Sex Differences in the Phosphorylation of Mitochondrial Proteins Result in Reduced Production of Reactive Oxygen Species and Cardioprotection in Females

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Rationale: Although premenopausal females have a lower risk for cardiovascular disease, the mechanism(s) are poorly understood.

Objective: We tested the hypothesis that cardioprotection in females is mediated by altered mitochondrial protein levels and/or posttranslational modifications.

Methods and Results: Using both an in vivo and an isolated heart model of ischemia and reperfusion (I/R), we found that females had less injury than males. Using proteomic methods we found that female hearts had increased phosphorylation and activity of aldehyde dehydrogenase (ALDH)2, an enzyme that detoxifies reactive oxygen species (ROS)-generated aldehyde adducts, and that an activator of ALDH2 reduced I/R injury in males but had no significant effect in females. Wortmannin, an inhibitor of phosphatidylinositol 3-kinase, blocked the protection and the increased phosphorylation of ALDH2 in females, but had no effect in males. Furthermore, we found an increase in phosphorylation of α-ketoglutarate dehydrogenase (αKGDH) in female hearts. αKGDH is a major source of ROS generation particularly with a high NADH/NAD ratio which occurs during I/R. We found decreased ROS generation in permeabilized female mitochondria given αKGDH substrates and NADH, suggesting that increased phosphorylation of αKGDH might reduce ROS generation by αKGDH. In support of this hypothesis, we found that protein kinase C–dependent phosphorylation of purified αKGDH reduced ROS generation. Additionally, myocytes from female hearts had less ROS generation following I/R than males and addition of wortmannin increased ROS generation in females to the same levels as in males.

Conclusions: These data suggest that posttranslational modifications can modify ROS handling and play an important role in female cardioprotection. (Circ Res. 2010;106:1681-1691.)

Key Words: gender difference ▪ cardioprotection ▪ mitochondria ▪ proteomics ▪ aldehyde dehydrogenase
protein levels or post translational modifications, and that PI3K is an important mediator of these effects. We report that females have altered posttranslational modification of several mitochondrial proteins, including ALDH2, a protein that has recently been reported to be involved in cardioprotection.25 Phosphorylation of ALDH2 and protection in females are blocked by inhibitors of PI3K. We further show reduced generation of ROS by α-ketoglutarate dehydrogenase (α-KGDH) in female mitochondria and less ROS production from female mitochondria and female cardiac myocytes on reoxygenation following anoxia. In addition, in vitro phosphorylation of purified α-KGDH reduces ROS production demonstrating a novel mechanism for reducing ROS production. Taken together, these data suggest that altered phosphorylation of mitochondrial proteins alters ROS handling in female mitochondria.

Methods

Animals

All animals (Charles River Laboratory) were treated in accordance with NIH Guide for the Care and Use of Laboratory Animals (1996). Adult male and female Sprague–Dawley rats were sexually mature (11 to 13 weeks old). Ovariectomized female Sprague–Dawley rats were purchased from Charles River laboratory and used 3 weeks after surgery. Estradiol pellets (Innovative Research of America), administered at a dose of 6 μg per day, were implanted in males for 2 weeks before the study.

Myocardial Ischemia

The in vivo left coronary artery occlusion was performed as described in the Online Data Supplement, available at http://circres.ahajournals.org. Langendorff-perfused hearts were also studied and infarct size and left ventricular developed pressure were measured as described in the Online Data Supplement.

Mitochondrial and Cardiomyocyte Isolation

Mitochondria and cardiomyocytes were isolated as described in the Online Data Supplement.

H₂O₂ Production and Aldehyde Dehydrogenase Activity

Hydrogen peroxide (H₂O₂) production from isolated heart mitochondria or myocytes was monitored fluorimetrically by measurement of oxidation of Amplex red to fluorescent resorufin (Invitrogen, Carlsbad, Calif). Aldehyde dehydrogenase (ALDH) activity was measured as described in the Online Data Supplement.

Proteomics

Details of the Western blot, 2D differential gel electrophoresis (2D-DIGE) (24 and 11 cm), and phospho-proteomics detection are provided in the Online Data Supplement.

Statistics

Data are presented as means±SE. Statistics were performed using ANOVA analysis followed by a Tukey post hoc test for multiple comparison or t test for comparison between 2 groups.

Results

Females Exhibit Less Ischemia and Reperfusion Injury

There are no significant male/female differences in hemodynamics during baseline perfusion. Heart rate was 278±18 bpm in males and 288±12 bpm in females. Baseline left ventricular developed pressure was not significantly different between males (152±12 cm water) and females (143±12 cm water). To assess male/female differences in ischemia and reperfusion (I/R) injury we examined whether there were sex differences in posts ischemic contractile function or infarct size. Figure 1 shows that females have less injury than males. Figure 1A shows that compared to male hearts, female hearts have significantly better posts ischemic recovery of rate-pressure product (expressed as a percentage of preischemic rate–pressure product [RPP]). Figure 1B shows that after 30 minutes of ischemia male hearts exhibited significantly more necrosis than females.

To assess the role of estradiol in the cardioprotection observed in females, we examined posts ischemic function and infarct size in intact females compared to ovariectomized (ovx) females. Figure 1C shows that hearts from ovx females had poorer recovery of posts ischemic function than hearts from intact females. Figure 1D shows that hearts from ovx females also exhibited significantly more necrosis than intact females. We also found (Figure 1E and 1F) that treatment of males with estradiol for 2 weeks reduced ischemic injury. We also performed studies to determine whether these male/female differences in I/R injury occurred in an in vivo model of left anterior descending coronary artery (LAD) occlusion. The LAD was occluded for 45 minutes and reperfused for 2 hours, and consistent with studies in perfused heart, we observed that females had significantly smaller infarcts (expressed as % of area at risk) than males (Figure 1G).

Mechanisms Responsible for Reduce Ischemic Injury in Females

We were interested in elucidating mechanisms involved in the protection observed in females. As mitochondria have been shown to be at the center of cardioprotection, we focused our attention on sex differences in mitochondrial proteins. To exam-
The basis for the sex differences in protection we used a proteomic approach to determine whether there were male/female differences in mitochondrial proteins. We first performed a series of experiments to assess the purity and integrity of the mitochondria (see Online Figure I). The mitochondria show enrichment of mitochondrial markers and lack cytosolic markers (see Online Figure I).

### Two-Dimensional Differential Gel Electrophoresis

The protection observed in females is likely to be mediated by either changes in protein levels or changes in posttranslational modifications. To determine differences in protein levels and posttranslational modifications, mitochondria from male and female hearts were separated by 2D fluorescence difference gel electrophoresis (DIGE). Figure 2 shows a representative 2D-DIGE in which the male samples are indicated in red and female samples in green. If the peptide is present in males and females at similar levels and with the same posttranslational modifications, it will be yellow (equal green and red). Peptide spots in red are present at higher levels in males, whereas spots in green are present at higher levels in females. Using the Progenesis software (Nonlinear Dynamics, Durham, NC) a comparison be-
tween male and female mitochondrial proteins revealed significant differential expression and/or migration of 25 peptides, which corresponded to 20 proteins because of multiple locations of some peptides resulting from posttranslational modifications (Figure 2 and the Table). The proteins that were identified by the software as significantly different between males and females, as well as some nearby spots that might be attributable to posttranslational modifications (spots 26 to 30), were extracted and identified by mass spectrometry using MALDI TOF/TOF (matrix-assisted laser desorption/ionization tandem time of flight) (Table). Most of the proteins identified are related to metabolism, including control points in the Krebs cycle \( \alpha \)-KGDH, and several elements of the electron transport chain (NADH dehydrogenase [ubiquinone], cytochrome c oxidase subunit VIb isoform 1, and ATP synthase subunit \( \epsilon \)). These differences could be attributable to differences in protein expression or to differences in posttranslational modifications.

