Glycogen Synthase Kinase 3 Inhibition Slows Mitochondrial Adenine Nucleotide Transport and Regulates Voltage-Dependent Anion Channel Phosphorylation

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Abstract—Inhibition of glycogen synthase kinase (GSK)-3 reduces ischemia/reperfusion injury by mechanisms that involve the mitochondria. The goal of this study was to explore possible molecular targets and mechanistic basis of this cardioprotective effect. In perfused rat hearts, treatment with GSK inhibitors before ischemia significantly improved recovery of function. To assess the effect of GSK inhibitors on mitochondrial function under ischemic conditions, mitochondria were isolated from rat hearts perfused with GSK inhibitors and were treated with uncoupler or cyanide or were made anoxic. GSK inhibition slowed ATP consumption under these conditions, which could be attributable to inhibition of ATP entry into the mitochondria through the voltage-dependent anion channel (VDAC) and/or adenine nucleotide transporter (ANT) or to inhibition of the F1F0-ATPase. To determine the site of the inhibitory effect on ATP consumption, we measured the conversion of ADP to AMP by adenylate kinase located in the intermembrane space. This assay requires adenine nucleotide transport across the outer but not the inner mitochondrial membrane, and we found that GSK inhibitors slow AMP production similar to their effect on ATP consumption. This suggests that GSK inhibitors are acting on outer mitochondrial membrane transport. In sonicated mitochondria, GSK inhibition had no effect on ATP consumption or AMP production. In intact mitochondria, cyclosporin A had no effect, indicating that ATP consumption is not caused by opening of the mitochondrial permeability transition pore. Because GSK is a kinase, we assessed whether protein phosphorylation might be involved. Therefore, we performed Western blot and 1D/2D gel phosphorylation site analysis using phos-tag staining to indicate proteins that had decreased phosphorylation in hearts treated with GSK inhibitors. Liquid chromatographic–mass spectrometric analysis revealed 1 of these proteins to be VDAC2. Taken together, we found that GSK-mediated signaling modulates transport through the outer membrane of the mitochondria. Both proteomics and adenine nucleotide transport data suggest that GSK regulates VDAC and that VDAC may be an important regulatory site in ischemia/reperfusion injury. (Circ Res. 2008;103:983-991.)

Key Words: mitochondria ■ GSK-3 ■ VDAC ■ adenine nucleotide transport

Ischemic heart disease is a major cause of morbidity and mortality worldwide. This has motivated a search for new approaches to reduce ischemic injury, propelled by the discovery of ischemic preconditioning (IPC).1 Considerable data suggest that IPC leads to the release of adenosine, opioids, or bradykinin,2,3 which bind to G protein–coupled receptors and initiate a signaling cascade that involves activation of phosphatidylinositol-3 kinase and downstream kinases such as Akt, endothelial nitric oxide synthase, protein kinase C, mTOR (mammalian target of rapamycin), p70S6 kinase, extracellular signal-regulated kinase, and glycogen synthase kinase (GSK)-3β.4–6

GSK-3, first identified as a regulator of glycogen metabolism, is also an important regulator of cell function, including gene expression and cell cycle, survival, and apoptosis.7 GSK-3 belongs to the serine/threonine kinase family of proteins, and the 2 known isoforms are GSK-3α (51 kDa) and GSK-3β (47 kDa), which share 98% homology and similar substrate specificities.

The activity of GSK-3β is regulated by phosphorylation.7 GSK-3β is active under normal resting conditions when it is not phosphorylated, and phosphorylation at serine 9 leads to its inactivation. The activity of GSK-3β is regulated by several signaling pathways as a number of kinases (Akt, protein kinase C, protein kinase A, and others) can phosphorylate GSK-3β on serine 9 and inactivate it.

In addition to its role in cell survival, proliferation, and differentiation, GSK-3β has been proposed to be involved in the cardioprotective effects of IPC8,9 and various forms of pharmacological cardioprotection.10–14 Our laboratory8 previ-
ously demonstrated that the cardioprotection elicited by IPC is mediated by the inhibition of GSK via phosphatidylinositol (PI)3-kinase. It has also been reported that opioid-induced cardioprotection occurs via inactivation of GSK-3β by phosphorylation at serine 9 through PI3-kinase and TOR-dependent pathways. Protective effects have been observed when GSK-3β inhibitors were administered immediately before ischemia, 24 hours before ischemia, or immediately on reperfusion. GSK inhibition is also protective during heart failure.

Recently GSK-3β has been described in mitochondria, and GSK inhibition is thought to block opening of the mitochondrial permeability transition pore (mPTP) in cardiomyocytes. However, the molecular targets of GSK-3β in mitochondria and the precise physiological effects of GSK-3β inhibition on mitochondrial function have not been fully elucidated.

The present study was designed to identify downstream targets of GSK-3 and to link the phosphorylation of downstream targets to physiological effects on mitochondrial function. We propose that cardioprotection resulting from inhibition of GSK-3 is partly mediated by modulation of mitochondrial bioenergetics.

**Materials and Methods**

**Animals**
Male Sprague–Dawley rats were used in this study. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Johns Hopkins University.

**Langendorff Rat Heart Preparation**
Hearts were perfused as described previously. Details are provided in the online data supplement, available at http://circres.ahajournals.org.

