Glycogen Synthase Kinase-3 Inactivation Is Not Required for Ischemic Preconditioning or Postconditioning in the Mouse


Abstract—The inactivation of glycogen synthase kinase-3β (GSK-3β) is proposed as the event integrating protective pathways initiated by preconditioning and other interventions. The inactivation of GSK-3 is thought to decrease the probability of opening of the mitochondrial permeability transition pore. The aim of this study was to verify the role of GSK-3 using a targeted mouse line lacking the critical N-terminal serine within GSK-3β (Ser9) and the highly homologous GSK-3α (Ser21), which when phosphorylated results in kinase inactivation. Postconditioning with 10 cycles of 5 seconds of reperfusion/5 seconds of ischemia and preconditioning with 6 cycles of 4 minutes of ischemia/6 minutes of reperfusion, similarly reduced infarction of the isolated perfused mouse heart in response to 30 minutes of global ischemia and 120 minutes of reperfusion. Preconditioning caused noticeable inactivating phosphorylation of GSK-3. However, both preconditioning and postconditioning still protected hearts of homozygous GSK-3 double knockin mice. Moreover, direct pharmacological inhibition of GSK-3 catalytic activity with structurally diverse inhibitors before or after ischemia failed to recapitulate conditioning protection. Nonetheless, cyclosporin A, a direct mitochondrial permeability transition pore inhibitor, reduced infarction in hearts from both wild-type and homozygous GSK-3 double knockin mice. Furthermore, in adult cardiac myocytes from GSK-3 double knockin mice, insulin exposure was still as effective as cyclosporin A in delaying mitochondrial permeability transition pore opening. Our results, which include a novel genetic approach, suggest that the inhibition of GSK-3 is unlikely to be the key determinant of cardioprotective signaling in either preconditioning or postconditioning in the mouse. (Circ Res. 2008;103:307-314.)

Key Words: postconditioning ▪ preconditioning ▪ GSK-3 ▪ mPTP

Ischemic preconditioning is a powerful and established modulator of intrinsic myocardial resistance to lethal ischemia.1 Its clinical application in patients with acute myocardial infarction is limited as a result of the inability to predict the moment of coronary artery occlusion. More recently, Zhao et al described the concept of postconditioning as a novel strategy for protecting the heart against reperfusion-injury.2 They demonstrated that a similar regimen of brief periods of ischemia after a lethal index ischemic insult was as protective as preconditioning. This phenomenon has subsequently been confirmed in a variety of animal models, both in vivo and in isolated heart preparations,3 as well as in preliminary clinical interventional studies.4,5

The endogenous cellular and molecular mechanisms involved in cardioprotection against ischemia have been extensively investigated in the field of preconditioning.6 Because pre- and postconditioning are temporally remote, the signaling pathways might be expected to differ considerably.2,7 However, recent evidence suggests that the early reperfusion phase after lethal ischemia is crucial in mediating the cardioprotective effects of preconditioning.8,9 Furthermore, many of the mimetics and kinases of preconditioning have also been implicated in postconditioning.10,11 Finally, there is increasing evidence that the final step of both signaling pathways is inhibition of the mitochondrial permeability transition pore (mPTP),12–14 whereby pore opening results in cell death. Glycogen synthase kinase-3β (GSK-3β) has been reported as a common target of converging protective signals in preconditioning, immediately proximal to the mPTP, that inhibits pore opening in cardiomyocytes.15 Using both adult rat cardiomyocytes and neonatal rat cardiomyocytes, Juhaszova et al16 demonstrated that numerous preconditioning pathways converged to inactivate GSK-3β by Ser9 phosphorylation. Although the importance of GSK-3 in preconditioning is
Figure 1. Experimental protocols for assessment of infarction and GSK-3 signaling. A, In all experiments, hearts were subjected to 30 minutes of global ischemia and 120 minutes of reperfusion. Preconditioning (PreC) was performed with 4 cycles of 4 minutes of ischemia and 6 minutes of reperfusion. Postconditioning was performed with 10 cycles of 5 seconds of reperfusion/ischemia (5 seconds PostC) and with 4 cycles of 30 seconds of reperfusion/ischemia (30 seconds PostC). Arrows indicate time points for protein analysis. Hearts were harvested before ischemia, at the end of ischemia, and at the indicated time after reperfusion with or without pre- and postconditioning. B, Hearts in the Pre SB and Pre BIO groups were perfused with 3 μmol/L SB216763 and 100 nmol/L BIO for 15 minutes before ischemia, respectively. Post SB15 and Post SB120 hearts were perfused with 3 μmol/L SB216763 for 15 minutes and 120 minutes after ischemia, respectively. Hearts in the CsA group were perfused with 0.2 μmol/L CsA for 10 minutes after ischemia.