Some of the male/female differences are clearly attributable to posttranslational modification as indicated by the multiple locations of the same protein (eg, ALDH2, pyruvate dehydrogenase [PDH]-E1\( \alpha \)). As shown in inset A to Figure 2, for PDH-E1\( \alpha \), females show an increase in the spot at the higher isoelectric point (pl) (spot 5) and a decrease in the spot at a lower pl value (spot 7) consistent with a less of an acidifying posttranslational modification such as phosphorylation. We confirmed the increase in phosphorylation of PDH-E1\( \alpha \) using ProQ Diamond staining (Online Figure II). Because phosphorylation of the E1 subunit of PDH decreases the activity of PDH, the increase in the phosphorylation of this subunit in males would account for the relative reduction in glucose metabolism that we observed previously in males compared to females. This would also be consistent with a study showing increased expression of PDH-kinase in males.

Multiple spots were also identified for the E2 subunit of PDH (spots 2, 26, 27, and 28). Only spot number 2 was significantly elevated in females, but all were identified by mass spectrometry as the E2 component of PDH. Multiple spots were also identified as the E2 subunit of \( \alpha \)-KGDH (spots 3, 29 and 30). The increase in migration at these more acidic pl values in females for the E2 components of PDH and \( \alpha \)-KGDH suggest an increase in phosphorylation in females in these subunits. We confirmed phosphorylation of \( \alpha \)-KGDH in females using an antibody that recognized protein kinase (PK)C substrate phosphorylation sites (data not shown).

Role of Aldehyde Dehydrogenase 2 in Cardioprotection

Interestingly, we also noted multiple spots at different pl values for mitochondrial ALDH2. As shown in inset B in Figure 2, females show a decrease in ALDH2 at high pl values (spot 16) and an increase at low pl values (spot 18) consistent with an increase in a post-translational modification such as phosphorylation in females. We also performed 2D DIGE comparing intact females to ovx females and found that compared to intact females, ovx females showed many of the same protein changes as males (see Online Figure III). For example, we found that ovx females had less phosphorylation of ALDH2 than hearts from non-ovx females. Interestingly, a recent study showed that PKCc can phosphorylate ALDH2 and that hearts with increased phosphorylation of ALDH2 had reduced infarct size. We therefore ran 1D and 2D gel electrophoresis and probed with an antibody that recognizes PKC substrate phosphorylation sites. We then stripped and then reprobed with an antibody for total ALDH2 to quantitated the ratio of phosphoALDH2 to total ALDH2. Representative gels for male and female are shown in Figure 3A. We also used mass spectrometry to confirm that these spots contained ALDH2. Figure 3B summarized quantitative data on the ratio of phosphoALDH2 to total ALDH2, showing that mitochondria from female hearts have increased phosphorylation of ALDH2. We confirmed that consistent with the increase in phosphorylation that mitochondria from females had an increase in ALDH activity (Figure 3C). Interestingly treatment with estradiol resulted in an increase in phosphorylation of ALDH2 in males (Figure 3B). Consistent with an
increase in PKC dependent phosphorylation of ALDH2 in females mitochondria we showed that mitochondria from female hearts have increased mitochondrial PKCe (Figure 3D).

Chen et al also identified an activator of ALDH2, called Alda-1, which was shown to be cardioprotective. We hypothesized that if phosphorylation and activation of ALDH2 is important in the protection that we observed in female, then addition of Alda-1 should improve recovery of function and reduce infarct size in males to a greater extent than in females (because females already have phosphorylated ALDH2). Consistent with this hypothesis, we observed that Alda-1 reduced post ischemic contractile dysfunction in males but not in females (Figure 4A). Alda-1 also reduced infarct size in male (Figure 4B), but not in female hearts. We similarly hypothesized that a PKC activator such as DOG (1,2-dioctanoyl-sn-glycerol), might be more protective in males than in females. We therefore perfused male and female hearts with DOG and consistent with the hypothesis we observed that DOG treatment improved recovery of function and decreased infarct size in male, but not in female hearts (Figure 4C and 4D). In addition we also tested whether a PKC inhibitor would block the protection that we observe in females. As shown in Figure 4E and 4F, we find that Ro-317549, a PKC inhibitor blocked the protection observed in females.

We were also interested in determining whether the changes such as the increase in phosphorylation of ALDH2 observed in females at baseline persisted during I/R. We therefore performed 2D-DIGE after 30 minutes of ischemia and 10 minutes of reperfusion and as shown in Online Figure IV, many of the changes such as the increase in phosphorylation of ALDH2 in females persisted during I/R.