**Isolated Mitochondria Protocols**
Mitochondria were isolated as described previously. The online data supplement provides details of the assays.

**Proteomics**
Details of the Western blots, 1D/2D gel electrophoresis, and phosphoprotein detection are provided in the online data supplement.

**Results**

**GSK-3 Inhibitors Increase Recovery of Contractile Function After Ischemia**
We previously reported that GSK-3 inhibitors improved recovery of function and reduced necrosis following ischemia. In this study, we examined the mechanisms involved, after confirming the protective effects of 2 GSK-3 inhibitors SB216763 and SB415286. Dose–response experiments were performed to establish effective concentrations. As shown in Figure 1, either 3 µmol/L SB216763 or 10 µmol/L SB415286 significantly enhanced recovery of left ventricular developed pressure during reperfusion, to 48.9±6.2% and 56.8±8.1% of baseline compared with 34.7±7.5% in control hearts (P<0.05). Previous work from our laboratory and others has shown that GSK-3 inhibitors also reduce necrosis.

![Graph showing LVDP recovery](http://circres.ahajournals.org/)

**GSK-3 Inhibitors Reduce the Rate of ATP Consumption in Isolated Mitochondria**
We previously found that addition of recombinant Bcl-2 to mitochondria resulted in reduced breakdown of ATP under deenergized conditions when the mitochondrial F1F0-ATPase runs in reverse. This reduced rate of ATP consumption was attributed to altered ATP transport into the mitochondria. To determine whether GSK-3 inhibitors also decreased ATP consumption under deenergized conditions, we studied mitochondria rapidly deenergized using the uncoupler dinitrophenol. Under these conditions, mitochondria from hearts treated with GSK inhibitors consumed less ATP than control mitochondria (Figure 2A). Similarly, GSK inhibitors significantly reduced ATP consumption when sodium cyanide was used to stop mitochondrial respiration (Figure 2B). In the physiologically relevant model of mitochondrial deenergization, mitochondria were allowed to consume all of the oxygen in an oxygraph chamber, and then the consumption of ATP during the ensuing anoxic period was measured. ATP consumption was linear in both control- and GSK inhibitor–treated mitochondria, and after 60 minutes of anoxia, the amount of ATP consumed was significantly less in the GSK inhibitor–treated mitochondria (Figure 2C). Mitochondria from ischemically preconditioned myocardium showed a similar slowing of ATP consumption (Figure 1 in the online data supplement). This slowing of ATP consumption could be accomplished by attenuating ATP entry into the mitochondria through the voltage-dependent anion channel (VDAC) and/or the adenine nucleotide transporter (ANT) or by inhibition of the mitochondrial F1F0-ATPase.

To determine whether this effect is caused by inhibition of the mitochondrial F1F0-ATPase, mitochondria were sonicated to disrupt the mitochondria and expose the F1F0-ATPase. We found (Figure 3A) that ATPase activity in the sonicated mitochondrial fragments was similar in the presence or absence of uncoupler, as expected, if we had fully disrupted the mitochondria, and GSK inhibitors had no effect on ATP consumption by the sonicated mitochondrial fragments. Only
in the intact mitochondria was there an inhibitory effect of GSK inhibitors. Thus, the effect of GSK inhibitors does not appear to be attributable to direct inhibition of F$_1$F$_0$-ATPase activity.

The Effect of GSK-3 Inhibitors on Mitochondrial Adenine Nucleotide Metabolism Is Not Attributable to a Direct Effect on the mPTP

To examine whether the effect of GSK inhibitors on adenine nucleotide metabolism is caused by a direct effect on the mPTP, we examined the effect of cyclosporin A (CsA) on ATP consumption under deenergized conditions. To establish that an effective dose of CsA was being used, we measured mitochondrial swelling induced by calcium, and we found that 200 $\mu$mol/L CsA markedly reduced mitochondrial swelling in response to calcium compared to control. However, this concentration of CsA had no effect on adenine nucleotide transport (Figure 3B). Thus GSK inhibitors are inhibiting transport through physiological transport mechanisms, and there is no evidence that adenine nucleotide entry into the mitochondria during early anoxia is related to mPTP opening. However, inhibiting physiological transport could indirectly protect the mitochondria and reduce the probability of mPTP opening as injury progresses.

GSK-3 Inhibitors Reduce Adenine Nucleotide Transport Through the Outer Mitochondrial Membrane

To further explore the mechanism whereby GSK inhibitors slow ATP consumption under deenergized conditions, an assay was developed that isolated effects of outer mitochondrial membrane transport from effects on ANT or the F$_1$F$_0$-ATPase. This assay took advantage of adenylate kinase, which resides in the intermembrane space. Under deenergized conditions, comparable to the conditions used to measure mitochondrial ATP consumption but in the presence of oligomycin to block matrix adenine nucleotide metabolism, ADP was added to isolated mitochondria, and AMP generation was measured. If the effect of GSK inhibitors is on adenine nucleotide transport across the outer mitochondrial membrane, then there should be less AMP consumed and less ADP remaining after 2.5 minutes of incubation was measured. GSK inhibitors have no effect on ATPase activity in sonicated mitochondria. Bar graph showing ATP consumption (% of initial ATP) under deenergized conditions with and without GSK inhibitors. Results are means±SEM (n=6). *P<0.05 vs intact control; #P<0.05 vs intact SB216763 treated; $\zeta$P<0.05 vs intact + DNP, SB 216763-treated.
AMP produced in the mitochondria treated with GSK inhibitors compared to control. There is significantly less ADP consumed (Figure 4A) and less AMP produced (Figure 4B) in the GSK inhibitor–treated mitochondria, as compared to the untreated mitochondria. In a separate series of experiments, we assessed the effect of GSK inhibitors on adenylate kinase activity in sonicated mitochondria where the outer membrane would be disrupted, and GSK inhibitors had no effect on ADP consumption or AMP production (Figure 4A and 4B, right). The data suggest that GSK inhibitors do not inhibit adenylate kinase and that the ATP-sparing effect of GSK inhibitors is related to decreased adenine nucleotide transport across the outer mitochondrial membrane.

