Acute inhibition of GSK causes mitochondrial remodeling

Tiffany Nguyen,1* Renee Wong,1* Guanghui Wang,2 Marjan Gueck,2 Charles Steenbergen,3 and Elizabeth Murphy1

1Systems Biology Center, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland; 2Proteomics Core Facility, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland; and 3Department of Pathology, Johns Hopkins Medical Institute, Baltimore, Maryland

Submitted 13 January 2012; accepted in final form 20 March 2012

Nguyen T, Wong R, Wang G, Gueck M, Steenbergen C, Murphy E. Acute inhibition of GSK causes mitochondrial remodeling. Am J Physiol Heart Circ Physiol 302: H2439–H2445, 2012. First published March 30, 2012; doi:10.1152/ajpheart.00033.2012.—Recent data have shown that cardioprotection can result in the import of specific proteins into the mitochondria in a process that involves heat shock protein 90 (HSP90) and is blocked by geldanamycin (GD), an HSP90 inhibitor. To test the hypothesis that an alteration in mitochondrial import is a more widespread feature of cardioprotection, in this study, we used a broad-based proteomics approach to investigate changes in the mitochondrial proteome following cardioprotection induced by inhibition of glycogen synthase kinase (GSK)-3. Mitochondria were isolated from hearts treated with a GSK-3 inhibitor, SB 216763 (SB) for 15 min before isolation of mitochondria. Mitochondrial extracts from control and SB-perfused hearts were labeled with isotope tags for relative and absolute quantification (iTRAQ), and differences in mitochondrial protein levels were determined by mass spectrometry. To test for the role of HSP90-mediated protein import, hearts were perfused in the presence and absence of GD for 15 min before perfusion with SB followed by mitochondrial isolation and iTRAQ labeling. We confirmed that treatment with GD blocked the protection afforded by SB treatment in a protocol of 20 min of ischemia and 40 min of reperfusion. We found 16 proteins that showed an apparent increase in the mitochondrial fraction following SB treatment. GD treatment significantly blocked the SB-mediated increase in mitochondrial association for five of these proteins, which included annexin A6, vinculin, and pyruvate kinase. We also found that SB treatment resulted in a decrease in mitochondrial content of eight proteins, of which all but two are established mitochondrial proteins. To confirm a role for mitochondrial import versus a change in protein synthesis and/or degradation, we measured changes in these proteins in whole cell extracts. Taken together, these data show that SB leads to a remodeling of the mitochondrial proteome that is partially GD sensitive.

Cardioprotection by preconditioning has been shown to be mediated by a number of signaling pathways, and inhibition of GSK-3 has been shown in many studies (12, 16, 18, 21, 23, 32, 33, 37, 38) to be an important component of cardioprotection. There are two isoforms of GSK-3, GSK-3α and GSK-3β, and recent data show that these two isoforms regulate different functions in the heart (10, 38). In unstimulated cells, GSK is dephosphorylated and in an active form, which can phosphorylate downstream targets, typically resulting in their inactivation (10, 24). Activation of phosphoinositide-3-kinase or other upstream kinases results in GSK phosphorylation and inactivation (10, 24). Cardioprotection by preconditioning has been shown to result in increased GSK-3β phosphorylation, and GSK inhibitors reduce cell death when they are added before ischemia or at the start of reperfusion (16, 18, 21, 23, 32, 33). Because GSK-3 inhibitors block both isoforms, the relative roles of GSK-3α and GSK-3β in cardioprotection are unclear. Despite the inability to differentiate between the relative role of each isoform, GSK-3 inhibitors have been repeatedly shown to result in cardioprotection and are therefore useful tools for inducing cardioprotection (16, 18, 21, 23, 32, 33).

Using isotope tags for relative and absolute quantification (iTRAQ) and mass spectrometry, we found that mitochondrial fractions isolated from hearts treated with a GSK-3 inhibitor, SB 216763 (SB), showed an increase in 16 proteins and a decrease in eight proteins. Of the proteins that showed an increase in the mitochondrial fraction about half were blocked by GD, suggesting a role for HSP90 in the increase in mitochondrial association of these proteins.

**METHODS**

* Animals. All animals were treated and cared for in accordance with the Guide for the Care and Use of Laboratory Animals [National Institutes of Health (NIH) Publication No. 85-23, Revised 1996], and protocols were approved by the Institutional Animal Care and Use Committee. Adult male Sprague-Dawley rats (180–230 g) were used.