### Table. Two-Dimensional Differential Gel Electrophoresis Results

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Protein Name</th>
<th>Molecular Mass (Da)</th>
<th>Theoretical Protein pI</th>
<th>Peptide Count</th>
<th>Change in Female</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(Q5SGE0) Leucine-rich PPR motif–containing protein</td>
<td>157 808</td>
<td>6.20</td>
<td>4</td>
<td>Increase 42%</td>
<td>0.030</td>
</tr>
<tr>
<td>2</td>
<td>(P08461) Dihydrolipoylsine-residue acetyltransferase component of pyruvate dehydrogenase complex</td>
<td>67 116</td>
<td>8.76</td>
<td>2</td>
<td>Increase 26%</td>
<td>0.042</td>
</tr>
<tr>
<td>3</td>
<td>(Q01205) Dihydrolipoylsine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex</td>
<td>48 925</td>
<td>8.89</td>
<td>9</td>
<td>Increase 42%</td>
<td>0.017</td>
</tr>
<tr>
<td>4</td>
<td>(O68FXO) Isocitrate dehydrogenase [NAD] subunit β</td>
<td>42 612</td>
<td>8.89</td>
<td>6</td>
<td>Increase 82%</td>
<td>0.027</td>
</tr>
<tr>
<td>5</td>
<td>(P26284) Pyruvate dehydrogenase E1 component subunit α</td>
<td>43 888</td>
<td>8.49</td>
<td>12</td>
<td>Increase 42%</td>
<td>0.042</td>
</tr>
<tr>
<td>6</td>
<td>(P26284) Pyruvate dehydrogenase E1 component subunit α</td>
<td>43 888</td>
<td>8.49</td>
<td>3</td>
<td>Decrease 33%</td>
<td>0.050</td>
</tr>
<tr>
<td>7</td>
<td>(P26284) Pyruvate dehydrogenase E1 component subunit α</td>
<td>43 888</td>
<td>8.49</td>
<td>5</td>
<td>Decrease 37%</td>
<td>0.040</td>
</tr>
<tr>
<td>8</td>
<td>(O63065) Pyruvate dehydrogenase [lipoamide] kinase isozyme 1</td>
<td>49 392</td>
<td>8.09</td>
<td>4</td>
<td>Decrease 21%</td>
<td>0.050</td>
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<tr>
<td>9</td>
<td>(P51650) Succinate-semialdehyde dehydrogenase</td>
<td>52 669</td>
<td>6.40</td>
<td>4</td>
<td>Decrease 37%</td>
<td>0.020</td>
</tr>
<tr>
<td>10</td>
<td>(O62425) NADH dehydrogenase [ubiquinone] 1 α subcomplex subunit 4</td>
<td>9321</td>
<td>9.52</td>
<td>2</td>
<td>Decrease 31%</td>
<td>0.009</td>
</tr>
<tr>
<td>11</td>
<td>(P80430) Cytochrome c oxidase subunit Vb isoform 1 (COX Vb-1)</td>
<td>10 293</td>
<td>8.96</td>
<td>4</td>
<td>Decrease 57%</td>
<td>0.013</td>
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<tr>
<td>12</td>
<td>(P29419) ATP synthase α chain</td>
<td>8249</td>
<td>9.34</td>
<td>6</td>
<td>Decrease 51%</td>
<td>0.040</td>
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<tr>
<td>13</td>
<td>(P84817) Mitochondrial fission 1 protein (Fis1 homolog) (Fis1)</td>
<td>17 041</td>
<td>8.56</td>
<td>5</td>
<td>Decrease 28%</td>
<td>0.042</td>
</tr>
<tr>
<td>14</td>
<td>(P07483) Fatty acid-binding protein, heart (H-FABP)</td>
<td>14 766</td>
<td>5.90</td>
<td>5</td>
<td>Decrease 19%</td>
<td>0.027</td>
</tr>
<tr>
<td>15</td>
<td>(P15651) Short-chain specific acyl-CoA dehydrogenase</td>
<td>45 022</td>
<td>8.96</td>
<td>9</td>
<td>Decrease 31%</td>
<td>0.040</td>
</tr>
<tr>
<td>16</td>
<td>(P11884) Aldehyde dehydrogenase, mitochondrial precursor (ALDH-E2)</td>
<td>56 966</td>
<td>6.63</td>
<td>5</td>
<td>Decrease 31%</td>
<td>0.042</td>
</tr>
<tr>
<td>17</td>
<td>(P11884) Aldehyde dehydrogenase, mitochondrial precursor (ALDH-E2)</td>
<td>56 966</td>
<td>6.63</td>
<td>11</td>
<td>Decrease 31%</td>
<td>0.050</td>
</tr>
<tr>
<td>18</td>
<td>(P04797) Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)</td>
<td>36 090</td>
<td>8.14</td>
<td>3</td>
<td>Decrease 37%</td>
<td>0.003</td>
</tr>
<tr>
<td>19</td>
<td>(P42123) L-lactate dehydrogenase B chain (LDH-B)</td>
<td>36 874</td>
<td>5.70</td>
<td>5</td>
<td>Decrease 34%</td>
<td>0.013</td>
</tr>
<tr>
<td>20</td>
<td>(P60711) Actin, cytoplasmic 1 (β-actin)</td>
<td>42 612</td>
<td>5.22</td>
<td>9</td>
<td>Decrease 49%</td>
<td>0.002</td>
</tr>
<tr>
<td>21</td>
<td>(P62738) Actin, aortic smooth muscle (α-actin-2)</td>
<td>42 381</td>
<td>5.23</td>
<td>8</td>
<td>Decrease 64%</td>
<td>0.003</td>
</tr>
<tr>
<td>22</td>
<td>(P62738) Actin, aortic smooth muscle (α-actin-2)</td>
<td>42 381</td>
<td>5.23</td>
<td>10</td>
<td>Decrease 59%</td>
<td>0.001</td>
</tr>
<tr>
<td>23</td>
<td>(P16409) Myosin light polypeptide 3</td>
<td>22 256</td>
<td>5.03</td>
<td>8</td>
<td>Decrease 30%</td>
<td>0.017</td>
</tr>
<tr>
<td>24</td>
<td>(P51667) Myosin regulatory light chain 2, ventricular/cardiac muscle isoform (MLC-2v)</td>
<td>18 852</td>
<td>4.86</td>
<td>7</td>
<td>Decrease 32%</td>
<td>0.011</td>
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<tr>
<td>25</td>
<td>(P08461) Dihydrolipoylsine-residue acetyltransferase component of pyruvate dehydrogenase complex</td>
<td>67 116</td>
<td>8.76</td>
<td>7</td>
<td>Increase 2%</td>
<td>NS</td>
</tr>
<tr>
<td>26</td>
<td>(P08461) Dihydrolipoylsine-residue acetyltransferase component of pyruvate dehydrogenase complex</td>
<td>67 116</td>
<td>8.76</td>
<td>5</td>
<td>Decrease 10%</td>
<td>NS</td>
</tr>
<tr>
<td>27</td>
<td>(P08461) Dihydrolipoylsine-residue acetyltransferase component of pyruvate dehydrogenase complex</td>
<td>67 116</td>
<td>8.76</td>
<td>3</td>
<td>No difference</td>
<td>NS</td>
</tr>
<tr>
<td>28</td>
<td>(Q01205) Dihydrolipoylsine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex</td>
<td>48 925</td>
<td>8.89</td>
<td>2</td>
<td>Increase 2%</td>
<td>NS</td>
</tr>
<tr>
<td>29</td>
<td>(Q01205) Dihydrolipoylsine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex</td>
<td>48 925</td>
<td>8.89</td>
<td>2</td>
<td>Increase 4%</td>
<td>NS</td>
</tr>
</tbody>
</table>

Proteins identified by MALDI TOF/TOF with 2 or more different peptides. N=4 in each group.
Role of PI3K

Because estrogen has been reported to signal by activation of the PI3K pathway, we were interested in determining if inhibition of the PI3K pathway with wortmannin (WM) would block the protection and/or block the increased phosphorylation of ALDH2 that was observed in females. As shown in Figure 4G and 4H, addition of WM, 10 minutes before ischemia, increased postischemic contractile dysfunction and increased infarct size in females but not in males. Because WM blocked the protection observed in females, we were interested in determining whether the increase in phosphorylation of ALDH2 would also be blocked by WM. As shown in Figure 3B, there was significantly less phosphorylation of ALDH2 in female hearts treated with WM compared to female hearts without WM. These data suggest that addition of WM concomitantly blocks the increased phosphorylation of ALDH2 and the protection observed in females. Figure 3B also shows that WM had no effect on phosphorylation in males.

Role of α-KGDH in Cardioprotection

In Figure 2, we observed male/female differences in the isoelectric shift of α-KGDH. We confirmed phosphorylation of α-KGDH in females using an antibody that recognized PKC substrate phosphorylation sites (data not shown). We were interested in identifying potential functional consequences of the modification of this dehydrogenase. NAD is a substrate for α-KGDH and it has been shown that α-KGDH can generate ROS,

tent with previous studies showing that high NADH levels increase ROS production by α-KGDH. These data suggest that α-KGDH in female mitochondria is less susceptible to generation of ROS under conditions of high NADH/NAD.

As high NADH levels are present during anoxia or ischemia and at the start of reoxygenation, and as the start of reoxygenation is a time in which production of ROS increases, we examined whether there were male/female differences in ROS production following anoxia and reoxygenation. Using Amplex red we measured H2O2 production by mitochondria from males and females under normoxic conditions, and we found no significant difference between the groups (Figure 5B). We next examined whether there were male/female differences in ROS production in mitochondria that were subjected to anoxia and reoxygenation. We observed that on reoxygenation, after 30 minutes of anoxia, ROS generation by male mitochondria is markedly increased compared to normoxic mitochondria, whereas ROS generation was only mildly increased in females (see Figure 5C). The ROS production in male mitochondria on reperfusion was significantly greater than in female mitochondria.