Materials and Methods

Detailed methodology is provided in the online data supplement, available at http://circres.ahajournals.org. Key techniques involved adaptations of previously published protocols, including those for the perfusion and assessment of infarction in isolated murine hearts,20,21 immunoblot analysis,20,21 isolation of murine ventricular myocytes,22 and assay of the mitochondrial permeability transition.23

GSK-3 Knockin Mice

The targeting strategy used to generate GSK-3αβ/β knockin (KI) mice, in which the PKB/Akt phosphorylation sites on GSK-3α (Ser21) and GSK-3β (Ser9) are changed to Ala, has been described previously.18

Pre- and Postconditioning Experiments

Protocol 1

Our objective in this protocol was to assess the potential for postconditioning (PostC) to limit infarct size and compare it to preconditioning (PreC) in our model. C57BL/6 mice were divided into 4 study groups, as shown in Figure 1A. In the control group, there was no additional intervention. Hearts in the PreC group underwent 4 cycles of 4 minutes of ischemia/6 minutes of reperfusion before the 30 minutes of global ischemia. Hearts in the PostC group were subjected to 1 of 2 protocols to determine the optimum strategy: in the 30 seconds PostC and 5 seconds PostC groups, 4 cycles of 30 seconds of reperfusion/30 seconds of ischemia and 10 cycles of 5 seconds of reperfusion/5 seconds of ischemia were performed at the end of 30 minutes of index ischemia, respectively.

Protocol 2

GSK-3αβ KI mice were used to determine the dependence of preconditioning cardioprotection on GSK-3 inhibition. GSK-3 wild-type (WT) and KI mice were assigned to control, PreC, and PostC with repetitive 5 seconds of reperfusion/ischemia groups as described in protocol 1.

Protocol 3

In these experiments, the effects on infarct size of direct pharmacological inhibition of GSK-3 activity before and after ischemia were examined (Figure 1B). Two inhibitors, SB216763 (3 μmol/L) (Sigma) and 6-bromoisouindirubin-3′-oxime (BIO) (100 nmol/L) (Calbiochem), were used based on concentrations required to achieve dephosphorylation of glycogen synthase. C57BL/6 mice were divided into five groups (Figure 1B): (1) standard ischemia/reperfusion (control); pretreatment with (2) 3 μmol/L SB216763 (Pre SB) or (3) 100 nmol/L BIO (Pre BIO) for the last 15 minutes of stabilization; and treatment postischemia with (4) 3 μmol/L SB216763 for the first 15 minutes of reperfusion (Post SB15) or (5) 3 μmol/L SB216763 for 2 hours (Post SB120). SB216763 and BIO were dissolved in dimethyl sulfoxide (DMSO) and then diluted with perfusion buffer so that the final concentration of DMSO was 0.05%.

Protocol 4

These experiments set out to determine whether the cardioprotective effect of direct pharmacological inhibition of mPTP opening at reperfusion is preserved in the GSK-3αβ KI mouse. After 30 minutes of global ischemia, GSK-3 WT and KI hearts were subjected to either infusion of 0.2 μmol/L cyclosporin A (CsA) (Sigma) for 10 minutes at the moment of reperfusion or normal buffer (control) (Figure 1B). CsA was dissolved in DMSO at a final concentration of less than 0.05%. Similar experiments were also performed in isolated adult murine myocytes to visualize mPTP opening directly.

Results

Pre- and Postconditioning the Isolated Murine Heart: Protocol 1

The PreC protocol was as used previously in this model.20 Postconditioning was examined using 2 reperfusion patterns to determine the optimum strategy (30 seconds PostC and 5 seconds PostC). The protocols, described in Figure 1A, resulted in significant reductions of infarction only in the case of PreC and 5 seconds PostC (see protocol 1 in Table II in the online data supplement), which was reflected by improved contractile recovery in these groups (see protocol 1 of supplemental Table I).
Despite improved recovery in the PreC and 5 seconds PostC groups, there was only a slight effect on GSK-3α/β phosphorylation (see Figure 2A and 2B, respectively). On close examination GSK-3β, but not GSK-3α, was phosphorylated after preconditioning (phospho-/total GSK-3β 335.3±38.1% of control baseline; \( P<0.05, n=5 \)) and then dephosphorylated during ischemia to a level similar to that of control (150.5±82.4% of control baseline and 95.1±52.1% of time-matched control ischemia; \( P=NS, n=5 \) for both). During reperfusion, GSK-3β demonstrated some rephosphorylation that was comparable in preconditioned and control-reperfused hearts (Figure 2A) and far less than that seen with insulin. Postconditioning did not significantly alter GSK-3α/β Ser21/9 phosphorylation compared to control (Figure 2B). Furthermore, we examined the phosphorylation status of Tyr 279/216 of GSK-3α/β and found these sites similarly phosphorylated under all conditions (data not shown).