GSK-3 Inhibitors Reduce Phosphorylation of VDAC

Because the functional data suggest that GSK inhibitors are slowing adenine nucleotide transport through the outer mitochondrial membrane, and VDAC is the channel that is responsible for this transport, we explored the effect of GSK inhibitors on VDAC phosphorylation. We used several approaches. First, we used an antibody that recognizes proteins that are phosphorylated at serine/threonine residues of Akt substrates, and we performed Western blotting to determine whether GSK inhibition affects protein phosphorylation. We observed a dramatic change in a protein band at \( \approx 32 \) kDa. As shown in Figure 5A (top), there is much less phosphorylation of this band when the hearts were treated with a GSK inhibitor than in untreated hearts. The molecular mass of VDAC is 32 kDa. The gel was stripped and reprobed with

![Figure 4](image)

**Figure 4.** GSK inhibitors slow the rate of ADP consumption (A) and the rate of AMP production (B) by cyanide-treated mitochondria. Results are means \( \pm \) SEM (n = 6). *P < 0.05 vs control.

![Figure 5](image)

**Figure 5.** Effect of GSK inhibition on phosphorylation of a 32 kDa protein. A, Top, Representative 1D gel showing the amount of 32-kDa phosphorylated Akt substrate protein in control and GSK inhibitor–treated heart extracts. Middle, The gel reprobed with VDAC antibody, showing no significant difference in total VDAC expression. Gel densitometry was performed for quantitation, and the ratio of 32-kDa phospho-protein to total VDAC is plotted. *P < 0.05 vs control. B, Whole heart homogenates were further separated using 2D gel electrophoresis. In the upper images, control homogenate (left) and GSK inhibitor–treated homogenate (right) were probed with VDAC antibody. Then, the membranes were stripped and reprobed for phosphorylated Akt substrate protein (lower images). There is phosphorylated protein in the VDAC region in control but not in GSK inhibitor–treated homogenate. The region of interest is circled and shown at higher magnification at the bottom.
antibody to VDAC, and the phosphorylated protein overlaps with VDAC, and, as shown in Figure 5A (bottom), there is the same amount of VDAC in each lane. Next, we performed 2D gel electrophoresis to verify this finding when proteins are better separated. As shown in Figure 5B, the location of VDAC in the 2D gel was established with VDAC antibody (upper images), and phosphorylation was assessed using phospho-Akt substrate antibodies (lower images). The lower images show VDAC phosphorylation in the control extracts on the left but not in the GSK inhibitor–treated extracts on the right. To examine this further, we used Phos-tag to identify phosphorylated proteins, and we performed 1D and 2D gel electrophoresis. In the 1D gels, the GSK inhibitors significantly reduced protein phosphorylation in the 32-kDa region. To validate this finding, 2D gel electrophoresis was performed with narrow pH range strips that focused the VDAC region more clearly, and then we stained with Phos-tag. Figure 6 shows several spots that had high levels of phosphorylation in untreated heart extracts but very little phosphorylation in GSK inhibitor–treated heart extracts. Mass spectrometry demonstrated that VDAC2 is present in all 3 spots in the 2D gel.

**VDAC Can Be Phosphorylated by Either Akt or GSK-3β**

To determine whether Akt or GSK-3β can phosphorylate VDAC in vitro, we partially purified VDAC and performed an in vitro kinase assay using recombinant active Akt and recombinant GSK-3β. VDAC was partially purified using a hydroxyapatite/celite column as used by others.17 We then performed an in vitro kinase assay and measured the extent of phosphorylation using Pro-Q Diamond staining. Although there was some endogenous phosphorylation, this was further increased by either Akt or GSK-3β (Figure 7A). We also examined the ability of recombinant active Akt to phosphorylate VDAC using isolated mitochondria. Recombinant Akt added to the medium, in the presence of ATP, increased phosphorylation of the ≈32-kDa protein band (Figure 7B). Thus external Akt can phosphorylate the protein, indicating that the phosphorylation site is on the outside of the mitochondria, consistent with the location of VDAC.