Our data show that females have altered posttranslational modification of α-KGDH and less ROS production. To examine the hypothesis that increased phosphorylation of α-KGDH might be causally involved in the decrease in α-KGDH-mediated ROS production, we tested whether in vitro phosphorylation of purified α-KGDH might alter ROS production of α-KGDH. The NetPhosK database (http://www.cbs.dtu.dk/services/NetPhosK/) identified PKC as a likely kinase to phosphorylate α-KGDH.

Initially we wanted to verify that recombinant, active PKCe can induce in vitro phosphorylation of α-KGDH. As shown in
Figure 6A, addition of recombinant active PKCε to purified α-KGDH resulted in phosphorylation of α-KGDH. To determine whether increased phosphorylation of α-KGDH alters ROS generation, we phosphorylated α-KGDH with PKCε and measured ROS generation using Amplex Red. As illustrated in Figure 6B and 6C, phosphorylated α-KGDH exhibits significantly less ROS production when CoA, α-KG and NADH are added. Addition of NAD prevents the increase in ROS production by both phosphorylated and nonphosphorylated α-KGDH.

Because we found that WM blocked the protection in females (see Figure 4), we were interested in determining whether the reduced ROS production observed in females after anoxia and reoxygenation can be blocked by WM. We treated male and females cardiac myocytes with 100 nmol/L WM just before simulated ischemia (30 minutes) and we observed that ROS production after simulated ischemia was significantly higher in females myocytes in the presence of WM compared to the absence of WM (slopes: female WM, 4.7±1.0; female control, 1.4±0.2; P<0.05). In contrast, WM had no effect on ROS production in males on reoxygenation following simulated ischemia (slopes: male WM, 3.4±1.4; male control, 4.7±1.0; P>0.05) and males were similar to females plus WM (Figure 7A through 7C).

**Discussion**

In this study we report that females have less I/R injury than males both in vivo and in an isolated perfused heart model. Because mitochondria play a central role in ischemia reperfusion injury, we examined male/female differences in the mitochondrial proteome and identified a number of mitochondrial proteins that have male/female differences in post-translational modification. In particular we find that males have increased phosphorylation of the PDH-E1 subunit and females have increased phosphorylation of ALDH2, and the E2 subunit of α-KGDH.

PDH catalyzes the conversion of pyruvate to acetyl-CoA. PDH activity is inhibited by phosphorylation of the PDH-E1α subunit. The sex differences in phosphorylation of PDH-E1α are consistent with a recent report that females have decreased mRNA expression of PDH kinase. The increased phosphory-
lation of PDH-E1α in males is consistent with our previous finding that compared to females, males have a lower ratio of carbohydrate/fatty acid metabolism. Churchill et al. showed that PKC translocation to the mitochondria during reperfusion resulted in inhibition of PDH and increase injury. Activation of PDH has been shown to be beneficial. Thus a decrease in phosphorylation (and an increase in activity) of PDH in females would be expected to reduce ischemic injury. In addition to

**Figure 5.** H$_2$O$_2$ production from male and female rat heart mitochondria measured by Amplex red. A, Permeabilized mitochondria with 0.12 mmol/L reduced form of coenzyme A of α-ketoglutarate as a substrate, NADH is added as indicated. B, Intact normoxic mitochondria with glutamate/malate (G/M) as substrates. C, Reoxygenated mitochondria with G/M as substrates (after 30 minutes of anoxia). *P<0.05.

**Figure 6.** Effect of in vitro phosphorylation of α-ketoglutarate dehydrogenase. A) In vitro phosphorylation of α-KGDH by PKC; B) ROS generated from α-KGDH in presence of NAD or NADH with or without prephosphorylation by PKC; C) Plot of the slope of ROS generation from α-KGDH in presence of NAD with or without PKC pretreatment. *P<0.05.
being a key regulator of metabolism, PDH and α-KGDH are responsible for a significant amount of ROS generated by mitochondria.28,29,32 Interestingly we find male/female differences in phosphorylation of α-KGDH. In the absence of NAD, oxygen can act as an electron acceptor for α-KGDH, thereby generating superoxide. Also the lipoamide dehydrogenase that is present in α-KGDH and PDH is capable of functioning as an NADH oxidase leading to \( \text{H}_2\text{O}_2 \) generation.28 The generation of ROS by αKGDH is dependent on the NADH/NAD ratio; a high ratio enhances ROS formation. This observation led us to speculate that the modification of α-KGDH observed in females might attenuate the ROS generation by α-KGDH. Consistent with this hypothesis we found reduced ROS formation in female mitochondria after addition of α-ketoglutarate and CoA-SH with addition of NADH. To further test this hypothesis we phosphorylated purified α-KGDH by addition of active PKC and showed that addition of substrates and NADH to phosphorylated α-KGDH resulted in less ROS generation than was observed with nonphosphorylated α-KGDH. NADH levels at the start of reperfusion are higher and therefore might be expected to cause a larger increase in ROS via α-KGDH in males than in females.

Studies in Figure 5 confirmed this hypothesis. We also find that WM blocks the reduction in ROS observed in females following I/R (Figure 7).

Our finding of less ROS generation in female mitochondria is also consistent with other reports in the literature.21–24 However, our data provide a mechanism for the reduced ROS generation in females. We propose that sex differences in post-translational modification of α-KGDH result in altered ROS generation, particularly under conditions of high NADH/NAD, conditions that occur at the start of reperfusion after ischemia, which is a key time for cardioprotection.

We also made the novel observation that females have increased phosphorylation and activation of ALDH2. ALDH2 and succinate-semialdehyde dehydrogenase are both involved in detoxifying toxic aldehydes such as 4-hydroxy-2-nonenal (HNE) which is an end product of lipid peroxidation. A recent study showed that PKC\( \varepsilon \) activation induces increased phosphorylation of mitochondrial ALDH2, which results in a decrease in ischemic injury.25 HNE has also been reported to regulate mitochondrial uncoupling, at least in part by interaction with the adenine nucleotide translocator.33 Interestingly, a number of the mitochondrial male/female differences observed are involved in regulating ROS homeostasis. We report altered posttranslational modification of α-KGDH, which is an important generator of mitochondrial ROS. We also report increased phosphorylation and activation of ALDH2, which has been shown to be cardioprotective. These data are consistent with a growing number of studies reporting that mitochondria from females generate less ROS and that hearts from females show less oxidative damage.21–24 Protein changes associated with oxidative stress were shown to be greater in aged males compared to aged females suggesting higher ROS in males.34

The reduced I/R injury that we observe in females appears to be mediated by the PI3K pathway as the protection was blocked by treatment with WM. Mitochondria from females also have increased PKC\( \varepsilon \). Shinmura et al also showed that PKC\( \varepsilon \) depen-
dent signaling is altered in oxv female mice and is responsible for the loss of ischemic preconditioning.35 Estrogen has been shown to activate PI3K16 and activation of PI3K has been shown to be important in cardioprotection18; thus these data provide a plausible mechanism for the protection observed in females. The finding that the PI3K pathway mediates protection is also consistent with the observation that a number of proteins exhibit male/female differences in post translational modification. Interestingly we find that WM blocked the increase in phosphorylation of ALDH2. These data support the conclusion that the increased phosphorylation of ALDH2 is modulated by the PI3K pathway and is an important mediator of the cardioprotection observed in females.

