene mutations associated with rhabdomyolysis and myoglobinuria; severe deficiency associated with liver failure, cardiomyopathy, and early death; currently an active area for clinical trials (<a href="http://www.clinicaltrials.gov">http://www.clinicaltrials.gov</a>)</td>
<td>6, 59, 91</td>
</tr>
<tr>
<td>ECHS1, FH</td>
<td>oxidation and β-oxidation of fatty acids, Krebs cycle</td>
<td>marker of carcinogenesis; biallelic mutations impair CNS development; serves as a tumor suppressor gene; deficiency associated with renal cell cancer syndrome and multiple smooth muscle tumors</td>
<td>67, 135, 11</td>
</tr>
<tr>
<td>IVD</td>
<td>amino acid metabolism</td>
<td>deficiency associated with isovaleric acidemia which is considered a severe, potentially life-threatening condition in neonates</td>
<td>42</td>
</tr>
<tr>
<td>MPST</td>
<td>H2S synthesis</td>
<td>product hydrogen sulphide is a gasotransmitter that in physiological concentrations is cytoprotective in cardiovascular system and neurons; a modulator of inflammation and may have antiangcancer effects; 2 clinical trials in progress associated with kidney disease or peripheral arterial disease (<a href="http://www.clinicaltrials.gov">http://www.clinicaltrials.gov</a>)</td>
<td>20, 79, 105, 138</td>
</tr>
<tr>
<td>PCCA</td>
<td>oxidation and beta-oxidation of fatty acids</td>
<td>mutations in gene coding for PCC associated with propionic acidemia with associated metabolic instability that can lead to neurological impairment and cardiomyopathy and increased copy number per cell associated with more aggressive form of breast cancer</td>
<td>23, 82, 94</td>
</tr>
<tr>
<td>UQCRSF1</td>
<td>oxidative phosphorylation</td>
<td></td>
<td>95</td>
</tr>
</tbody>
</table>

Protein and inbred rat strain abbreviations are those noted in Table 2. Functions are determined via use of Ingenuity Pathway Analysis (http://www.ingenuity.com/) and Refs. 93 and 105.

References

-Refs. 93 and 105.

-References than did strains that differed in survival time (DA and FHH).

-References not all identified proteins will be discussed. Rather, we will focus primarily on those proteins that were in common for DA and FHH and were different than BN for all three experimental groups. At the same time we will attempt to represent most metabolic pathways associated with identified proteins.

-References of proteins that were in common for DA and FHH and were different than BN for all three experimental groups. At the same time we will attempt to represent most metabolic pathways associated with identified proteins.

-References to other phenotypes. Based on the intactness of our mitochondrial preparations and the 100% mitochondrial localization of all identified proteins, we are confident that the majority of remaining unidentified proteins are associated with the mitochondria and at most represent but minor numbers of proteins from other cellular structures. Finally, although various fold increases or decreases were measured among the inbred rat strains for these proteins, it is difficult to extrapolate the effects of these fold-differences to overall effects on enzymatic products. The flow rates of products through the mitochondrial metabolic pathways depend not only on the expression levels of each enzyme, but also on their activity and substrate/product relationships (120).

-References to these metabolic pathways. At the same time we will attempt to represent most metabolic pathways associated with identified proteins.

-References to the Krebs cycle itself that oxidizes these molecules and transfers electrons and hydrogen ions (H+3) to oxidizing agents (coenzymes) NAD+ and FAD+; and 3) transfer of electrons and H+ to protein complexes of the respiratory chain wherein the energy released during transfer of electrons is conserved in the form of ATP (93). Because of space constraints not all identified proteins will be discussed. Rather, we will focus primarily on those proteins that were in common for DA and FHH and were different than BN for all three experimental groups. At the same time we will attempt to represent most metabolic pathways associated with identified proteins.