Thus, robust methods exist to pre- and postcondition the isolated mouse heart on the C57BL/6 background. These forms of conditioning were associated with variable, but low levels, of inhibitory phosphorylation of GSK-3α/β. We next examined whether this phosphorylation, albeit at a low level, had any influence on conditioning of WT and KI mice from the GSK-3 colony, ostensibly on the same C57BL/6 background.

**Characterization of GSK-3α/β-Dependent Signaling in Inactivation-Resistant Hearts**

GSK-3 KI mice were phenotypically similar to WT animals from the same colony. In particular, there were no differences in baseline developed pressure or coronary flow (see protocol 2 of supplemental Table I) or body weight and heart size (see protocol 2 of supplemental Table II). Myocardial content of PKB/Akt, GSK-3, and glycogen synthase protein was also comparable between genotypes (see Figure 3), consistent with existing data. The α and β isoforms of GSK-3 seem approximately equally abundant and unchanged by targeting. Insulin induced robust phosphorylation of PKB/Akt at Ser473 and found these sites similarly phosphorylated under all conditions (data not shown).

Having determined that GSK-3 within KI hearts is resistant to inactivation even in response to insulin (see Figure 3), they were next subjected to pre- and postconditioning. We used the protocols (PreC and 5 seconds PostC within protocol 1 of supplemental Tables I and II) optimized to reduce infarction, which were associated with residual GSK-3 phosphorylation (albeit at a much lower level than occurs with insulin; see Figure 2).

Surprisingly, both pre- and postconditioning protected hearts with inactivation-resistant (KI) forms of GSK-3 just as well as within colony controls containing inactivation-sensitive forms of GSK-3 (WT) (see protocol 2 of supplemental Tables I and II). The protection by both forms of conditioning was evident in infarction size on both WT and KI backgrounds. However, the WT mice were more sensitive to infarction than the KI mice and also probably outsourced C57BL/6 controls.

Based on these data, it seems that inhibition of GSK-3α/β through phosphorylation of the regulatory N-terminal serines is not essential to either pre- or postconditioning. However, given the indirect supporting evidence within the literature, it is possible that GSK-3 inhibition by another means contributed to protection. To examine this possibility we determined the effect of pharmacological inhibition of GSK-3 on infarction and posts ischemic recovery.

**Pharmacological Inhibition of GSK-3α/β: Protocol 3**

To determine whether direct inhibition of GSK-3 mimics conditioning, we used 2 structurally diverse ATP-competitive small molecule inhibitors. As seen in Figure 2, comparing insulin to other lanes, the majority of GSK-3 is in a nonphosphorylated active form and thereby amenable to inhibition.

In Figure 4, we used the same readout of GSK-3 activity as in Figure 3, i.e., the maintenance of glycogen synthase phosphorylation. Exposure to 3 \( \mu \)mol/L SB216763 or 100 nmol/L BIO for 15 minutes resulted in complete dephosphorylation of the GSK-3 sites within glycogen synthase, suggesting that these concentrations completely inhibit GSK-3 activity. We therefore used these concentrations and exposure times immediately before global ischemia to determine the relevance of the GSK-3 phosphorylation seen with preconditioning (see Figure 2A). Neither regime had a consistent effect on posts ischemic recovery or infarction size (see protocol 3 Pre BIO and Pre SB of supplemental Tables I and II). Next, we examined the relevance of the low levels of GSK-3 phosphorylation seen during reperfusion with postconditioning in Figure 2B by determining whether protection could be mimicked by GSK-3 inhibition during reperfusion. Because it was uncertain for how long the low levels of GSK-3 phosphorylation persisted during reperfusion, we examined infarction in response to 3 \( \mu \)mol/L SB216763 for the first 15 minutes (Post SB15) and throughout (Post SB120) reperfusion. As can be seen from the results in protocol 3 of supplemental Tables I and II, neither duration of GSK-3 inhibition recapitulated the protection seen with postconditioning.