**GSK-3 Inhibitors Increase Bcl-2 Binding to Mitochondria**

Previous work16 had demonstrated that cardiac overexpression of Bcl-2 protects the heart from ischemia/reperfusion injury, and this protection is associated with inhibited mitochondrial ATP consumption under deenergized conditions and with binding of Bcl-2 to VDAC. To determine whether GSK inhibitors are protective, at least in part, by enhancing Bcl-2 binding to VDAC, cell fractionation experiments were performed, and the amount of Bcl-2 in the mitochondrial and cytosolic fractions were determined by Western blotting. GSK inhibition causes a significant loss of Bcl-2 from the cytosol and a significant increase in the mitochondrial fraction (Figure 8A). This indicates that binding of Bcl-2 to mitochondrial targets increases in the presence of GSK inhibitors. To test whether this increased binding of Bcl-2 to mitochondria is specific binding to VDAC, we added equal

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**Figure 6.** Whole heart homogenate protein was separated by 2D gel electrophoresis and stained with Phos-tag to detect phosphorylated proteins. Three spots showing marked differences in phosphorylation level, in the 32-kDa region (shown in higher magnification) were analyzed by light chromatography–mass spectrometry, and VDAC-2 was identified by at least 4 peptide fragments, in all 3 spots.

**Figure 7.** A, In vitro phosphorylation of semipurified VDAC by Akt and GSK-3β. B, Increased 32 kDa Akt substrate phosphorylation in isolated mitochondria following addition of recombinant Akt (rAkt). *P<0.05 vs control.
amounts of recombinant Bcl-2 to GSK inhibitor–treated mitochondria and untreated mitochondria, immunoprecipitated VDAC, and measured the amount of Bcl-2 that was bound to VDAC. As shown in Figure 8B, there was significantly more Bcl-2 bound to VDAC in the GSK inhibitor–treated mitochondria. Thus, phosphorylation of VDAC may affect the binding affinity for Bcl-2, which may regulate outer mitochondrial membrane transport.

**Discussion**

Previously, our group has demonstrated that IPC results in phosphorylation and inactivation of GSK-3β and that this is mediated by the PI3-kinase pathway. Furthermore, pretreatment with GSK-3 inhibitors is approximately as protective as IPC. Others have shown that GSK-3β is involved in a variety of forms of pharmacological preconditioning, and the GSK-3β inhibitors are protective when added at the start of reperfusion. This suggests that GSK-3β may play a central role in a final pathway of cardioprotection, as suggested by Sollott and colleagues. Furthermore, previous work has suggested that the effects of GSK-3β inhibition are primarily focused on the mitochondria, although the molecular target has not been determined.

The present study suggests a possible molecular target for GSK-3β in the mitochondria that may be involved in cardioprotection and a possible mechanism whereby protection is achieved. The molecular target appears to be VDAC in the outer mitochondrial membrane and phosphorylation may be a mechanism for regulating VDAC activity, either directly or by altering the binding of Bcl-2. There are 3 VDAC isoforms in mammalian cells, which are primarily responsible for transport of molecules up to 5000 kDa across the outer mitochondrial membrane. Transport of anions is highly voltage-sensitive, with high conductance at low voltages and low conductance when voltage is increased. In contrast, cation conductance is relatively voltage-insensitive. Nucleotides such as ATP are readily permeable when VDAC is in the open state and are virtually impermeant when VDAC is closed. Ablation of the mouse VDAC1 gene results in altered mitochondrial sensitivity for ADP in muscle, but because VDAC2 and VDAC3 are also present at high levels in the heart, the mice are viable. Mice deficient for VDAC3 are viable, but deficiency of VDAC2 is embryologically lethal. Our proteomic studies suggest that VDAC2 is a primary GSK target in the outer mitochondrial membrane.

Several investigators have suggested that VDAC can be an important regulator of mitochondrial function. Under conditions of anoxia, there is evidence of inhibition of mitochondrial anion exchange. Others have found evidence for VDAC regulation of mitochondrial function under conditions that stimulate apoptosis. In the apoptosis studies, it has been suggested that early in the apoptotic program, the outer mitochondrial membrane becomes impermeable to small molecules such as ATP and creatine phosphate and that this leads to the eventual loss of outer mitochondrial membrane integrity and cytochrome c release, which can be prevented by antiapoptotic Bcl-2 family members. Although the conditions and mechanisms are different in our study, the findings support the concept that VDAC transport can be regulated, and plays a role in cell injury. Under conditions leading to apoptosis, there is some controversy concerning Bcl-2 regulation of VDAC. Some reports suggest that during apoptosis, VDAC closes and that Bcl-2 is protective by maintaining VDAC in an open configuration, whereas others suggest that Bcl-2 promotes VDAC closure. This could be related to the specific experimental conditions or the timing of the measurements. Regardless of the controversy, our data as well as the apoptosis literature support the concept that altered VDAC conductance can be involved in the evolution of cell injury.

Although our data suggest that VDAC phosphorylation under the control of GSK-3 is an important mechanism that alters mitochondrial function under energy deficient conditions, others suggest that mitochondrial protein phosphorylation is not involved in the protective effect of IPC. One might expect that IPC and GSK inhibition would involve many of the same mechanisms because IPC inhibits GSK through phosphorylation. However, this thorough study by Clarke et al was performed on extracts following mitochondrial purification, and we found that we had to use whole-tissue extracts, immediately placed in detergent, to see the phosphorylation clearly. If we purified the mitochondria and then prepared extracts, the phosphorylation was barely detectable. This suggests that the phosphorylation is highly labile, presumably because the phosphatases must be sepa-
rated from the substrate rapidly to preserve the phosphorylated state. To minimize this problem, we used a rapid isolation procedure for preparing mitochondria and analyzing mitochondrial function. It is possible that even under these conditions, our mitochondrial functional assays are underestimating the magnitude of the effect that is present in situ.