In summary, female Sprague–Dawley rat hearts have less I/R injury than males. Consistent with reduced I/R injury in females, we find that mitochondria from females have a number of posttranslational modifications in mitochondrial enzymes involved in regulating ROS generation and oxidative metabolism, and females have reduced ROS generation on reoxygenation. Online Figure V presents a model in which estrogen activation of PI3K and PKC leads to phosphorylation of ALDH2, which decreases toxic aldehydes generated by ROS. Activation of PI3K also increases phosphorylation of α-KGDH which reduces ROS generation under conditions of high NADH which occur with I/R. Taken together, these data provide a mechanistic basis for the protection observed in females.

Acknowledgments

We thank the National Heart, Lung, and Blood Institute Laboratory of Animal Medicine and Surgery, especially Randy Cleveenger, Karen Keenan, and Kenneth Jeffries, for their support. We thank Dr Mochly-Rosen for Alda-1. We thank Drs Junhui Sun and Renee Wong for helpful comments.

Sources of Funding

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Disclosures

None.

References

Novelty and Significance

What Is Known?

- Females have less cardiovascular disease than males.
- Low levels of reactive oxygen species (ROS) are important in cell signaling whereas high levels of ROS can contribute to cell death and data suggest that female mitochondria produce less ROS than males.
- Phosphorylation of mitochondrial aldehyde dehydrogenase (ALDH)2, an enzyme that detoxifies ROS generated aldehyde adducts, has been shown to reduce ischemia/reperfusion (I/R) injury.

What New Information Does This Article Contribute?

- This study provides novel information in support of the hypothesis that cardioprotection in females involves a phosphatidylinositol 3-kinase (PI3K)-mediated decrease in ROS production and better detoxification of ROS byproducts.
- Our data show male/female differences in phosphorylation of α-KGDH, a major source of ROS, and that α-KGDH from female mitochondria produces less ROS.
- Our data show male/female differences in phosphorylation and activity of ALDH2.

This study examined the mechanistic basis for reduced I/R injury in females. We provide novel data demonstrating male/female differences in phosphorylation of 2 proteins involved in ROS handling, ALDH2 and α-KGDH. We further show that phosphorylation of these proteins is mediated by PI3K and that phosphorylation is important in the protection observed in females. We demonstrate that inhibition of PI3K blocks both the protection in females and phosphorylation of ALDH2. We further show that females have increased activity of ALDH2 and that an activator of ALDH2 is more protective in males than in females. We also show that the increase in phosphorylation of α-KGDH reduces ROS generation by this enzyme and consistent with this playing an important role in protection in females, we find that females generate less ROS following ischemia than males and that inhibition of PI3K results in an increase in postischemic ROS production in females to a level similar to that observed in males. These studies provide important new insights into male/female differences in handling of ROS and show a role for altered enzyme activity in protection in females. These studies could have important clinical implications for understanding the basis of gender differences in cardiac disease.
Sex Differences in the Phosphorylation of Mitochondrial Proteins Result in Reduced Production of Reactive Oxygen Species and Cardioprotection in Females
Claudia J. Lagranha, Anne Deschamps, Angel Aponte, Charles Steenbergen and Elizabeth Murphy

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Supplemental Material:

Animals

All animals (Charles River Laboratory) were treated in accordance with Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 1996). All protocols were approved by the Institutional Animal Care and Use Committee. Adult male and female Sprague-Dawley rats were sexually mature (11-13 weeks old). Ovariectomized female rats were obtained from Charles River Laboratory.

In vivo myocardial ischemia

The left coronary artery occlusion model has been described previously. Briefly, male and female rats were anesthetized with ketamine (95 mg/kg) plus xylazine (5.2 mg/kg) injected intramuscularly, and administered glycopyrrolate (0.02 mg/kg) intramuscularly. The animals were intubated under direct visualization, passing an endotracheal tube into the trachea. Anesthesia was maintained with 1-3% isoflurane in oxygen, administered through a precision vaporizer and a positive pressure ventilator. Body temperature was carefully monitored with a rectal probe and was maintained at 37.0 °C throughout the experiment by using circulating heated water blankets. After respiratory stabilization, a skin incision was made, and the ribs were gently spread to expose the left side of the heart and visualize the left anterior descending coronary artery (LAD). An 8-0 Vicryl suture (Ethicon, Inc.) was placed around the LAD and the ends were threaded through a silicone tube to form a snare. Coronary artery occlusion was produced by tightening the snare and was verified by the presence of epicardial cyanosis in the ischemic zone. Typical ECG changes immediately after LAD occlusion were used as signs of successful induction of anterior wall ischemia. After 45 min occlusion, reperfusion was achieved by loosening the snare. All rats were subjected to a 45 min LAD occlusion and 2 h reperfusion. After 2 hours the LAD was re-occluded in the same location as before. Evans blue dye (5 ml of a 7.0% solution; Sigma-Aldrich) was injected through the carotid artery catheter into the heart for delineation of the ischemic zone from the non-ischemic zone. The heart was rapidly excised and was cut into 3 or 4 cross-sectional pieces then incubated in 1.0% 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich) in 0.1 M phosphate buffer adjusted to pH 7.4 for 15 minutes at 37°C for demarcation of the viable and nonviable myocardium within the risk zone. The areas of infarction, area at risk (AAR), and non-ischemic left ventricle were assessed using NIH Image J.

Langendorff Perfused Hearts

After intraperitoneal injection of sodium pentobarbital, a transverse abdominal incision was made, the vena cava was exposed, and heparin sodium was administered intravenously. The heart was quickly excised, placed in ice cold Krebs-Henseleit (K-H) buffer (120 mM NaCl, 4.6 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, 1.25 mM CaCl2, 25 mM NaHCO3, 11 mM glucose), and connected to the perfusion cannula via the aorta. A water filled latex balloon was inserted into the left ventricle to measure hemodynamic parameters using a Powerlab 4/25 and Chart v5.5 software (AD Instruments, Colorado Springs, CO). Hearts were stabilized for 20 minutes with K-H buffer gassed with 95% O2 and 5% CO2, followed by 30 minutes of global ischemia and 1.5 hours of reperfusion. Rate pressure product (left ventricular developed pressure, LVDP × heart rate) was used as a measure of function. Hearts were maintained at 37°C for the duration of the protocol. The hearts that received wortmannin (WM) were stabilized for 10 minutes with K-H buffer then perfused for 10 minutes with WM (100nM) and subjected to 30 minutes of global ischemia followed by reperfusion as previously published. The hearts that received Alda-1 (aldehyde dehydrogenase activator) were stabilized for 10 minutes with K-H buffer then perfused for 10 minutes with Alda-1 (20μM) prior to 30 minutes of global ischemia and for 10 min immediately at the onset of reperfusion as previously published. The hearts that
received DOG (1,2-dioctanoyl-sn-glycerol) were stabilized for 15 minutes with K-H buffer then perfused for 5 minutes with DOG (3μM) followed by 10 min of washout prior to 30 minutes of global ischemia as previously published⁴. The hearts that received Ro-31-7549 (PKC inhibitor) were stabilized for 10 minutes with K-H buffer then perfused for 10 minutes with (875nM) prior to 30 minutes of global ischemia. At the end of reperfusion infarct size was measured with 2,3,5-triphenyltetrazolium chloride (TTC) as described previously⁵,⁶. Hearts were initially perfused with TTC and then incubated in TTC for an additional 15 minutes at 37°C. The hearts were subsequently fixed in formalin and 4-6 cross sectional slices were taken. These slices were imaged on a Leica Stereoscope and the percentage of infarct (white area) to viable tissue (red area) was analyzed using ImageJ software. Area of infarct was expressed as a percentage of total ventricles.

