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Inhibition of glycogen synthase kinase 3 β prevents peroxide-induced collapse of mitochondrial membrane potential in rat ventricular myocytes

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Abstract

1. Preconditioning has been proposed to protect the myocardium by inhibiting glycogen-synthase kinase (GSK) 3 β . The aim of the present study was to test whether transfection of ventricular myocytes with inactive GSK3 β would mimic preconditioning and whether a constitutively active form of GSK3 β would prevent protection by an opioid receptor agonist.

2. Isolated ventricular myocytes from adult rats were infected with live adenovirus containing either a wild-type (wtGSK), constitutively active (caGSK) or dominant-negative (dnGSK) GSK3 β plasmid. Cells were loaded with tetramethylrhodamine ethyl ester (TMRE) and exposed to H₂O₂ (100 μ mol/L) for 40 min before mitochondrial membrane potential ($\Delta\Psi_m$) was assessed using flow cytometric analysis.

3. Fluorescence intensity was reduced in H₂O₂-treated cells compared with untreated cells, presumably because oxidant injury opened mitochondrial permeability transition pores, causing mitochondria to lose TMRE. The selective GSK3 β inhibitor SB216763, as well as the Δ -opioid receptor agonist [D-Ala²-D-Leu⁵]-enkephalin (DADLE) (1 μ mol/L), protected cells against peroxide-induced loss of $\Delta\Psi_m$.

4. Cells transfected with dnGSK (1 μ mol/L) were equally protected against peroxide stress, when given throughout the TMRE and H₂O₂ treatment, confirming a protective effect of GSK3 β with a highly selective inhibition. Cells transfected with wtGSK did not show any difference in responses to H₂O₂, SB216763 or DADLE compared with untransfected cells, suggesting that adenovirus infection itself had no effect. In contrast, caGSK-transfected myocytes could no longer be protected with DADLE, suggesting a role for GSK3 β between the surface receptor and the mitochondria.

5. These experiments confirm that inhibition of GSK3 β protects the myocytes, but also that the preconditioning mimetic DADLE loses its protective effect when a constitutively active GSK3 β is present.

Introduction

Ischaemic preconditioning (IPC) is a powerful endogenous intervention that reduces ischaemic injury in the heart. The protective mechanism of IPC is thought to involve inhibition of the formation of mitochondrial permeability transition pores (mPTP) at the onset of reperfusion.¹ These pores, which open at reperfusion,² uncouple the mitochondria, blocking their ability to regenerate ATP, and also cause the release of pro-apoptotic cytochrome *c* into the cytosol. Various receptor agonists, such as Δ -opioid receptor agonists,³ bradykinin⁴ and adenosine mimetics,⁵ given at reperfusion are also thought to protect through inhibition of mPTP. Juhaszova *et al.*⁶ proposed that IPC inhibits mPTP formation by inhibiting glycogen synthase kinase (GSK) 3 β activity through phosphorylation. Inhibition of GSK3 β then inhibits mPTP formation. Indeed, pharmacological inhibition of GSK3 β mimics IPC.⁷ We⁵ and others⁴ have shown that administration of the selective and potent GSK3 β inhibitor SB216763 at the onset of reperfusion reduces infarct size in several species, including rabbits and rats.

There are two known GSK3 isoforms, GSK3 α (51 kDa) and GSK3 β (47 kDa). Together, they contribute to many cellular processes, such as transcription, metabolism, cell division, adhesion and apoptosis,⁸ as well as cardiac hypertrophy⁹ and heart failure.¹⁰ Both GSK3 isoforms are active in resting cells because of their basal phosphorylation of Tyr^{279/216}, but become inactivated after phosphorylation of Ser²¹ (GSK3 α) and Ser⁹ (GSK3 β).⁸ Various reports using RNA interference⁶ and phosphorylation studies of the corresponding residues have demonstrated that it is the β - isoform that mediates protection in cardiomyocytes.¹¹ However, it was reported recently that a transgenic mouse expressing a constitutively active (ca) form of GSK3 β could still be preconditioned.¹²

Because there is always a question of specificity when pharmacological inhibitors are used and genetically modified animals can experience compensatory changes during their development, in the present study we used a genetic approach to influence GSK3 β activity in a more rapid manner and developed a cell model in which rat cardiomyocytes were exposed to H₂O₂ to induce formation of mPTPs. We transfected isolated cardiomyocytes with adenoviruses coding for different GSK3 β variants and tested a dominant negative (dn) form for its protective potential and caGSK3 β for its ability to block Δ -opioid receptor-mediated protection.