* Isolated heart preparation. Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium. The abdominal cavity was exposed with a transverse incision, and heparin was administered...
to the inferior vena cava. The heart was quickly isolated and placed in 

ice-cold Krebs-Heinslein buffer containing (in mM) 25 NaHCO3, 120 
NaCl, 11 glucose, 4.7 KCl, 1.2 KH2PO4, 1.2 MgSO4, and 1.25 CaCl2 
to arrest the heart. The aorta was cannulated, and the heart was 

perfused via the aorta. A water-filled latex balloon was inserted into 

the left ventricle to measure hemodynamic parameters using a Pow-

erLab 2/25 and Chart v5.5 software (AD Instruments, Colorado 
Springs, CO). All hearts were perfused with Krebs-Heinslein buffer 
gassed with 95% O2-5% CO2 and maintained at 37°C. Hearts were 

perfused in the presence and absence of 3 

GSK-3 inhibitor. After stabilization with control perfusate, hearts 

were treated 

before SB perfusion and ischemia and reperfusion. In other studies, 

hearts were treated ±SB and ±GD and taken for isolation of mito-

chondria or tissue extractions before ischemia.

Mitochondria isolation. Mitochondria were isolated by differential 

centrifugation according to standard procedures (29). After a brief 

perfusion to rinse out the blood, hearts were minced in (in mM) 225 
mannitol, 75 sucrose, 5 MOPS, 0.5 EGTA, and 2 taurine (pH 7.25) 
(buffer B) and homogenized by Polytron. To digest the contractile 

proteins, trypsin (0.001 g/0.1 g wet tissue) in 

(buffer A) and homogenized by Polytron. To digest the contractile 

proteins, trypsin (0.001 g/0.1 g wet tissue) in 

buffer B was added to the 

homogenate for 5 min on ice. Digestion was stopped by addition of 

0.2% BSA. The homogenate was centrifuged at 500 

g, and the 

resulting supernatant was spun at 11,000 g to pellet the mitochondria. 
The final mitochondrial pellet was resuspended in buffer B with 0.2% 

BSA.

iTRAQ labeling of SB heart mitochondria. Before all labeling, 

mitochondrial samples were separated on NuPAGE 4–12% Bis-Tris 
gels (Invitrogen) and stained with Coomassie Blue to ensure similar 
protein bands in all groups. We performed a Western blot using anti-
mitochondrial reference protein [voltage-dependent anion channel 
(VDAC)] (Santa Cruz Biotechnology, Santa Cruz, CA) and appropri-
ate secondary antibody to ensure that similar mitochondrial content 
was isolated and analyzed between all treatment groups (Fig. 2). 

Peptide labeling with iTRAQ was performed as described previously 
(6). In brief, individual mitochondrial samples were lysed in buffer 
containing 8 M urea, 2 M thiourea, 4% CHAPS (wt/vol), 75 mM 
NaCl, 50 mM Tris-HCl, 1 mM PMSF, 2 mM glycerophosphate, 1 mM 
sodium orthovanadate, 2 mM sodium fluoride, and 10 mM sodium 
pyrophosphate (pH 8.0) and sonicated on ice. Lysates were spun at 
14,000 g at 4°C for 30 min, and a Bradford assay was performed on 
the supernatant to obtain an optimal protein concentration of 5–8 
mg/ml. The heart mitochondrial lysates (100 μg of each sample) were 
reduced with 5 mM dithiothreitol at 37°C for 1 h and alkylated with 
80 mM iodoacetamide at 37°C for 1 h. Trypsin digestion (protein 

earned enzyme ratio of 25 to 1) was performed overnight at 37°C and 

desalted on Oasis HLB 1 cm3 cartridges (Waters, Milford, MA) per 

manufacturer’s instructions. Eluents were dried completely and re-

constituted in 30 μl of iTRAQ dissolution buffer. One vial each of 
iTRAQ-8plex reagents 113, 115, 117, and 119 was used to label WT 
samples, whereas one vial each of iTRAQ-8plex reagents 114, 116, 
118, and 121 was used to label SB samples at room temperature for 
2 h. After the samples were labeled, the samples were combined and 
diluted to a total of 25 ml with 0.1% trifluoroacetic acid before desalting on Oasis HLB 6 cm3 cartridges (Waters) and dried 

completely.