**Cardiomyocyte isolation**

Adult cardiomyocytes were isolated from the left ventricle of male and female Sprague-Dawley rats. Rats were anesthetized with pentobarbital sodium and heparinized; hearts were excised, mounted on a Langendorff apparatus and perfused with Ca²⁺ free normal Tyrode solution. Blendzyme Type IV (0.042 mg/ml) (Roche Applied Science, Indianapolis, IN) was then added to the perfusate at 37°C. After 5–8 min, the ventricles were removed, cut, and shaken gently at 34°C in the blendzyme IV solution. This digestion was repeated 4-6 times as needed.

**Simulated ischemia model**

The cardiac myocytes were subjected to simulated ischemia by pelleting the myocytes as described previously⁷. Briefly, freshly isolated myocytes from male or female rat heart (2x10⁴ cells) were divided into experimental groups and treated with or without 100nM of wortmannin (WM) for 10 min and then covered with mineral oil and incubated for 30 min.

**Mitochondria isolation**

For mitochondria isolation, hearts were cannulated and perfused with K-H buffer for 5 minutes to washout the blood. The buffer was aerated with 95% O₂ and 5% CO₂, at pH of 7.4 at 37°C. Freshly isolated mitochondria were prepared from hearts by differential centrifugation as described previously⁸. After separation of the fractions, mitochondrial and cytosolic proteins were submitted to a western blotting protocol to verify the purity of the isolation procedure using prohibitin and LDH antibody as markers for mitochondria and cytosolic compartments respectively.

**Mitochondrial oxygen consumption**

Measurement of mitochondrial respiration was performed at 25°C in a chamber (600 µL) connected to a Clark-type O₂ electrode (Instech) and O₂ monitor (Model 5300, YSI, Inc). The mitochondria were incubated in respiration buffer containing (in mmol/L) KCl 120, MOPS 5, EGTA 1, KH₂PO₄ 5, and 0.2% BSA. After addition of glutamate/malate (10/2 mmol/L), state 3 respiration was measured by addition of ADP (0.5 mmol/L). On depletion of ADP, state 4 respiration was determined.⁸

**H₂O₂ production**

Hydrogen peroxide (H₂O₂) production from isolated heart mitochondria or cardiac myocytes was measured fluorimetrically by measurement of oxidation of Amplex Red to fluorescent resorufin (Invitrogen kit). After ischemia, myocytes (2x10⁶ cells) were resuspended in a Media 199. Mitochondria were incubated at 1.75 mg/ml mitochondrial protein in buffer containing 120 mM KCl, 5 mM MOPS, 10 mM Tris and 5mM KH₂PO₄ (pH 7.25). All incubations also contained 50 μM Amplex Red, 5 U/mL of horseradish peroxidase. The increase in
fluorescence at an excitation of 544 nm and an emission of 590 nm was monitored. Standard curves were generated using known amounts of hydrogen peroxide. Hydrogen peroxide was measured in mitochondria (10 mM glutamate + 2 mM malate + ADP) during normoxia. The mitochondria were incubated until the oxygen was consumed and were left anoxic for 30 minutes followed by reoxygenation.

In another series of studies we measured H$_2$O$_2$ generation from α-ketoglutarate dehydrogenase. Permeabilized mitochondria (1% Triton) were incubated at a concentration of 1.0 mg/ml in buffer containing 120 mM KCl, 5 mM MOPS, 10 mM Tris and 5 mM KH$_2$PO$_4$ (pH 7.25). 50 μM Amplex Red, 5 U/mL of horseradish peroxidase, and 0.12mM HS-CoA were added. The reaction was started with the addition of 5mM of α-ketoglutarate. The increase in fluorescence (544nm excitation, 590nm emission) was monitored, and after 5 minutes, 2 μM of NADH was added.

**ALDH activity**

Enzymatic activity of mitochondrial ALDH was determined spectrophotometrically by monitoring the reductive reaction of NAD+ to NADH at 340 nm as previously described. ALDH2 assays were carried out at 25 °C in 60 mM Na-pyrophosphate buffer (pH 8.5) with 5 mM pyrazole. To this volume, 200 μM propionyl aldehyde and 250 μg of mitochondrial protein lysate were added. To start the reaction, 2 mM NAD was added and the accumulation of NADH was monitored for 3 min. ALDH activity were expressed as μmol/min/mg protein.

**Western blotting**

Hearts from male and female rats were perfused with K-H buffer for 5 minutes to washout the blood and freeze clamped for western blot analysis. Frozen tissue was pulverized in liquid nitrogen using mortar and pestle until completely powdered. Samples were suspended in standard lysis buffer. Protein concentrations were determined using Bradford assay and bovine serum albumin as a standard. Equal amounts of proteins of each sample (20 μg) were separated by 4-12% Bis-Tris Gel (Invitrogen, USA). The resolved proteins were electrophoretically transferred to nitrocellulose membranes at 30 V for 1 hour using a NuPAGE transfer buffer with 10% methanol added. Gel transfer efficiency and equal load was verified using reversible Ponceau staining. After blocking for 1 hour at room temperature with SuperBlock blocking buffer (Thermo scientific, Rockford, Illinois) membranes were incubated for 1 hour at room temperature with anti-phospho Serine/Threonine PKC substrate (rabbit, Cell signaling,1:1,000 dilution). The membrane was washed and a secondary antibody rabbit-HRP (Cell signaling,1:5,000 dilution) was added for 1 hour. After the final washes, the membranes were incubated with Western lightning Plus-ECL (PerkinElmer, Shelton, CT) and exposed to X-ray films. After exposure, membranes were stripped with Re-Blot Plus Mild Solution (Chemicon, Temecula, CA). The same protocol was followed as above with the primary antibody being aldehyde dehydrogenase-2 (1:1000, Santa Cruz Biotech, Santa Cruz, CA) and secondary being a donkey anti goat-HRP antibody (1:5000, Santa Cruz Biotech, Santa Cruz, CA). Optical densities for each band were obtained using ImageJ.

Equal amounts of α-KGDH from porcine hearts (cat # K1502, lot 075K7501; Sigma, St. Louis, MO) with and without phosphorylation by PKCe (PKCe kinase cat # 7492, Cell Signaling Technologies, Beverly, MA) were separated by 4-12% Bis-Tris Gel (Invitrogen, USA). The resolved proteins were electrophoretically transferred to nitrocellulose membranes at 10 V overnight using a NuPAGE transfer buffer with addition of 10% methanol. Gel transfer efficiency and equal load was verified using reversible Ponceau staining. After blocking for 2 hour at room temperature with BSA, membranes were incubated for 3 hours at room temperature with anti-phospho serine PKC substrate (Cell Signaling Technologies, Beverly, MA,1:1000 dilution). After washing, membranes were subsequently incubated with anti-rabbit horseradish peroxidase conjugated secondary antibody (1:5000, Cell Signaling Technologies, Beverly, MA) for 1 h at
room temperature. Proteins were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL) and visualized by autoradiography. The same protocol was used to analyze and quantify PKCε (1:1000, Santa Cruz Biotech, Santa Cruz, CA) in mitochondria from male and female samples. Optical densities for each band were obtained using ImageJ.