-References to the Krebs cycle itself that oxidizes these molecules and transfers electrons and hydrogen ions (H+3) to oxidizing agents (coenzymes) NAD+ and FAD+; and 3) transfer of electrons and H+ to protein complexes of the respiratory chain wherein the energy released during transfer of electrons is conserved in the form of ATP (93). Because of space constraints not all identified proteins will be discussed. Rather, we will focus primarily on those proteins that were in common for DA and FHH and were different than BN for all three experimental groups. At the same time we will attempt to represent most metabolic pathways associated with identified proteins.
this study) and then transferred to the matrix through the inner mitochondrial membrane via a transporter (39). Within the matrix, these fatty acyl-carnitines are further processed wherein the fatty acyl moiety is transferred to CoA to reform fatty acyl-CoA via the enzyme CPT2. The levels of CPT2 isoforms varied dramatically between BN and DA/FHH rats. These isoform-specific differences, noted with this and other proteins, suggests a remarkable “fine-tuning” mechanism that undoubtedly involves other enzymes and that allows a very precise regulation of mitochondrial functions. Such specificity has been reported in myocardocyte proteomic responses to preconditioning (8). CPT2 deficiency is the most common inborn error of fatty acids oxidation and results in reduced transfer of long-chain fatty acid into mitochondria, and its deficiency compromises the ability to use long-chain fatty acids during exercise (96). To date, 40 point mutations or microrearrangements have been described for the human CPT2 gene (15, 124). The large number of CPT2 isoforms detected in the current study is consistent with such genetic diversity. Furthermore, there are multiple phosphorylation sites on CPT2, and the detected isoforms appear to differ primarily in charge, which is consistent with different levels of phosphorylation. Whereas there is no literature concerning phosphorylation of CPT2, it is known that phosphorylation of CPT1 occurs and that phosphorylation increases CPT1 activity (66). CPT2 activity decreased in rat heart after chronic stress (78), but its association with responses to severe hemorrhage has not been documented.

The next procedure in the processing of intramitochondrial fatty acids is the actual β-oxidation process that involves four enzymatic steps (39, 93); some of which [very long chain (ACADVL), long chain (ACADL), and short chain (ACADS) dehydrogenases (Table 2, Supplemental Table S2, Fig. 2)] were identified in this study and which catalyze the first step in β-oxidation. In rats cardiac ACADVL activity, but not protein content, was decreased after 30 min of ischemia (78). To date, 40 point mutations or microrearrangements have been described for the human CPT2 gene (15, 124). The large number of CPT2 isoforms detected in the current study is consistent with such genetic diversity. Furthermore, there are multiple phosphorylation sites on CPT2, and the detected isoforms appear to differ primarily in charge, which is consistent with different levels of phosphorylation. Whereas there is no literature concerning phosphorylation of CPT2, it is known that phosphorylation of CPT1 occurs and that phosphorylation increases CPT1 activity (66). CPT2 activity decreased in rat heart after chronic stress (78), but its association with responses to severe hemorrhage has not been documented.

The next procedure in the processing of intramitochondrial fatty acids is the actual β-oxidation process that involves four enzymatic steps (39, 93); some of which [very long chain (ACADVL), long chain (ACADL), and short chain (ACADS) dehydrogenases (Table 2, Supplemental Table S2, Fig. 2)] were identified in this study and which catalyze the first step in β-oxidation. In rats cardiac ACADVL activity, but not protein content, was decreased after 30 min of ischemia (81). Such findings could be consistent with our current observations, which did not show hemorrhage-associated changes in ACADVL. Differences in expression of these dehydrogenases among the rat strains might suggest preferences for specific fatty acids as substrates. In humans, ACADVL deficiency, although rare (75), is also associated with multiple disease phenotypes of varying severity with one early-onset form being associated with cardiomyopathy and a high mortality (Table 3; Refs. 4, 75, 81).

During β-oxidation FADH$_2$ is produced and almost immediately passes its electrons to the electron-transferring flavoprotein (ETF). The ETF-subunit of this protein was increased about twofold in DA and FHH vs. BN. ETF ultimately will transfer these electrons to the respiratory chain via ubiquinone oxidoreductase and coenzyme Q (39, 93). One enzyme catalyzing the next step of β-oxidation, short-chain enoyl-CoA hydratase (ECHS1), was identified with its three isoforms being expressed to a much greater degree (~3-fold) in DA and FHH than in BN. Increased cardiac ECHS1 protein expression in a mouse model of cardioprotection (134) provides corroborating evidence that ECHS1 could also be involved in strain-dependent differential survival to hemorrhage.