Strong cation exchange chromatography of iTRAQ-labeled peptides. The 
dried peptides were reconstituted in 200 μl of strong cation exchange chromatography solvent A containing 5 mM KH2PO4 and 25% acetonitrile (ACN; pH 2.67) and injected onto a PolySULFOETHYL A strong cation exchange chromatography column (4.6 mm inner diameter x 20 cm length, 5 μM particle size, 300 Å pore size). Chromatography was performed on an Agilent HP1100 system at 1 ml/min flow rate using the following gradient: 0–70% solvent B [5 mM KH2PO4, 25% ACN, 500 mM KCl (pH 2.67)] for 35 min; 70–100% solvent B for 1 min; and 100% solvent B held for 4 min. UV absorbance was as
monitored at 214 nm and fractions were collected at 1.5-mI intervals, which were subsequently dried completely. Fractions were desalted on Oasis HLB 1 cm³ cartridges (Waters) before mass spectrometry (MS) identification.

**Liquid chromatography-MS/MS analysis.** All dried fractions were analyzed on a LTQ-Orbitrap Velos mass spectrometer (Thermo-Fisher Scientific) interfaced with an Eksigent nano-LC-Ultra 1D plus system (Eksigent Technologies, Dublin, CA) using higher-energy collision-induced dissociation. Briefly, samples were loaded onto an Agilent Zorbax 300SB-C18 trap column (0.3 mm i.d. × 5 mm length, 5 μm particle size) at a flow rate of 5 μl/min for 10 min. Reversed-phase C₁₈ chromatographic separation of peptides was carried out on a preparative BioBasic C₁₈ PicoFrit column (75 μm i.d. × 10 cm length; New Objective, Woburn, MA) at 250 nl/min using the following gradient: 2–5% B for 1 min; 5–35% B for 39 min; 35–85% B for 3 min; and 85% B held for 5 min (solvent A, 0.1% formic acid in 100% water; solvent B, 0.1% formic acid in 100% ACN). LTQ-Orbitrap Velos settings were as follows: spray voltage 1.5 kV, 1 microscan for MS1 scans at 30,000 resolution (fwhm at m/z 400), 1 microscan for MS2 at 7,500 resolution (fwhm at m/z 400); full MS mass range, m/z 105–2,000; MS/MS mass range, m/z 100–2,000. The LTQ-Orbitrap Velos was operated in a data-dependent mode, that is, one MS1 FTMS scan for precursor ions followed by six data-dependent higher-energy collision-induced dissociation-MS2 scans for precursor ions above a threshold ion count of 10,000 with collision energy of 45%.

**Database search and iTRAQ quantification.** Proteome Discoverer (Thermo Scientific) was used to preprocess the raw MS data file and to submit the search to our six processor Mascot cluster at NIH (http://biospec.nih.gov; version 2.3) using the following criteria: database, Swiss-Prot; taxonomy, Rattus norvegicus (rat); enzyme, trypsin; maximum allowed miscleavages, 2; fixed modifications, carbamidomethylation (+57.021 Da), NH2-terminal iTRAQ8plex (+304.205 Da); variable modifications, methionine oxidation (+15.994 Da); MSpeptide mass tolerance as 25 ppm; MS/MS fragment mass tolerance as 0.05 Da. Peptides reported were accepted only if they met the false discovery rate of 1%. iTRAQ quantification was also performed in Proteome Discoverer, and the ratios of iTRAQ reporter ion intensities in MS/MS spectra (m/z: 113.1–121.1) from raw data sets were used to calculate fold changes in protein expression between control and SB treated.

**Data analysis.** All data are presented as means ± SE. Statistics were performed using a one-way ANOVA analysis followed by a Bonferroni post hoc test. A value of P ≤ 0.05 was considered significant.