**The Effect of CsA: Protocol 4**

Although we were able to pre- and postcondition the mouse heart, none of our pharmacological or genetic manipulations
Figure 2. A, GSK-3 phosphorylation time course in buffer-perfused C57BL/6 hearts subjected to preconditioning (PreC) or control perfusion (Con). Heart samples were collected just before ischemia (PreC-Base or Con-Base), at the end of 30 minutes of ischemia (PreC-Isch30 or Con-Isch30) and at 15 minutes of reperfusion (PreC-Rep or Con-Rep) with or without ischemic preconditioning. Perfusion of a nonischemic heart with insulin (5 U/L; Actrapid, Novo Nordisk) for 15 minutes was used as positive control for GSK-3 phosphorylation. Heart samples were snap frozen in liquid nitrogen for immunoblotting analysis. Representative immunoblots are shown with quantitative analyses of repeat experiments expressed as the ratio of phosphorylated to total protein. Data are expressed as the means±SEM (n=5). *P<0.05 vs control baseline.

B, GSK-3 phosphorylation time course in buffer-perfused C57BL/6 hearts subjected to postconditioning (PostC-Rep) or control reperfusion (Con-Rep). Heart samples were collected just before ischemia (Con-Base), at the end of 30 minutes of ischemia (Isch-30), and at 2, 5, and 15 minutes of reperfusion, with or without ischemic postconditioning (PostC-Rep or Con-Rep). Perfusion of a nonischemic heart with insulin (5 U/L; Actrapid, Novo Nordisk) for 15 minutes was used as positive control for GSK-3 phosphorylation. Heart samples were snap frozen in liquid nitrogen for immunoblotting analysis. Representative immunoblots are shown with quantitative analyses of repeat experiments expressed as the ratio of phosphorylated to total protein. Data are expressed as the means±SEM (n=5). *P<0.05 vs control baseline.
of GSK-3 activity had an effect on postischemic recovery or infarction. The mouse heart has been used by a variety of investigators to elucidate cardioprotective signaling, but we remained concerned that the GSK-3–mPTP signaling axis was not operative in our model and in particular on the background of the GSK-3 colony. We therefore examined the effect of CsA. Once again, the WT hearts had greater infarcts than KI hearts (see protocol 4 of supplemental Table II). However, CsA reduced infarction and improved contractile recovery similarly in both genotypes (see protocol 4 of supplemental Tables I and II), suggesting mPTP opening at reperfusion does contribute to infarction in this model but is not influenced by varied manipulations of GSK-3 activity.

To confirm more directly that GSK-3 inactivation did not influence mPTP, the timing of opening was assayed in cardiomyocytes isolated from adult KI mice using confocal laser analysis, as previously described.23 The time to mPTP opening was significantly increased in cells exposed to CsA in both GSK-3 WT cardiomyocytes (from 91.6±6.6 to 144.2±6.4 seconds; P<0.05) and KI cardiomyocytes (from 127.4±10.3 to 196.3±18.7 seconds; P<0.05). Insulin exposure was as effective as CsA in delaying mPTP opening in both genotypes (WT, 136.0±6.8 seconds; KI, 194.5±17.2 seconds; P<0.05 compared to control baseline). This demonstrates that, even in the absence of inactivatable GSK-3, signals downstream of insulin are still able to act on the mPTP. Furthermore, the time to mPTP opening in isolated cardiomyocytes subjected to laser-induced oxidative stress was longer in KI cells compared to WT cells. This was true at baseline and after treatment with insulin or CsA (P<0.05 in each study group). This is consistent with the results that the KI heart is more resistant to global ischemia than WT.

Discussion

The purpose of the present study was to use a novel mouse line to confirm that the inhibition of GSK-3 is a necessary step in the signal transduction cascade that leads to protection in pre- and postconditioning. Contrary to our expectations, mouse hearts with only noninhibitable forms of GSK-3 could still be protected by either form of conditioning. Furthermore, in myocytes isolated from these mice, insulin was still able to delay mPTP opening. Moreover, documented pharmacological inhibition of GSK-3 with structurally diverse agents present before, or after, ischemia failed to recapitulate the protection observed with conditioning. Thus, in the murine heart, the inhibition of GSK-3 is neither necessary nor sufficient to cause pre- or postconditioning.

Notably, our results differ from those of Juhaszova et al15 who previously demonstrated that isolated cardiomyocytes from transgenic mice expressing a noninhibitable isoform of GSK-3 showed no protection. These discrepancies may be explained by differences in the genetic background or by the use of different agents to inhibit GSK-3. It is possible that GSK-3 plays a different role in cardiomyocytes isolated from different mouse strains or that different agents have different effects on GSK-3 activity.