**Functional Consequences of GSK Signaling in the Mitochondria**

One of the downstream effects of GSK inhibition is delayed opening of the mPTP in response to oxygen radicals, and opening of the mPTP has been suggested to be a causative event in cell death during myocardial ischemia/reperfusion injury. This effect on mPTP opening could be a direct or indirect effect of GSK inhibition. Because VDAC does not appear to be necessary for mPTP formation, it seems unlikely that altered phosphorylation of VDAC would necessarily prevent mPTP formation. Also, the studies of Sollott and colleagues show that mPTP opening occurs even in the presence of GSK inhibitors, just more slowly. In accordance with these observations, our present study has also revealed that CsA treatment does not change the rate of ATP hydrolysis following deenergization, indicating an effect independent of mPTP opening. Alternatively, GSK inhibitors could alter mitochondrial function in a way that makes the mPTP less likely to form or open, possibly by limiting the factors that are known to activate the mPTP, oxidant stress, and high calcium concentrations. If GSK inhibitors either reduced mitochondrial calcium uptake, reduced endogenous oxygen radical production, or both, this could account for the ability of GSK inhibitors to slow mPTP opening. The importance of calcium loading in mPTP opening in situ has been questioned and ROS may be a more important factor in intact tissue.

One mechanism whereby GSK inhibitors could reduce mitochondrial calcium loading would be by inhibiting ATP influx through VDAC during ischemia. During ischemia, ATP is produced by anaerobic glycolysis, and this ATP can enter the mitochondria and be hydrolyzed by the matrix F$_1$F$_0$-ATPase. This is a significant ATP-consuming process; when ischemic myocardium is treated with oligomycin, ATP consumption slows, by as much as 50%. Although inhibition of this process may be protective by preserving ATP, this is not likely to be its major effect. Rather, mitochondrial ATP hydrolysis is used to preserve the mitochondrial membrane potential (Δψ), and direct measurement of Δψ in isolated myocytes subjected to simulated ischemia shows that Δψ falls much more rapidly in the presence of oligomycin than in its absence, indicating that the matrix F$_1$F$_0$-ATPase is using cytosolic ATP to slow the decay of Δψ. Because Δψ is the primary driving force for calcium uptake into the mitochondria, it is not surprising that there is virtually complete elimination of mitochondrial calcium uptake in isolated myocytes subjected to simulated ischemia in the presence of oligomycin, whereas mitochondrial calcium uptake is observed under similar conditions in the absence of oligomycin. Although this calcium uptake may be transient and may not directly open the mPTP, it could facilitate mPTP opening in response to other stimuli.

Another possible mechanism whereby inhibition of VDAC transport by GSK inhibitors may be protective under anoxic conditions is by reducing oxygen radical production during early reperfusion. Agents that reduce Δψ by a small amount are cardioprotective, such as mitochondrial K-ATP channel openers, presumably because production of oxygen radicals is enhanced when the electrochemical proton gradient is high. However, there is no consensus that a decrease in Δψ is critical for the protective effect of K-ATP channel openers. A decrease in Δψ during ischemia could result in less oxygen radical production during early reperfusion, which would reduce the probability of mPTP opening. Therefore, GSK inhibitors could be protective by allowing Δψ to decrease during ischemia, reducing mitochondrial calcium loading during ischemia and reducing oxygen radical production during reperfusion, both of which would tend to reduce the probability of mPTP opening and thereby be protective. Others have suggested that the protective effect of IPC involves suppression of oxidant stress. These effects on calcium and oxidant stress would not be direct effects on the mPTP but rather effects on mPTP activators.

One interesting aspect of this study is that we found that GSK inhibitors reduced VDAC phosphorylation using an antibody that detects proteins phosphorylated at Akt sites. This could be because the antibody is not entirely specific for Akt phosphorylation sites and also recognizes other serine/threonine phosphorylations. However, it is also possible that VDAC is phosphorylated at multiple sites. GSK-3β is known to prefer substrates that have already been phosphorylated by other kinases. Thus, it is possible that physiological regulation of VDAC is achieved through a dual phosphorylation mechanism involving Akt as the priming kinase and GSK-3β as the secondary kinase that is required for regulation of VDAC function. An alternative possibility is that VDAC is phosphorylated by Akt and that GSK-3β regulates the phosphatase that dephosphorylates VDAC. If GSK-3β inactivates the phosphatase that dephosphorylates VDAC, then GSK inhibitors could decrease VDAC phosphorylation by increasing the activity of the phosphatase. Further characterization of the phosphorylation sites of VDAC will be necessary to resolve this issue.

Although dephosphorylation of VDAC by GSK inhibition may be protective by altering channel conductance directly, an alternative mechanism would involve differential protein binding to the phosphorylated versus dephosphorylated VDAC. In a previous study, we found that Bcl-2 overexpression inhibited mitochondrial ATP consumption under deenergized conditions, similar to what we report here with GSK inhibitors. One possible explanation for the similar effect of Bcl-2 overexpression and GSK inhibition is that an important mechanism of VDAC regulation involves Bcl-2 binding, and this binding can be increased either by overexpression or by altering affinity, and 1 mechanism for altering affinity may be through phosphorylation. The data presented here suggest that Bcl-2 affinity for mitochondria, and for VDAC specifically, is increased by GSK inhibitors, consistent with a regulatory role for VDAC phosphorylation. Others have provided convincing evidence that Bcl-2 binding to a component of the mPTP is critical for cardioprotection and...
that GSK-3β is a critical regulator of this interaction. Another possibility is that binding of hexokinase to VDAC could have important regulatory properties. The binding of hexokinase to VDAC appears to require activated Akt, although it is not clear whether phosphorylation plays a role.