Other points of entry to the Krebs cycle. Succinyl-CoA is one intermediate within the Krebs cycle that primarily derives from α-ketoglutarate (Fig. 2). However, succinyl-CoA can also be formed from odd-numbered fatty acids and from some amino acids such as valine (93). Most fatty acids have an even number of carbons, but some odd-numbered fatty acids are ingested in the diet (93). These are catabolized in a manner similar to even-numbered fatty acids until there remains a three-carbon chain, propionyl-CoA, which is carboxylated by the enzyme detected in this study, propionyl-CoA carboxylase (PCCA, PCCB). Increases in subunits of this enzyme in both DA and FHH rats relative to BN (~2-fold for PCCA) suggest the possibility of increased production of succinyl-CoA via this pathway. This succinyl Co-A may stay within the Krebs cycle and be converted to succinate (Fig. 2), or it can also be used as a source of CoA by the 3-oxoacid CoA transferase enzyme.

---

**Fig. 2.** Diagram of mitochondrion showing functional relationships of identified cardiac mitochondrial proteins. All abbreviations are found in Table 2 or Supplemental Table S2. All proteins shaded in gray were identified in this study.
Generation of succinyl-CoA by catabolism of valine is a process that makes use of another identified enzyme, 3-hydroxyisobutyl-CoA hydrolase (HIBCH). This pathway also includes ECHS1 (Ref. 113). In addition to providing more substrates for the Krebs cycle, this latter pathway also ensures rapid removal of an intermediary of valine catabolism, methyacrylyl-CoA, that is toxic to cells (60), thereby providing a potential survival advantage to cardiomyocytes in DA and FHH vs. BN rats. Although there is no documented evidence as yet for any of these enzymes (PCCA, PCCB, HIBCH) being involved in responses to hemorrhage, it is known that branched chain amino acids such as valine (one substrate for these enzymes) protect against acute cardiac ischemic injury (74, 87).

A final source of substrate for entry into the Krebs cycle is catabolism of a number of amino acids that include leucine, arginine, and proline (93). For leucine there are six steps to generation of acetyl CoA (98): one step involves the identified enzyme isovaleryl-CoA dehydrogenase (IVD), which shares similar structural and functional features with the above-noted acyl-CoA dehydrogenases (ACADVL, ACADL, ACADS; 83). Leucine and other branched-chain amino acids protect against myocardial ischemic injury in rats (74) and improve cardiac function (87). This protein is increased in DA and FHH vs. BN (Table 2).

Krebs cycle. The Krebs cycle oxidizes supplied substrates to CO₂ and concomitantly transfers the energy released to the coenzyme electron carriers NAD⁺ and FAD⁺ (93). As the maximal rate of respiration is dependent on NADH production, changes in the Krebs cycle activity will ultimately affect ATP synthesis (109). Nine unique proteins and a total of 21 isoforms that represent enzymatic components of the Krebs cycle itself were identified as differentially expressed in our comparisons (Supplemental Table S2).

Isocitrate resulting from the enzymatic activity of ACO2 is subsequently converted to α-ketoglutarate by isocitrate dehydrogenase. NAD-dependent isocitrate dehydrogenase (IDH3) is an important rate-limiting step in the Krebs cycle (55, 70). It is considered to be a unidirectional enzyme regulated by multiple stimulatory (Ca²⁺, ADP, citrate) and inhibitory (ATP, NADH, and NADPH) regulators (30, 48). In heart mitochondria, NADP-dependent isocitrate dehydrogenase (IDH2) provides significant conversion of α-ketoglutarate to isocitrate (reverse flux) that may serve to provide a fine regulation of the Krebs cycle (30). IDH2 is a major source of intramitochondrial NADPH, which provides for regeneration of glutathione (GSH) from glutathione disulfide and thereby provides a mechanism of protection to superoxide anions (62). In the current study subunits of both the NAD- and NADP-dependent enzymes and multiple isoforms of each were detected and identified. The large fold-differences for at least one of the isoforms of IDH3 suggest that, if indeed substrate flow through the Krebs cycle is greater in DA and FHH than in BN rats, then this enzymatic step might be of importance. Moreover, IDH2 was of greater abundance in DA and FHH vs. BN, suggesting an additional mechanism to improve mitochondrial function in these two rat strains when subjected to oxidative stress.