Figure 3. Effects of insulin on PKB/Akt–GSK-3–glycogen synthase signaling cascade in GSK-3 WT and KI hearts. GSK-3 WT and KI hearts were harvested at 40 minutes after intraperitoneal saline (Con) or insulin injection. Representative immunoblots are shown with quantitative analyses of repeat experiments expressed as the ratio of phosphorylated to total protein. Data are expressed as the means±SEM (n=3). *P<0.05 vs control in each protocol; †P<0.05 KI vs WT in each protocol. N/D indicates not detectable.
GSK-3β were resistant to the protective effects of ischemic and pharmacological preconditioning.15 Transgenic GSK-3β expression in these hearts was approximately 8-fold greater than the physiological endogenous level introducing the possibility of loss of physiological substrate selectivity and induction of a misfolded protein response, whereas allelically encoded GSK-3β still remained potentially inhibitable by N-terminal phosphorylation. Furthermore, in these mice at 4 weeks, transgene expression is accompanied by a 4- to 5-fold upregulation of both atrial natriuretic factor and brain natriuretic peptide,25 which are cardioprotective peptides.26–28 Moreover, transgenic overexpression of GSK-3β has been associated with metabolic derangement29 and myocardial contractile dysfunction.30 These factors may in part explain the divergence in findings between the 2 genetic approaches taken to examine the same hypothesis.

The reason we adopted a genetic approach that differed from that taken by Juhaszova et al15 included the concerns outlined above, as well as the fact that little is known about the action of GSK-3α in the heart. The most notable difference between the 2 isoforms is that GSK-3α possesses a glycine-rich N-terminal extension of unknown function that is absent in GSK-3β, resulting in 5-kDa difference in molecular mass between isoforms.31 However, the isoforms share more than 98% homology within their kinase domain,19 seem approximately equally abundant in cardiac myocytes,32 in keeping with our findings, are identically inhibited by PKB/ Akt,31 cannot be selectively inhibited pharmacologically,31 and, most importantly, exhibit functional redundancy on systematic allelic disruption.31 Thus, although the emphasis to date has been on GSK-3β, it seems probable that inhibition of GSK-3α could similarly lead to protection or even that coincident inhibition of both kinases is required. To cover all of these possibilities, we chose to use GSK-3α/β double KI mice in our initial experiments to confirm their role in conditioning, before progressing to mice homologous for targeted alleles encoding noninhibitable kinases of each individual isoform. Because conditioning still occurred on the double KI background, however, the additional experiments became redundant.

Despite the evidence of functional redundancy between GSK-3α and GSK-3β in early mammalian development,31 the situation in the heart maybe more complex. For example, in zebrafish, a morpholino-based knockdown of GSK-3α results in a cardiac phenotype that differs from GSK-3β knockdown and cannot be rescued by GSK-3β overexpression.33 Similarly, although cardiac-restricted expression of GSK-3α and GSK-3β in mice results in a similar phenotype, there are biochemical distinctions in the coupled downstream signals and in the response to hemodynamic stress.34 Thus, it is possible GSK-3α and GSK-3β have distinct, and perhaps opposing, roles during myocardial ischemia that complicate our observations in a mouse line in which both alleles have been targeted in all cell types and tissues. Thus, our findings may have differed if we had examined mice in which only GSK-3β had been targeted. However, in other species at least, it seems such opposition does not prevent the reduction in infarction seen with pharmacological inhibitors that do not discriminate between the GSK-3 isoforms.16,17,35,36

Downstream of GSK-3α/β there are proposed links with certain components of the mPTP, including the voltage-dependent anion channel and adenine nucleotide translocator.15,37 GSK-3α/β is suggested as the immediate point of convergence for multiple preconditioning signaling pathways.15 However, more recent studies have suggested that voltage-dependent anion channel and adenine nucleotide translocator are not essential to functional mPTP formation38,39 and that there is no mitochondrial GSK-3β detectable

![Figure 4. Effects of GSK-3 inhibition on GSK-3 and glycogen synthase levels. C57BL/6 hearts were harvested after 15 minutes of perfusion with normal buffer (Control), insulin, SB216763 (SB), and BIO. Representative immunoblots are shown with quantitative analyses of repeat experiments expressed as the ratio of phosphorylated to total protein. Data are expressed as the means±SEM (n=3). *P<0.05 vs control.](image_url)
after preconditioning stimuli,\textsuperscript{14} raising more doubt regarding the dependency of mPTP function on upstream GSK-3 activity. CsA administered at the point of reperfusion is believed to inhibit mPTP opening by preventing binding of cyclophilin D to the adenine nucleotide translocator\textsuperscript{40} and has been shown significantly to reduce infarct size by numerous independent investigators.\textsuperscript{3,41–44} This correlates with the cardioprotection afforded by CsA in our study, demonstrated in both WT and KI animals, supporting the independence of mPTP function and its own candidacy as end effector in protective signaling.