---

**Table 1. Proteins in the mitochondrial fraction decreased with SB treatment**

<table>
<thead>
<tr>
<th>Localization and Description</th>
<th>Control</th>
<th>SE</th>
<th>SB</th>
<th>SE</th>
<th>P Value</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Q8R431) monoglyceride lipase</td>
<td>0.828</td>
<td>0.076</td>
<td>0.566</td>
<td>0.033</td>
<td>0.052</td>
<td>-31.6</td>
</tr>
<tr>
<td>(P04041) glutathione peroxidase l</td>
<td>0.956</td>
<td>0.041</td>
<td>0.747</td>
<td>0.077</td>
<td>0.054</td>
<td>-21.9</td>
</tr>
<tr>
<td>Mitochondria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(P20069) mitochondrial processing peptidase subunit-α</td>
<td>0.979</td>
<td>0.020</td>
<td>0.826</td>
<td>0.015</td>
<td>0.001</td>
<td>-15.7</td>
</tr>
<tr>
<td>(Q9WV9A) mitochondrial import inner membrane translocase subunit</td>
<td>1.083</td>
<td>0.041</td>
<td>0.923</td>
<td>0.036</td>
<td>0.027</td>
<td>-14.7</td>
</tr>
<tr>
<td>Tim11 A</td>
<td>1.128</td>
<td>0.048</td>
<td>0.962</td>
<td>0.021</td>
<td>0.020</td>
<td>-14.7</td>
</tr>
<tr>
<td>(Q63750) 39S ribosomal protein L23, mitochondrial</td>
<td>1.008</td>
<td>0.035</td>
<td>0.862</td>
<td>0.029</td>
<td>0.019</td>
<td>-14.5</td>
</tr>
<tr>
<td>(P21913) succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial</td>
<td>1.000</td>
<td>0.020</td>
<td>0.876</td>
<td>0.043</td>
<td>0.039</td>
<td>-12.4</td>
</tr>
<tr>
<td>(P51650) succinate-semialdehyde dehydrogenase, mitochondrial</td>
<td>1.021</td>
<td>0.023</td>
<td>0.899</td>
<td>0.026</td>
<td>0.013</td>
<td>-12.0</td>
</tr>
</tbody>
</table>

Values are expressed as percent change in SB compared with control; n = 4 in each group. Accession numbers are from the SWISSPROT/Uniprot database.
min before and during SB treatment and mitochondria were isolated for measurement of protein abundance using iTRAQ isobaric labeling, as in Tables 1 and 2. We reasoned that SB treatment should allow us to identify changes due to alterations in mitochondrial protein import mediated by HSP90.

**Proteins increased in the mitochondrial fraction.** Because we were testing the hypothesis that SB increases import of mitochondrial proteins, we initially focused on the 16 proteins that were increased in the mitochondrial fraction following SB treatment (Fig. 3). To determine whether these protein increases were due to alterations in mitochondrial import, we used GD to inhibit HSP90. Of the 16 proteins that showed an increase with SB treatment, 10 were also observed in the SB/GD treated group. Of the 10 proteins that increased in the mitochondria with SB treatment and that were observed in both the SB and SB/GD groups, the increase in five of these proteins was blocked by GD treatment. These five proteins are likely candidates for GD-mediated protein import, and they include annexin A6, vinculin, heat shock cognate 71, fructose-bisphosphate aldolase A, and pyruvate kinase.

To obtain additional information, we also used the iTRAQ method to examine changes in protein levels in the whole cell extract. The increase in mitochondrial abundance of these proteins could be due to increased import or association, reduced degradation, or altered solubility. If the increase in mitochondrial proteins is due to enhanced protein import or association, one would not expect a protein increase in the whole cell extract, whereas an increase in the whole cell extract would be expected with reduced degradation or altered solubility. As shown in Fig. 3, 15 of the 16 proteins increased with SB were detected in the whole cell extract; however, none showed an increase in abundance, which would be consistent with increased import for these cytosolic proteins and better retention of cytochrome c following SB treatment.

**Proteins decreased in the mitochondrial fraction.** Of the eight proteins decreased with SB treatment, none were blocked by GD, suggesting that the protein decreases were not dependent on HSP90 (Fig. 4). If the decreases were due to increased degradation or solubility changes, one would expect a concurrent decrease in total protein. Two of the eight proteins were not detected in the whole cell extract. Of the remaining six...