Multiple isoforms of another member of the Krebs cycle, fumarase, were identified and found to be about two- to threefold reduced in both DA and FHH vs. BN. Fumarase catalyzes the reversible conversion of fumarate to malate. Of relevance to our theme, it has been shown that exogenous fumarate increased survival in hemorrhaged rabbits (29) and was cardioprotective during ischemia (9, 71). Furthermore, in a fumarase cardiac knockout mouse model, endogenous cardiac fumarate concentrations increased (10) coincident with increased resistance to ischemia reperfusion injury (9). Associated with reduced fumarase activity was an increased anaplerotic use of amino acids to produce Krebs cycle metabolites and a fumarate-induced increase in antioxidants associated with the transcription factor Nrf2 (9). The increased use of amino acids to supply substrates to the Krebs cycle is consistent with observations in the current study wherein amounts of enzymes associate with supplying amino acid-derived substrates to the Krebs cycle (IVD, PCCA, PCCB) were increased in DA and FHH vs. BN. Hence, one might speculate that reduced fumarase activity in DA and FHH rats could similarly increase cardiac fumarate and provided associated cardioprotection during the global ischemia of hemorrhagic shock. Such a concept is further reinforced by the role of fumarase deficiency in conferring a survival advantage to human fibroblasts and renal cells that might contribute to enhanced tumorigenesis (Table 3; Ref. 11).

Respiratory chain. Seven unique proteins (16 total proteins including isoforms) associated with multiple components of the respiratory chain were identified (Table 2, Supplemental Table S2). Cardiac ischemia decreases electron transport function and oxidative phosphorylation (73). There were many differences in expression of these proteins among the rat strains, and often the isoforms for a given protein varied in different directions when two rat strains were compared (e.g., ubiquinol-cytochrome c reductase E, UQCRSF1). This latter protein is one of 11 subunits of a complex III monomer (ubiquinone to cytochrome C) that couples the transfer of electrons from ubiquinol to cytochrome c with the transport of protons from the mitochondrial matrix to the intermembrane space (93). Ischemia decreased complex III activity (72, 108) and specifically damaged the iron-sulfur protein complex (72). Of interest is the finding that temporary blockade of electron transport prior to ischemia is associated with improved mitochondrial respiration and cardiac function after ischemia and reperfusion (28). Is it possible that the dramatically decreased (up to 4.6-fold) levels of some isoforms of iron-sulfur protein in DA and FHH relative to BN might actually provide a similar cardioprotection through reduced production of ROS? Such a hypothesis is worthy of future research.

ATP synthesis. FₐFₛ-ATP synthase is a large protein complex that catalyzes the synthesis of ATP from ADP and Pᵢ. It consists of two components, a peripheral membrane protein Fₐ and an integral membrane protein Fₛ (93). Fₛ provides the ATP synthesis role for the complex, but isolated Fₐ actually catalyzes both the synthesis and hydrolysis of ATP. The FₐFₛ complex, however, binds ATP with a high affinity that drives the enzymatic activity toward the production of ATP (93). Subsequently, ATP must be released from Fₛ, and this is accomplished via the passage of protons through the Fₒ down an electrochemical gradient from the intermembrane space into the mitochondrial matrix (93).
The F₁ component has five different types of subunits, and a total of nine subunits; three alpha (α), three beta (β), one gamma (γ), one delta (δ), and one epsilon (ε). Each β-subunit has one catalytic site for ATP synthesis, but the β-subunits are different in conformation and in ATP/ADP binding sites (93). We detected two isoforms each of α- and β-subunits. Three such isoforms were twofold greater in DA vs. BN rats. It is known that phosphorylation of both F₁ α- and β-subunits occurs and affects both structure and function of the F₀F₁ complex (63). In this study, the isoforms of both F₁ α- and β-subunits appear (based on location on the gel) to involve differences in molecular size. For F₁ α, one isoform (spot #642) migrated closer to the anode, which is consistent with its being phosphorylated.