Conclusions
The data suggest that VDAC is an important site of regulation of mitochondrial metabolism and function under deenergized conditions. GSK inhibition decreases adenine nucleotide entry through the outer mitochondrial membrane, most likely through VDAC, under anoxic or similar conditions. This reduction in adenine nucleotide transport is likely to result in a decrease in Δψ, less mitochondrial calcium loading during anoxic conditions, and less endogenous oxygen radical production during reoxygenation/reperfusion. Furthermore, GSK inhibitors alter the phosphorylation status of VDAC and alter the affinity for Bcl-2, which may also contribute to the decrease in transport through VDAC. All of these factors, less calcium loading, less oxidant stress, and more Bcl-2 binding, likely contribute to the cardioprotective effect of GSK inhibitors.

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Disclosures
None.

References


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Detailed Methods

**Animals:** Male Sprague-Dawley rats (250-275 g, Harlan Sprague-Dawley) were used in this study. They were provided with food and water *ad libitum*. Rats were treated humanely and all experimental procedures were approved by the Institutional Animal Care and Use Committee of Johns Hopkins University.

**Langendorff Rat Heart Preparation:** After sufficient anesthesia was achieved with sodium pentobarbital (80 mg/kg b/w i.p. injection) (Abbott Laboratories, North Chicago, IL) and the rat was anticoagulated with heparin sodium (500 IU/kg body weight, i.v. injection) (Elkin-Sinn Inc., Cherry Hill, NJ), rat hearts were excised, cannulated, and perfused with Krebs-Henseleit buffer containing (in mmol/L) NaCl 120, KCl 5.9, MgSO4 1.2, CaCl2 1.25, NaHCO3 25, and glucose 11. The buffer was aerated with 95% O2 and 5% CO2, to give a pH of 7.4 at 37°C as described previously (1). Hearts were randomly assigned to one of three groups: control (0.01% DMSO), SB 216763 (3 µM), and SB 415286 (10 µM). All hearts were perfused for stabilization for 30 min; for the GSK-3β inhibitor groups, the inhibitor was present for the last 15 min. Dose response studies were done to determine the optimal concentration of both SB216763 and SB415286. The protocol consisted of 20 minutes global ischemia, followed by 40 minutes of reperfusion. LVDP, ± LVdp/dt, and heart rate were continuously monitored via a water-filled balloon inserted into the left ventricle. Recovery of contractile function was assessed by measurement of LVDP during reperfusion and was expressed as a percentage of preischemic, pretreatment LVDP.
Isolated Mitochondria Protocols: Freshly isolated mitochondria were prepared from hearts with or without SB treatment, by differential centrifugation (2-3). Briefly, at the end of perfusion, the left ventricle was dissected out and placed in Buffer A (in mM: 180 KCl, 2 EGTA, 5 MOPS, 0.2% BSA; pH: 7.25). The tissue was then digested with trypsin (0.0001 g/0.1 g tissue) in 0.7 ml of ice-cold Buffer B (in mM: 225 Mannitol, 75 sucrose, 5 MOPS, 0.5 EGTA, 2 Taurine; pH: 7.25) and finally homogenized with Buffer B with a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) using a Polytron. To further separate the heart mitochondria from other cellular components and tissue debris, a series of differential centrifugations were performed in a Microfuge 22R centrifuge (Beckman Coulter, Fullerton, CA) at 4°C. The crude pellet was then suspended in 400 µl Buffer (in mM: 137 KCl, 10 HEPES, 2.5 MgCl₂; pH: 7.2) at 4°C.

To characterize the integrity of the isolated mitochondria, the ADP-dependence of mitochondrial respiration was assessed at 25°C in a chamber containing respiration buffer (in mM) KCl 120, MOPS 5, EGTA 1, KH₂PO₄ 5, and 0.2% BSA, pH 7.25 and connected with a Clark-type O₂ electrode (Instech) and O₂ monitor (Model 5300, YSI, Inc) (1). After addition of glutamate/malate (10 mM/2 mM), state 3 respiratory rate was measured by addition of ADP. Once the ADP had been converted to ATP, the state 4 respiratory rate was determined.

Several protocols were used to examine mitochondrial adenine nucleotide metabolism under ischemia-like conditions. In the first protocol, mitochondrial ATP uptake and hydrolysis was assessed by adding ATP (875 µM) to mitochondria in the presence of 2,4-dinitrophenol (DNP, 50 µM), an uncoupler that rapidly dissipates the mitochondrial membrane potential. After 5 minutes, oligomycin (1 µg/ml) was added to block ATP hydrolysis and the supernatant was obtained by brief centrifugation. ATP remaining in the supernatant was measured (1).
The second protocol for assessing mitochondrial adenine nucleotide metabolism involved using cyanide to block respiration and allow the mitochondrial membrane potential to gradually dissipate. Mitochondrial ATP uptake was measured in the presence of sodium cyanide (NaCN, 2 mM), which was more similar to ischemic conditions than uncoupler and de-energized the mitochondria more slowly. After 20 minutes of reaction time, oligomycin (1 μg/ml) was added and the supernatant was isolated by brief centrifugation, and the ATP remaining in the supernatant was assayed.