---

**Table 2. Proteins in the mitochondrial fraction increased with SB treatment**

<table>
<thead>
<tr>
<th>Localization and Description</th>
<th>Control</th>
<th>SE</th>
<th>SB</th>
<th>SE</th>
<th>P Value</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(P63017) heat shock cognate 71 kDa protein</td>
<td>1.031</td>
<td>0.029</td>
<td>1.129</td>
<td>0.022</td>
<td>0.036</td>
<td>10.0</td>
</tr>
<tr>
<td>(P16617) phosphoglycerate kinase 1</td>
<td>0.993</td>
<td>0.021</td>
<td>1.127</td>
<td>0.045</td>
<td>0.036</td>
<td>13.5</td>
</tr>
<tr>
<td>(P84078) ADP-ribosylation factor 1</td>
<td>1.032</td>
<td>0.023</td>
<td>1.178</td>
<td>0.043</td>
<td>0.024</td>
<td>14.2</td>
</tr>
<tr>
<td>(P05065) fructose-bisphosphate aldolase A</td>
<td>1.073</td>
<td>0.045</td>
<td>1.239</td>
<td>0.030</td>
<td>0.022</td>
<td>15.5</td>
</tr>
<tr>
<td>(P35704) peroxiredoxin-2</td>
<td>0.925</td>
<td>0.047</td>
<td>1.071</td>
<td>0.035</td>
<td>0.047</td>
<td>15.8</td>
</tr>
<tr>
<td>(P34058) heat shock protein HSP 90-β</td>
<td>1.030</td>
<td>0.052</td>
<td>1.202</td>
<td>0.010</td>
<td>0.018</td>
<td>16.7</td>
</tr>
<tr>
<td>(P11980) pyruvate kinase isozymes M1/M2</td>
<td>1.086</td>
<td>0.031</td>
<td>1.270</td>
<td>0.070</td>
<td>0.053</td>
<td>17.0</td>
</tr>
<tr>
<td>(P47858) 6-phosphofructokinase, muscle type</td>
<td>1.019</td>
<td>0.016</td>
<td>1.202</td>
<td>0.032</td>
<td>0.002</td>
<td>17.9</td>
</tr>
<tr>
<td>(P35213) 14-3-3 protein-β/α</td>
<td>1.032</td>
<td>0.026</td>
<td>1.249</td>
<td>0.051</td>
<td>0.009</td>
<td>21.1</td>
</tr>
<tr>
<td>(P85972) vinculin</td>
<td>1.117</td>
<td>0.053</td>
<td>1.371</td>
<td>0.080</td>
<td>0.038</td>
<td>22.8</td>
</tr>
<tr>
<td>(P05213) tubulin α-1B chain</td>
<td>1.001</td>
<td>0.022</td>
<td>1.246</td>
<td>0.068</td>
<td>0.014</td>
<td>24.5</td>
</tr>
<tr>
<td>(P48037) annexin A6</td>
<td>1.067</td>
<td>0.026</td>
<td>1.330</td>
<td>0.080</td>
<td>0.021</td>
<td>24.7</td>
</tr>
<tr>
<td>(P10630) eukaryotic initiation factor 4A-II</td>
<td>0.978</td>
<td>0.046</td>
<td>1.250</td>
<td>0.053</td>
<td>0.008</td>
<td>27.8</td>
</tr>
<tr>
<td>(P48675) desmin</td>
<td>0.942</td>
<td>0.047</td>
<td>1.241</td>
<td>0.088</td>
<td>0.024</td>
<td>31.7</td>
</tr>
<tr>
<td>(Q6ZWN5) 40S ribosomal protein S9</td>
<td>1.196</td>
<td>0.125</td>
<td>1.942</td>
<td>0.226</td>
<td>0.028</td>
<td>62.4</td>
</tr>
<tr>
<td>Mitochondria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(P62897) cytochrome c, somatic</td>
<td>1.114</td>
<td>0.061</td>
<td>1.429</td>
<td>0.061</td>
<td>0.011</td>
<td>28.3</td>
</tr>
</tbody>
</table>

Values are expressed as percent change in SB compared with control; n = 4 in each group. Accession numbers are from the SWISSPROT/Uniprot database.
proteins, none showed a decrease in the whole cell extract. For the two cytosolic proteins, this could be explained by an SB-mediated decrease in mitochondrial association of these proteins. SB did not significantly change the total protein levels for the four established mitochondrial proteins. A possible explanation for this is that we are able to detect a smaller decrease in the enriched mitochondrial fraction than in the nonenriched whole cell extract. Another possibility is that these proteins are exported from the mitochondria, but they have not yet been degraded due to the short treatment period.

**Geldanamycin-mediated protein contents in the mitochondria.**
Because the presence of GD results in attenuation of the SB-mediated increase in mitochondrial proteins, we next wanted to evaluate whether GD alone might have an effect on protein levels in the mitochondria. As shown in Figs. 3 and 4, none of the proteins that showed a significant change following SB treatment (Tables 1 and 2) showed a change with GD treatment in the absence of SB. These results suggest that GD alone does not have an effect on SB-mediated mitochondrial protein changes.

**DISCUSSION**
A number of studies have shown that cardioprotection alters import of proteins into the mitochondria and/or association of proteins with the mitochondria (4, 8, 11, 27, 30). These proteins have been identified on a candidate basis. To gain further insight into this process, we used a nonbiased proteomic approach to measure changes in proteins associated with the mitochondria following treatment with a cardioprotective drug SB 216763. We find that a 15-min SB treatment decreases eight mitochondrial or mitochondria-associated proteins and increases 16 mitochondrial-associated proteins. These changes in protein abundance could be due to altered degradation, stability, or association with the mitochondria. To gain further insight, we performed additional studies in which hearts were perfused with SB + GD to block HSP90-mediated protein import into the mitochondria and we also measured changes in protein levels in whole cell extracts from SB-treated hearts.