2D Gel Electrophoresis-DIGE (Two-dimensional difference in gel electrophoresis) in 24cm gels

Mitochondrial proteins isolated from rat heart were resuspended in 50 μl lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS and 15 mM Tris-HCl, at pH to 8.5. The total amount of protein concentration in each sample was determined using a modified Bradford assay, using a protein determination reagent (USB Corporation, OH, USA). Four hundred picomoles of CyDye (Cy3, Cy5 and Cy2) were used to label 50 μg of male, female and standard samples as suggested in the manufacturer's protocol. The samples were brought up to a 440 μL volume using rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS (w/v), 10 μl De-streak reagent (v/v) (GE Healthcare), and 1% (pH 3-10 NL) Pharmalyte (v/v), 13 mM DTT and trace amounts of bromophenol blue and then loaded onto an immobilized gradient strip (24 cm Immobiline DryStrip, pH 3-10NL, GE Healthcare/Amersham Biosciences, Piscataway, New Jersey).

Isoelectric focusing was achieved by active rehydration for ~10 h at 30 V followed by stepwise application of 250, 500, 1000, and 8000 V for a total of ~70000 Vh (Ettan IPG Phor, GE Healthcare/Amersham Biosciences, Piscataway, New Jersey). Immobiline DryStrip gel strips were equilibrated in 10 mL of SDS equilibration solution containing 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, trace amounts of bromophenol blue and 1.0% DTT for 10 min followed by a second 10 min incubation in a solution containing 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, trace amounts of bromophenol blue 2.5% iodoacetemide. The strips were briefly washed in electrophoresis buffer (25 mM Tris (pH 8.3), 192 mM glycine, and 0.2% SDS), applied to 10-15% SDS-PAGE gels (Nextgen gels, Ann Arbor, Michigan) and sealed with 0.5% agarose containing bromophenol blue. Electrophoresis was performed in an Ettan DALT-12 tank (GE Healthcare/Amersham Biosciences, Piscataway, New Jersey) in electrophoresis buffer until the dye front advanced completely (~2500 Vh). The gels were imaged using the Typhoon 9400 Variable Mode Imager (GE Healthcare/Amersham Biosciences, Piscataway, New Jersey) and were then placed in 500 mL of a fixative solution containing 30% ethanol and 7.5% acetic acid. After two short water rinses the gels were placed in 500 mL of Coomassie Blue protein gel stain for total protein visualization (EZBlue gel staining reagent, Sigma, Saint Louis, Missouri) for 48 hours. Finally, the gels were washed in de-staining solution for 3 hours, changed every hour, before scanning for total proteins.

2D-DIGE image analysis and mass spectrometry.

Cy2, Cy3, Cy5, Pro-Q and Coomassie Blue images were collected using a Typhoon scanner in fluorescence mode (GE Healthcare/Amersham Biosciences, Piscataway, New Jersey). The Cy2 images were scanned at an excitation wavelength of 520/40 (maximal/bandwidth) using a blue laser while the Cy3 images were scanned with an excitation wavelength of 580/30 using a green laser. The Cy5 images were scanned using a 670/30 excitation wavelength and a red laser. Pro-Q images were collected using the excitation wavelength set at 580/30 using a green laser. For total protein staining we used Coomassie blue and the images were scanned at an excitation wavelength at 610/30. Analysis for the differences between male and female proteins was performed using Progenesis SameSpots software (Nonlinear Inc, Durham, North Carolina). Spots of interest were matched to the Coomasie stained image. Spots of interest were processed by the fully automated Spot Handling Workstation (GE Healthcare/Amersham Biosciences, Piscataway, New Jersey).
Peptide extracts were analyzed using the Proteomics Analyzer (ABI 4700 MALDI TOF/TOF). Peptides were analyzed using the ABI GPS Explorer software (Applied Biosystems, Foster City, California) and full scan mass spectrometry spectra were obtained first followed by MS/MS spectra. Protein identification was carried out using the search engine MASCOT.\(^{12-14}\)

**Pro-Q Diamond**

To analyze phosphoproteins in 24 cm gel we used Pro-Q Diamond stain (Molecular Probes, Eugene, OR) which selectively stains phosphate groups attached to tyrosine, serine, or threonine residues. After the second dimension the gels were placed in a fixative solution containing 30% methanol and 7.5% acetic acid. The gels were rocked in this solution for 8 hours and the solution was replaced for overnight rocking. The gels were then washed 4 times with 500 mL of warm water for 30 min. The gels were stained with 500 mL of Pro-Q-Diamond stain, for 2 hours while shaking. The gels were then de-stained in 500 mL Pro-Q-Diamond Destain solution (Molecular Probes, Eugene, Oregon) every hour for a total of 3 hours. Finally, the gels were washed twice in water, (ten minutes each) and were imaged using the Typhoon 9400 (GE Healthcare/Amersham Biosciences, Piscataway, New Jersey) in fluorescence mode. After imaging, the gels were placed in Coomassie blue (EZBlue gel staining) for 48 hours rocking.\(^{12,13}\)

**2D Gel Electrophoresis, Western blotting in 11 cm gels and Peptide Identification**

Whole heart extracts were suspended in standard lysis buffer (7 M urea, 2 M thiourea and 4% CHAPS) and then cleaned using acetone precipitation. The samples (400 µg) were brought to 200 µL in rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS (w/v), 0.2% Bio-Lyte (pH 3-10 Bio-Rad, Hercules, California), 4 mM DTT and trace amounts of bromophenol blue) and then loaded onto an immobilized gradient strip (11 cm ReadyStrip IPG, pH 4-7, Bio-Rad, Hercules, California).

Isoelectric focusing was performed as previously described for the large 2D gels except that the total voltage hours were ~28000 Vh. ReadyStrip IPG strips were equilibrated as detailed previously. After equilibration the strips were briefly washed in electrophoresis buffer and applied to 8-16% Criterion Precast Tris-HCL gels (Bio-Rad, Hercules, California), sealed with 0.5% agarose containing bromophenol blue and run for 2 hours at 150V.

The proteins were electrophoretically transferred to nitrocellulose membranes at 10 V during 10 hours at 4°C. Gel transfer efficiency and equal loading was verified using MemCode reversible protein stain (Thermo scientific, Rockford, Illinois). After blocking for 1 hour at room temperature with SuperBlock blocking buffer (Thermo scientific, Rockford, Illinois) membranes were incubated for 3 hours at room temperature with anti-phospho serine PKC substrate (rabbit, Cell signaling, 1:1,000 dilution). The membrane was washed and a secondary antibody conjugated with infrared Dye (LI-COR, at 1:10,000) was added for 1 hour. After the final washes, the membranes were scanned and analyzed using the Odyssey™ Infrared Imager (LI-COR, USA).