Surprisingly, GSK-3 KI hearts appeared less sensitive to infarction than WT mice, both in response to global ischemia in the whole heart and at the cellular level in response to oxidative stress. One possible explanation for this may be attributable to compensatory signaling pathways other than GSK-3 in KI mice. However, we did not observe any difference between genotypes in Akt, p38, extracellular signal-regulated kinase, p70S6 kinase, and PKC-\(\epsilon\) signaling, all of which have been reported as important kinases in cardioprotective signaling (data not shown).\textsuperscript{10,11}

Our results using pharmacological inhibitors of GSK-3 in the mouse heart differ from those previously reported in other species (rats\textsuperscript{16,17,35} or rabbit\textsuperscript{36}). Most importantly, this may reflect species difference, because we observed a protective effect in parallel studies of the isolated ischemic rat heart pretreated with BIO and SB216763 (percentage infarct size: 29.7\(\pm\)2.9\% \([P<0.05]\) and 38.4\(\pm\)4.3\% versus 43.6\(\pm\)3.9\% in control, respectively) (Y Nishino, MS Marber, unpublished data, 2008). Other potential explanations for the discrepancy, including experimental preparations (global versus regional ischemia\textsuperscript{17,35,36} or ex vivo versus in vivo\textsuperscript{17}) and inhibitor concentration (eg, 3 \(\mu\)mol/L versus 1 \(\mu\)mol/L SB216763\textsuperscript{36}), should be considered and inhibitors titrated based on their effects on signaling in that specific model and species. However, the use of 2 structurally diverse pharmacological inhibitors, together with the persistence of the conditioning effects, in the double KI mice would support the validity of our results in the mouse.

In the present study, the isolated Langendorff-perfused murine heart model was used to obtain contractile parameters of cardiac function, without confounding influences of neurohormonal and other compensatory factors. We acknowledge the use of isolated hearts with relatively short durations of reperfusion as a limitation of the study which might affect 5-triphenyl tetrazolium chloride staining for infarct measurement. Two postconditioning regimens were tested against an established murine preconditioning protocol. Postconditioning with 5-second cycles of reperfusion/ischemia was equally effective as preconditioning in limiting infarct size and preserving hemodynamic parameters in response to global ischemia, whereas 30-second cycles of postconditioning, known to be protective in some species,\textsuperscript{2,45–47} proved ineffective. These findings provide confirmatory evidence that the effectiveness of ischemic postconditioning is highly dependent on the number and duration of the interruptions to reperfusion, with a trend toward longer reperfusion-ischemia cycle lengths required in animals of increasing size (ie, 5 to 10 seconds in mice\textsuperscript{7,42} versus 1 minute in humans\textsuperscript{4}).

In conclusion, our findings based on a combination of genetic and pharmacological interventions do not support the premise that GSK-3 inhibition is essential to pre- or postconditioning of isolated mouse hearts.

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**Disclosures**

None.

**References**


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Materials and Methods

All experiments were performed in accordance with United Kingdom Home Office Guidance on the Operation of Animals (Scientific Procedures) Act 1986, published by Her Majesty’s Stationary Office, London.

GSK-3 knock-in mice

The targeting strategy used to generate GSK-3α/β knock-in mice, in which the PKB/Akt phosphorylation sites on GSK-3α (Ser21) and GSK-3β (Ser9) are changed to Ala, has been described previously.1 GSK-3 wild type (WT) and homozygous GSK-3 double knock-in mice (KI) were in-bred, and from the same colony, but for practical reasons not littermates. Efficacy of GSK-3 modulation was assessed by insulin stimulation of the PKB/Akt-GSK-3 signalling pathway.

Mice were fasted for 8 hours and injected intraperitoneally with insulin (150 mU/g; Actrapid, Novo Nordisk) or saline solution. At 40 min after injection hearts were rapidly extracted and snap frozen in liquid nitrogen. Immunoblotting was performed for PKB/Akt, GSK-3 and glycogen synthase.

Perfusion of isolated murine hearts

Adult male mice were anesthetized with pentobarbital (300 mg/kg) and heparin (150 units) intraperitoneally. Hearts were rapidly isolated, mounted onto a Langendorff apparatus and retrogradely perfused at a constant pressure of 80 mmHg with Krebs-Hensleit buffer (in mmol/L: 118.5 NaCl, NaHCO₃, 4.75 KCl, 0.18 KH₂PO₄, 1.19 MgSO₄, 11.0 D-glucose, and 1.41 CaCl₂) equilibrated with 95% O₂ and 5% CO₂ at 37°C. A fluid-filled balloon inserted into the left ventricular cavity monitored contractile function. The balloon was gradually inflated until the left ventricular end-diastolic pressure (LVEDP) was between 2 and 8 mmHg. Atrial pacing was performed at 580 bpm and coronary flow (CF) measured by timed collection of perfusate.