The third protocol was to de-energize the mitochondria by consuming the oxygen in the oxygraph chamber, in the presence of a mixture of ADP and ATP. After state 3 and state 4 respiratory rates were measured, and the mitochondria were in state 4, equal amounts of ADP and ATP (875 μM) were added. ADP was added to stimulate oxygen consumption and ATP was added so that ATP consumption could be measured once the mitochondria became anoxic. Once the oxygen level in the oxygraph chamber reached zero, samples were taken at 0, 20, 40, and 60 minutes of anoxia, and oligomycin (1 μg/ml) was added to stop the reaction. Then the mitochondria were separated from the media by centrifugation and the supernatant was isolated. Finally ATP was measured in the supernatant fraction.

**Sonicated mitochondria protocol:** For some experiments, mitochondria were sonicated to disrupt the mitochondria and generate submitochondrial particles with the F$_1$F$_0$-ATPase exposed (4). The sonicated mitochondria was prepared by cyclic episodes of sonication in ice (15 seconds) using Sonic Cleaner 1.5 Qt (Fisher Scientific, Pittsburg, PA), followed by vortexing, and each cycle was repeated 10 times. ATP hydrolysis by the mitochondrial fragments was measured by adding ATP (875 μM) to the mitochondrial fragments, with or without DNP (50 μM). After 2.5 minutes, oligomycin (1 μg/ml) was added to block ATP hydrolysis and the
supernatant was obtained by brief centrifugation. ATP remaining in the supernatant was measured.

**Adenylate kinase assay protocol:** Another method for measuring adenine nucleotide transport across the outer mitochondrial membrane took advantage of the presence of adenylate kinase in the intermembrane space, between the inner and outer mitochondrial membranes. This enzyme converts ADP into AMP and ATP. Initially mitochondria were de-energized by using NaCN (2 mM) and at the same time F$_1$F$_0$-ATPase activity was blocked with oligomycin (1 μg/ml). After 20 min, ADP was added for an additional 10 minutes, followed by brief centrifugation to separate the mitochondria from the media, and the supernatant was isolated and assayed both for ADP remaining in the media and AMP produced by adenylate kinase.

**Cyclosporin protocols:** To check the involvement of the mitochondrial permeability transition pore in GSK-3 mediated regulation of adenine nucleotide transport, cyclosporin A (Calbiochem, La Jolla, CA), 200 μM in 0.01% ethanol, was perfused, with or without GSK inhibitor for 15 min. Mitochondria were isolated and ATP hydrolysis was measured by adding ATP (875 μM) to mitochondria with DNP (50 μM). After 5 minutes, oligomycin (1 μg/ml) was added to block ATP hydrolysis and the supernatant was obtained by brief centrifugation. ATP remaining in the supernatant was measured.

The effective dose of cyclosporin A was determined by using a mitochondria swelling assay, described previously (5-6). Briefly, the hearts were perfused with cyclosporin A as described above, and mitochondria were isolated. Mitochondrial pellets were resuspended in EGTA free buffer (in mM; 225 Mannitol, 75 Sucrose, 5 MOPS, 2 Taurine, at pH 7.25). About 300 μg of mitochondria were then added to the assay buffer (in mM; 120 KCl, 10 Tris-HCl, 5 MOPS, 5 KH$_2$PO$_4$, at pH 7.25) and energized with glutamate/malate (10 mM /2 mM). The
absorbance was monitored at 540 nm. After 2-3 minutes, 250 μM calcium was added and the absorbance was recorded for an additional 8-10 minutes.

**Heart and Mitochondria Preparation for Western Blot:** Whole cell samples, the mitochondria-enriched fraction, or cytosolic fraction were prepared by differential centrifugation from the frozen heart. Whole heart homogenate protein was separated by 1D gel electrophoresis. After transfer to a PVDF membrane, the membrane was incubated with antibody that recognizes proteins that are phosphorylated at serine or threonine residues at Akt phosphorylation sites (cell signaling), total VDAC (cell signaling) in Tris-Buffered Saline (pH 7.4) with 1% TWEEN 20 (TBS-T) with 5% BSA or nonfat dry milk at 4°C overnight. Membranes were incubated with the secondary antibody, appropriate horseradish peroxidase–conjugated IgG in TBS-T with 5% nonfat dry milk for 1 hour at room temperature. Immunoreactive protein was visualized using an enhanced chemiluminescence analysis kit (GE HealthCare, Piscataway, NJ).

**1D and 2D Gel Phosphorylation Site Analysis:** Whole heart homogenate protein was separated by 1D/2D gel electrophoresis and the gels were stained with phos-tag (Perkin Elmer Life and Analytical Sciences, Shelton, CT), which will stain all phosphorylated proteins.