By comparing changes in mitochondrial fractions to whole cell extracts, we made the novel observation that SB leads to increased mitochondrial association of many glycolytic enzymes, such as phosphofructokinase, aldolase, pyruvate kinase, and phosphoglycerate kinase. It has been suggested that glycolytic enzymes are not free in the cytosol, but are located on scaffolding proteins that can channel substrate from one enzyme to the next. This finding is interesting in light of data suggesting that glycolytic enzymes can localize on the mitochondria outer membrane where they can provide pyruvate directly to the mitochondria (17). Alterations in substrate selection can alter cardiovascular response to ischemia and reperfusion (31, 35). Although not observed in this study, the glycolytic enzyme hexokinase has been reported previously to localize to the mitochondria with cardioprotection (19, 30). The data in this study suggest that not only hexokinase but most glycolytic enzymes can be localized to the mitochondrial. We further show that HSP90 plays an important role in the mitochondrial localization of most of these glycolytic enzymes (excluding phosphoglycerate kinase).

We also found the GSK inhibition enhanced the mitochondrial localization of many cytoskeletal proteins, such as desmin, annexin 6, tubulin, and vinculin. Interestingly, the cytoskeletal network is also connected to the mitochondria (3, 9). For example, tubulin, which shows a SB-mediated increase in mitochondrial association, has been reported to bind to VDAC and regulate its activity (28). Hexokinase has also been reported to bind to VDAC (26). It is tempting to speculate that perhaps the cytoskeletal network, which may be regulated by GSK, might be involved in the mitochondrial association of these glycolytic enzymes.

Cytochrome c is the only definitive mitochondrial protein that showed an increase with SB treatment. Because protein turnover is thought to be slow relative to the 15-min treatment period, it is unlikely that the increased cytochrome c abundance can be attributed to reduced degradation, and there is no evidence of a decrease in cytochrome c in the total heart.
extract. Furthermore, because cytochrome c is a mitochondrial protein, its elevated levels are unlikely to be due to increased mitochondrial translocation or association, and this explanation is consistent with our observation that the SB-mediated cytochrome c increase was unaffected by GD treatment. The increase in abundance could be due to a change in solubility such that the protein is differentially extracted. As discussed, this difference in solubility could be due to alterations in post-translational modification or localization (e.g., membrane association, etc.). However, if the increase was due to solubility changes, one would expect a protein increase in the whole cell extract; however, no increase was observed. These data suggest that the SB treatment allows better retention of cytochrome c in the mitochondria fraction.

A number of proteins were detected in the mitochondrial fraction following SB treatment, but were not detected in either the SB + GD treatment group or in the whole cell extract. This lack of detection may be due to dynamic range issues that are inherent in mass spectrometry analysis. In a typical mass spectrometer, abundant proteins are more preferentially identified than less abundant ones. If a relatively low-abundance protein was in a fraction with high abundant proteins, it might not be detected. In the whole cell lysate, there are many contractile proteins that are present in high abundance, which could swamp out other proteins in the same fraction due to dynamic range issues.

Although it was not a major aim of this study, we also found that inhibition of GSK reduced the mitochondrial level of eight proteins. The cytosolic proteins glutathione peroxidase 1 and monoglyceride lipase both showed a decrease in mitochondrial association that was not HSP90 dependent. Glutathione peroxidase 1 has been reported to localize both to the mitochondria and cytosol (22), but additional studies are needed to determine how glutathione peroxidase 1 localization is regulated and its effects on the cell.

Summary. Taken together, the data suggest rapid changes in mitochondrial localization of proteins. Interestingly, about half of the enzymes in glycolysis show an increase in mitochondrial association after SB treatment. Mitochondria are reported to be regulated by the cytoskeleton, and GSK inhibition could alter mitochondrial cytoskeletal interactions. Consistent with this hypothesis, SB results in increased association of many cytosolic proteins. The rapid nature of these changes suggests that remodeling of mitochondria can have an important role in acute cardioprotection.

ACKNOWLEDGMENTS

R. Wong, T. Nguyen, G. Wang, M. Gucek, and E. Murphy were all supported by the National Heart, Lung, and Blood Institute Intramural program (ZIA-HL 006052). C. Steenbergen was supported by NIH Grant NIH-R01-039752.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