Infarction assessment in isolated murine hearts

Hearts were stabilised for 30 min after initiation of retrograde perfusion. For inclusion, all hearts had to fulfill the following criteria:- CF between 1.5 and 4.5 mL/min, initial heart rate > 300 bpm (unpaced), left ventricular developed pressure (LVDP) > 55 mmHg, time from
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thoracotomy to aortic cannulation < 3 min, and no persistent dysrhythmias during the stabilization period. All hearts underwent 30 min of global ischemia followed by 2 hours of reperfusion. At the end of reperfusion hearts were perfused for 1 min with 5 mL of 1 % triphenyl tetrazolium chloride (TTC) in PBS and then placed in an identical solution at 37 °C for 10 min. The atria were then removed, and the hearts were blotted dry, weighed, and stored at -20 °C. Hearts were subsequently thawed, placed in 2.5 % glutaraldehyde for 1 min and set in 5 % agarose. The agarose heart blocks were then sectioned from apex to base in 0.75 mm slices using a vibratome (Agar Scientific). Sections were compressed between glass plates and scan-imaged (Epson model G850A). After magnification, planimetry was carried out using image analysis software (Adobe Photoshop 7.0). Risk and infarct volumes were calculated from surface area analysis of whole myocardium and TTC-negative myocardium, respectively, multiplied by tissue thickness. After summation of individual slices, infarct volume was expressed as a percentage of ventricular volume. Infarct analysis was performed in all cases by an investigator blinded to the group assignments.

Pre- and post-conditioning experiments

Protocol 1: Our objective in this protocol was to assess the potential for postconditioning (PostC) to limit infarct size, and compare it to preconditioning (PreC) in our model. C57BL/6 mice were divided into 4 study groups, as shown in Figure 1A. In the Control group there was no additional intervention. Hearts in the PreC group underwent 4 cycles of 4 min ischemia/6 min reperfusion before the 30 min global ischemia. Hearts in the PostC group were subjected to one of two protocols in order to determine the optimum strategy:- in the 30sec PostC and 5sec PostC groups, 4 cycles of 30 sec reperfusion/30 sec ischemia and 10 cycles of 5 sec reperfusion/5 sec ischemia were performed at the end of 30 min index ischemia, respectively.

Protocol 2: GSK-3α/β knock-in mice were used to determine the dependence of pre- and post-conditioning cardioprotection on GSK-3 inhibition. GSK-3 WT and KI mice were assigned to Control, PreC and PostC with repetitive 5sec reperfusion/ischemia groups as described in Protocol 1.

Protocol 3: In these experiments the effects on infarct size of direct pharmacological inhibition of GSK-3 activity before and after ischemia were examined (Figure 1B). Two inhibitors, SB216763 (3 µmol/L) (Sigma) and 6-bromoindirubin-3’-oxime (BIO) (100
nmol/L) (Calbiochem), were employed based on concentrations required to achieve dephosphorylation of glycogen synthase. C57BL/6 mice were divided into five groups (Figure 1B):- (i) standard ischemia/reperfusion group (Control), (ii) pretreatment with 3 µmol/L SB216763 (Pre SB) or (iii) 100 nmol/L BIO (Pre BIO) for the last 15 min of stabilization, (iv) treatment posts ischemia with 3 µmol/L SB216763 for the first 15 min of reperfusion (Post SB15) or (v) 3 µmol/L SB216763 for 2 hours (Post SB120). SB216763 and BIO were dissolved in dimethyl sulfoxide (DMSO) and then diluted with perfusion buffer so that the final concentration of DMSO was 0.05 %.

Protocol 4: These experiments set out to determine if the cardioprotective effect of direct pharmacological inhibition of mPTP opening at reperfusion is preserved in the GSK-3α/β knock-in mouse. After 30 min global ischemia, GSK-3 WT and KI hearts were subjected to either infusion of 0.2 µmol/L Ciclosporin A (Sigma) (CsA) for 10 min at the moment of reperfusion, or normal buffer (Control) (Figure 1B). Ciclosporin A was dissolved in DMSO at a final concentration of less than 0.05 %. Similar experiments were also performed in isolated adult murine myocytes to visualise mPTP opening directly.