The nitrocellulose membrane was placed in water overnight. Spots of interest were cut from the membrane, placed in new tubes and were destained with water until the MemCode stain was removed. To digest the peptides 20 µl of trypsin solution (Promega, Wisconsin) was added to each vial containing the membranes and 80 µl of 25 mM ammonium bicarbonate was added to cover. The vials were placed in a 37°C water bath for overnight digestion. The trypsin digests were transferred to new sterile tubes and the vials were placed in a speed vac until completely dried. The digests were resuspended in 20 µl of 0.1% formic acid. Each digest was concentrated and cleaned using C18 resin in a tip (Millipore, Massachusetts) following manufacture guidelines for LC MS/MS analysis.
The trypsinized samples were analyzed using nanoLC-ESI-MS/MS with LTQ Orbitrap XL. An Eksigent® NanoLC-2D™ HPLC with a C18 column 75 μm i.d. x 10 cm was used to separate peptides at 300 nL/min with 42 min gradients from 2 to 95% acetonitrile in 0.1% formic acid. MS scans were performed on the linear trap and up to six of the most intense ions per cycle were fragmented and analyzed in the linear trap.

Database searching was performed using the Mascot search engine (Matrix Science, v.2.2). All the MS/MS datasets were searched against the Swiss Prot (Swiss Institute of Bioinformatics) *Rattus norvegicus* database (v14.6; 7254 sequences). Protein modifications were selected as carbamidomethyl (C) (fixed) and oxidation (M) (variable). Up to one missed cleavage was allowed. The following criteria were used to generate a high-confidence data set: at least two peptides must be identified for each protein, with an ion expectation less than 1, corresponding to a significance level of p<0.05.

**RESULTS**

*Purity of the mitochondria*

To assess the purity of the mitochondria we measured the enrichment of mitochondrial proteins. As shown in Supplemental Figure I, the mitochondria show enrichment of mitochondrial markers and lack of cytosolic markers. Additionally, we verified the integrity of the isolated mitochondria by measuring mitochondrial state 3 and state 4 O₂ consumption.

*Phosphorylation of PDH-E1α*

As shown in Figure 2, for PDH-E1α, females show an increase in the spot at the higher pI and a decrease in the spot at a lower pI value consistent with less of an acidifying post translational modification such as phosphorylation in females. We confirmed the increase in phosphorylation of PDH-E1-α using ProQ staining (Suppl. Fig II and Suppl. Table I). Because phosphorylation of the E1 subunit of PDH decreases the activity of PDH, this increase in phosphorylation in this subunit in males would account for the relative reduction in glucose metabolism that we observed previously in males compared to females.

*2D DIGE comparing females to ovex females*

Hearts from bilaterally ovariectomized females show more post-ischemic contractile dysfunction and have larger infarcts size than hearts from intact female (see Figure 1D and E). To examine the mechanism involved in the cardioprotection observed in females compared with ovariectomized females we performed 2D-DIGE. As shown in Supplemental Figure III and Supplemental Table II, we found that ALDH2 from intact females was shifted to a more acidic pI compared to Ovx. The more basic pI for ALDH2 in Ovx hearts was similar to the pI value observed in males. These data suggest that the increase in phosphorylation in ALDH2 in female is related to estrogen.

*2D DIGE Male vs Female after ischemia*

We were interested in examining whether the male-female differences observed at baseline were maintained during ischemia and reperfusion. We therefore performed a set of experiments using a proteomic approach to examined gender-specific differences after 30 minutes of ischemia and 10 minutes of reperfusion. As shown in Suppl Fig. IV and Suppl. Table III, after ischemia and reperfusion the increase in ALDH2 in females at the more acidic (phosphorylated) pI was still present, suggesting that the phosphorylation in this enzyme in female is maintain after ischemia. We also found that the decreases in fatty acid binding protein and glyceraldehydes-3-phosphate dehydrogenase observed in females at baseline were preserved following ischemia and reperfusion.
REFERENCES:
1. Tilak GS, Hsu LY, Hoyt RF, Jr., Arai AE, Aletras AH. In vivo T2-weighted magnetic resonance imaging can accurately determine the ischemic area at risk for 2-day-old nonreperfused myocardial infarction. *Invest Radiol.* 2008;43:7-15.
Supplement Figure I:
Evaluation of mitochondrial integrity. A) Western blot for mitochondria and cytosolic markers; B) Mitochondria oxygen consumption; C) Respiratory Control Ratio (RCR).
Supplement Figure II:
Mitochondrial phospho-proteomics: In gel phosphorylated proteins using ProQ Diamond staining. Spot numbers 1, 2 and 3 were identified as PDH-E1α. Spot numbers 4 and 5 were identified as branched-chain α-keto acid dehydrogenase E1α.
Supplemental Figure III:
2D DIGE of Ovx vs Female. Representative overlay of equal amounts of protein between Ovx (Cy5, red) and females (Cy3, green).
Supplement Figure IV:
2D DIGE of Male vs Female mitochondrial extracts after ischemia. Representative overlay of equal amounts of protein between males (Cy5, red) and females (Cy3, green).
Supplemental Figure V:
Proposed mechanism by which estrogen mediates cardioprotection.
**Supplemental Table I**

<table>
<thead>
<tr>
<th>Spot #</th>
<th>Protein Name</th>
<th>MW (Da)</th>
<th>Theoretical Protein pI</th>
<th>Peptides Count</th>
<th>Changes in female</th>
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<td>1</td>
<td>(P35486) Pyruvate dehydrogenase E1α</td>
<td>43888</td>
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<td>43888</td>
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<tr>
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<td>50418</td>
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**Supplemental Table I:**

Differences in phosphorylation identified by ProQ. Proteins identified with 2 or more different peptides by mass spectrometry using MALDI TOF/TOF. Three animals were in each group.

**Supplemental Table II**

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<th>Spot #</th>
<th>Protein Name</th>
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<td>4</td>
<td>(Q920L2) Succinate dehydrogenase [ubiquinone] flavoprotein subunit,</td>
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<td>(P11884) Aldehyde dehydrogenase,</td>
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<td>9</td>
<td>(P15651) Short-chain specific acyl-CoA dehydrogenase,</td>
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**Supplemental Table II:**

Protein differences identified by 2D-DIGE and MS in Ovex vs Female. Proteins identified with 2 or more different peptides mass spectrometry using MALDI TOF/TOF. Three animals were in each group.
Supplemental Table III

<table>
<thead>
<tr>
<th>Spot #</th>
<th>Protein Name</th>
<th>M.W (Da)</th>
<th>Theoretical Protein pI</th>
<th>Peptides count</th>
<th>Changes in female</th>
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<td>3</td>
<td>(Q9Z2L0) Succinate dehydrogenase [ubiquinone] flavoprotein subunit,</td>
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<td>6.75</td>
<td>7</td>
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</tr>
<tr>
<td>4</td>
<td>(Q9Z2L0) Succinate dehydrogenase [ubiquinone] flavoprotein subunit,</td>
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<td>6.75</td>
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<td>Decrease</td>
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<tr>
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<td>(Q9Z2L0) Succinate dehydrogenase [ubiquinone] flavoprotein subunit,</td>
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<td>6.75</td>
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<tr>
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<td>(Q9Z2L0) Succinate dehydrogenase [ubiquinone] flavoprotein subunit,</td>
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<td>(A0JPQ4) Tripartite motif-containing protein 72;</td>
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Supplemental Table III:
Protein differences identified by 2D-DIGE in Male vs Female after ischemia. Proteins identified with 2 or more different peptides mass spectrometry using MALDI TOF/TOF. Three animals were in each group.