There is accruing evidence that cardiac F₀F₁-ATP synthase activity is decreased by ischemia and in a paradoxical manner this decrease is cardioprotective (1, 129). During ischemia and attendant hypoxia, the above-noted electrochemical gradient dissipates, and the hydrolytic activity of F₀F₁-ATP synthase (i.e., F₀F₁-ATPase) predominates. Hence, this enzymatic activity becomes a major consumer of ATP (129). Decreasing this activity preserves ATP levels for maintaining mitochondrial membrane potential (1). Indeed ischemic preconditioning decreases F₀F₁-ATPase activity commensurate with its cardioprotective effects (1). Such observations highlight the importance of the various functional aspects of this mitochondrial protein complex. Differences observed in the current study reflect basal, non-ischemic levels of this complex.

H₂S and mitochondria. An exciting finding during the last decade has been the role of H₂S as a gasotransmitter (130, 132). As one of its many functions, H₂S has been shown to be cardioprotective during myocardial ischemia-reperfusion in a number of animal models (41, 104, 118) by a variety of mechanisms that include a reduced inflammatory response and apoptosis, preservation of mitochondrial function, and regulation of ion channels (41, 105, 139; Table 3, Supplemental Table S2). There are three enzymes involved in production of H₂S from cysteine, one of which is the mitochondrial enzyme MPTST identified in the current studies (111, 131). Indeed, recent evidence indicates that mitochondrial MPTST-derived H₂S enhances electron transport and ATP production at low concentrations (89). Hence, increased levels of MPTST protein in DA and FHH mitochondria relative to BN could provide one mechanism for improved StaH.

In conclusion, our data strongly indicate inbred rat strain-specific differences in cardiac mitochondrial proteins. Despite our limited ability to make conclusive statements concerning the involvement of any of these cardiac mitochondrial proteins in the ability of one inbred rat strain to survive hemorrhagic shock better than another, our results do provide information and generate hypotheses concerning such associations that can be tested in future experiments. The complexity of the biological responses to severe hemorrhage is readily reflected in the complexity of the between-strain differences we observed in MP and their isoforms. Significant increases in cardiac MP associated with β-oxidation, the Krebs cycle, oxidative phosphorylation, and ATP synthesis suggest the possibility for a differential functioning of these mitochondria in DA and FHH vs. BN rats.


46. Hansford RG, Johnson RN. The steady state concentrations of coenzyme A-SH and coenzyme A thioester, citrate, and isocitrate during...
56. Higuchi S, Matsushita S, Muramatsu T, Murayama M, Hayashida M. Alcohol and aldehyde dehydrogenase genotypes and drinking behav-
58. Hubert AJ, Else PL. Basal metabolic rate: history, composition, regu-
59. Isackson PJ, Bennett MJ, Vladutiu GD. Identification of 16 new disease-causing mutations in the CPT2 gene resulting in carnitine palmito-
Baron M, Cuevza JM. Breast carcinomas fulfill the Warburg hypothesis and provide metabolic markers of cancer progression. Carcinogenesis 26:
63. Kane LA, Youngman MJ, Jensen RE, Van Eyk JE. Phosphorylation of the Flj0185 ATP synthase beta subunit: functional and structural conse-
66. Kerner J, Distler AM, Minkler P, Parland W, Peterman SM, Hoppel CL. Phosphorylation of rat liver mitochondrial carnitine palmitoyltrans-
68. Klemcke HG, Baer DG, Pankrath VS, Cox A, Cortez DS, Garrett MR, Joe B, Ryan KL. Ischemic injury to mitochondrial electron transport in the aging heart: damage to the iron-sulfur protein subunit of cytochrome oxidase in subsarcolemmal mitochondria. Myocardial ischemia decreases oxidative phosphorylation through cyto-
chrome c reductase (UQCRFS1) gene amplification in primary breast cancer core
70. Otis M, Campbell S, Payet MD, Gallo-Payet N. Angiotensin II stim-


111. Shibuya N, Mikami Y, Kimura Y, Nagahara N, Kimura H. Aldehyde dehydrogenase 2 gene is a risk factor for myocardial infarction by 10.220.32.247 on July 10, 2017 http://physiolgenomics.physiology.org/ Downloaded from...