Immunoblotting
Samples were thawed and homogenized in protein extraction buffer (50 mmol/L Tris-HCl; pH 7.5, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% (w/v) Triton X-100, 0.1 % β-mercaptoethanol, 1 mmol/L sodium orthovanadate, 50 mmol/L sodium fluoride, 5 mmol/L sodium pyrophosphate and protease inhibitor tablets (Complete®, Roche Applied Science), (1 Tab/50 ml of buffer). One millilitre of extraction buffer was used per 100 mg of frozen tissue. Homogenates were centrifuged at 4 °C for 10 min at 13,000 rpm and the insoluble fraction discarded. Protein content was estimated using a Bio-Rad Protein Assay Kit. Twenty micrograms of protein were electrophoresed on a 10 % polyacrylamide gel and then blotted onto a polyvinylidene difluoride membrane. After blocking with 5 % non-fat milk, the membranes were incubated with the following primary antibodies at dilutions of 1:1000:- anti-total-GSK-3β (#9315), anti-phospho-GSK-3α/β (Ser21/9) (#9331), anti-phospho-GSK-3β (Ser9) (#9336), anti-total-PKB/Akt (#9272), anti-phospho-PKB/Akt (Ser473) (#9271) (all from Cell Signalling), anti-total-GSK-3α/β (#05-903) (Upstate), anti-total-glycogen synthase (#MAB3106) (Chemicon), anti-phospho-GSK-3α/β (Tyr 279 and 216) (#44-604G) (#9331) and anti-phospho-glycogen synthase (Ser 641 and 645) (#44-1092G) (Biosource).
Appropriate secondary antibodies were used and proteins visualized using an ECL Western blotting detection kit (Amersham).

**Isolation of adult mouse ventricular cardiomyocytes**

Adult male mice were injected with heparin sodium (250 units) and anesthetized with ketamine/xylazine/atropine. The hearts were rapidly excised, cannulated and perfused with (in mmol/L) 113 NaCl, 4.7 KCl, 0.6 KH₂PO₄, 0.6 Na₂HPO₄, 1.2 MgSO₄.7H₂O, 12 NaHCO₃, 10 KHCO₃, 30 taurine, 10 HEPES, and 5.5 glucose, saturated with 95% O₂-5% CO₂ at 37°C. The hearts were perfused at 3 ml/min with perfusion buffer for 4 min, then with perfusion buffer containing 0.9 mg/ml collagenase (Wort hington type II), 0.125 mg/ml hyaluronidase and 12.5 μmol/L CaCl₂ for 10 min. The ventricles were then cut into several pieces and shaken at 37°C with oxygenation for 10 min. The supernatant was collected and 5% foetal calf serum was added. After centrifugation at 600 rpm for 3 min, the cell pellet was suspended in 10 ml of perfusion buffer containing 12.5 μmol/L CaCl₂ and the calcium concentration was gradually restored to 1 mmol/L over 20 min. The myocytes were recentrifuged then seeded onto sterilized laminin-coated coverslips in MEM medium containing 10 units/ml penicillin, 10 μg/ml streptomycin, 5% foetal calf serum for incubation before use on the same day of isolation.

**Assay of mPTP opening**

The sensitivity of the mPTP to opening was assayed using a well-characterized and reproducible cellular model.² Live isolated myocytes were incubated with the fluorescent dye, tetra-methyl rhodamine methyl ester (TMRM, 3 μmol/L) for 15 minutes in microscopy buffer (i.e.: perfusion buffer containing 1.2 mmol/L CaCl₂), then washed and imaged using confocal microscopy. TMRM, a lipophilic cation, accumulates selectively into mitochondria according to the mitochondrial membrane potential. Continual confocal laser-scanning generates reactive oxygen species (ROS) from the TMRM within the mitochondria, which, after several minutes, provokes mPTP opening, as indicated by mitochondrial depolarization. Some wells were treated with Ciclosporin A (0.2 μmol/L), or with insulin (0.3 U/L) for 10 min before and during analysis. A total of 20-39 cells from 4-6 hearts were analyzed in separate experiments.

**Statistical analysis**
All data are presented as means ± SEM. Comparisons between groups were assessed for significance by analysis of variance (ANOVA) or repeated measures ANOVA, as appropriate. When significant differences were detected, individual mean values were compared by Bonferroni’s post hoc test which allowed for multiple comparisons. All statistical analyses were performed using SPSS for Windows (v12.0.2; SPSS). P values less than 0.05 were considered significant.
Reference List


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Values are means ± SEM. *P < 0.05 vs. Control in each protocol. †P < 0.05 vs. WT in each protocol.

Left ventricular developed pressure (LVDP), left ventricular end-diastolic pressure (LVEDP) and coronary flow (CF) were measured after 30 min of stabilization (Baseline), and at 60 min (Rep 60 min) and 120 min (Rep 120 min) after the onset of reperfusion.
### Online Table II. Summary of infarct size data

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Values are means ± SEM. I/R, infarct size as a percentage of area at risk.

*P < 0.05 vs. Control in each study group. †P < 0.05 vs. WT in each study group.