**Proteomics Analysis:** The whole heart tissue was homogenized in lysis buffer (7.8 M Urea, 2.2 M Thiourea and 0.28% CHAPS). Isoelectric focusing (IEF) was performed using IPG strips (Bio-RAD, Hercules, CA) with either a nonlinear pH range of 3–10 or nonlinear pH range 5-8. Rehydration buffer (7.8 M urea, 2.2 M thiourea, 0.28% CHAPS, 0.9% Triton X-100, 11.1% isopropanol, 13.9% water saturated isobutanol, 5.6% glycerol, protease inhibitor cocktail Complete TM (Roche), a few grains of bromophenol blue, 69 mM DTT, 0.6% v/v biolytes) was added to both subfractions. The homogenates were applied to the IPG strips and rehydration was performed overnight at room temperature in a re-swelling tray. Next, the strips were focused in a
Protein IEF Cell (Bio-RAD, Hercules, CA) according to the following protocol: 250 V; 1 h, 500V; 1 hand 8000 V until 60 kVCh was reached. Next the IPG strips were equilibrated in the SDS-equilibration buffer (50 mM tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS) with the addition of DTT (10 mg/ml) for 15 min, followed by 15 min in the same buffer with the addition of iodoacetamide (25 mg/ml). SDS-PAGE was performed (180 mm x 180 mm x 1 mm polyacrylamide gel (12%) using the Protean II XL Cell System (Bio-RAD, Hercules, CA). The IPG strips were sealed in place with a 1% agarose solution, together with a protein mass standard. The 2-DE was carried out at 200 V for about an hour.

After the 2-DE separation, the gels were fixed overnight in 40% ethanol and 10% acetic acid. Next, the gels were washed in distilled water. Coomassie staining was performed for a minimal of 24 h in a 34% methanol, 17% (NH₄)₂SO₄, 2% H₃PO₄, and 0.066% CBB G-250 solution. The stained gels were scanned with a Typhoon 9400 variable mode imager (GE HealthCare, Piscataway, NJ) at a resolution of 100 μm and images were compared using Progenesis Discovery software (Nonlinear Dynamics, Newcastle upon Tyne, U.K.). Normalization of the gels was based on the total staining density of the image.

Spots of interest were excised in duplicate from the CBB stained gels and identified by LC/MS as described by Hopper et al (2). Briefly, the gel plugs were destained using a destaining solution (1 g (NH₄)HCO₃, 175 ml distilled water, 75 ml acetonitrile) until colorless. Subsequently, the gel plugs were dried using a centrifugal evaporator. The gel plugs were incubated overnight with trypsin (ultra sequencing grade, Promega, Madison, WI) at room temperature. Trypsin digestion was stopped by adding a solution of 1:2 acetonitrile and 0.1% TCA to the gel plugs. The sample solution was co-crystallized with matrix (6 mg a-cyano
cinnamic acid, 1 ml ACNM) onto a 400 µm anchor chip plate and air-dried before loading into the LC-MS.

**ATP, ADP, and AMP assays:** Supernatants from mitochondrial suspensions were separated from the mitochondria by centrifugation. PCA (4%) was added to denature any enzymes present in the supernatant, and the solutions were neutralized to pH 5-5.5 using KOH/Tris HCl. Adenine nucleotides were assayed using standard spectrophotometric techniques (7-8). Briefly, the assays were performed in a triethanolamine (TRA) buffer containing 50 mM TRA, 10 mM MgCl₂, 5 mM EDTA, pH 7.4. For the ATP assay, the TRA buffer also contained 5 mM glucose and 0.75 mM NADP. Glucose-6-phosphate dehydrogenase was added and absorbance at 340 nm was monitored. This initial reaction measured and removed any glucose-6-phosphate present in the solution. Then hexokinase was added to measure ATP by conversion of ATP and glucose to glucose-6-phosphate and ADP, and subsequent conversion of glucose-6-phosphate and NADP to NADPH and 6-phosphogluconate. The production of NADPH is monitored at 340 nm, and the mM extinction coefficient is 6.22. For the ADP and AMP assays, the TRA buffer also contained 1 mM phosphoenolpyruvate (PEP), 0.1 mM ATP, and 0.9 mM NADH. The reaction was started by addition of pyruvate kinase and lactate dehydrogenase, which converts ADP and PEP to ATP and pyruvate, and then pyruvate and NADH is converted to NAD and lactate, and the loss of NADH from the reaction mixture is monitored spectrophotometrically. NADH and NADPH have the same extinction coefficient at 340 nm. To assay AMP, adenylate kinase is added after the previous reaction is completed. This converts AMP plus ATP into 2 ADP, and then the ADP is used as a substrate for the pyruvate kinase/lactate dehydrogenase reactions. ATP is added to the initial reaction buffer, and ATP is created during the ADP assay, and therefore there is an
excess of ATP for the AMP assay. For all experiments, initial and final ATP or ADP is measured, and consumption is calculated from the difference.

**Preconditioning Protocol:** Experiments were performed using mitochondria isolated from ischemically preconditioned hearts. We used a standard preconditioning protocol consisting of 4 cycles of 5 minutes ischemia and 5 minutes reperfusion, and harvested the mitochondria after the final reflow period. We prepared mitochondria as described above, and measured ATP consumption under de-energized conditions as for the GSK inhibitor treated mitochondria. Online Figure I shows that ischemic preconditioning has a similar effect to GSK inhibitors on ATP consumption, although the effect was not as large as with the GSK inhibitors. This may be because some of the effect of preconditioning was lost during the mitochondrial isolation.
Online Figure I.

Effect of Uncoupler

ATP Consumption (% of initial ATP)

Control

PC

*
REFERENCES