Methods

The present study was performed in accordance with The Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, DC, 1996).

Adult rat cardiomyocytes

Ventricular myocytes from adult Wistar rats were isolated as described previously.^{5,13} Immediately after isolation, cells were plated on laminin-coated four-chamber cover slips and incubated in medium 199 supplemented with 0.2% bovine serum albumin (BSA), 5 mmol/L creatine, 6.2 mmol/L L-carnitine and 5 mmol/L taurine (CCT-medium 199). In addition, 100 U/mL penicillin, 100 μ g/mL streptomycin, 10 μ mol/L cytosine β -D-arabinofuranoside and 250 μ g/mL amphotericin B were added to the medium. Cells were stored in incubators at 37°C in air enriched with 5% CO₂.

Phase contrast microscopy

Cardiomyocytes were cultured over 72 h on laminin-coated four-chamber coverslips as described above. After 2, 24, 48 and 72 h, cells were used for phase contrast microscopy to

evaluate cellular integrity under culture conditions. Photographs were taken using a Nikon Eclipse Ti microscope and NIS-Elements software (Nikon, Düsseldorf, Germany).

Adenoviral transfection

Adenoviruses containing either wild-type GSK3 β (wtGSK), dnGSK or caGSK were used. Cells were infected with the virus by incubation with the adenoviruses for 24 h, followed by 48 h incubation in virus-free medium.

Construction of GSK viruses

The wild-type cDNA of human GSK3 β (NCBI reference: NM_002093.3) was obtained from Open Biosystems (Huntsville, AL, USA). The 5' - and 3' -untranslated regions (UTR) were truncated by polymerase chain reaction (PCR) with primers GSKfRI (ggaattcgccaccatgtcagggcgccagaac) and GSKrEclXho (cctcgagctagcttctcatgatctgg). The S9A (ca) mutation was introduced by site-directed mutagenesis using primers GSK3bS9AFor (cgaattcgccaccatgtcagggcgccagaaccaccgcctttgc) plus GSKrXho. The K85R (dn) mutation was introduced by overlap extension PCR¹⁴ using primers GSKfRI, GSKrXho, GSKk85Rf (ctggtcgccatcaggaaagtattgcag) and GSKk85Rr (ctgcaatacttctcatggcgaccag). The fidelity of amplification was verified by PCR and the wt, ca and dn variants were inserted into the adenoviral shuttle vector pMA905, a derivative of pDC512 (Microbix Biopharmaceuticals, Toronto, ON, Canada) thus generating pMA1782 (wtGSK), pMA1808 (caGSK) and pMA1781 (dnGSK), respectively. The pMA905 vector is a bicistronic vector in which enhanced green fluorescent protein (EGFP) follows the internal ribosomal entry site of encephalomyelocarditis virus. Plasmids pMA1781, pMA1808 and pMA1782 were recombined with adenovirus genomic plasmid pBDHfrt Δ E1,3FLP in HEK293 cells, thus producing adenoviruses Ad.1781 (dnGSK), Ad.1808 (caGSK) and Ad.1782 (wtGSK). The viruses were amplified in HEK293 cells and purified using a standard CsCl purification procedure.¹⁵

Validation of GSK3 β protein expression after adenoviral transfection

Virus infection of adult rat ventricular myocytes was performed using either wtGSK, dnGSK or caGSK adenoviruses. After 24 h incubation in the presence of the virus, the medium was changed to virus-free medium and cells were incubated for a further 48 h. Protein lysates were then generated and standard sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot techniques were performed using antibodies against GSK3 β and the phosphorylated form of GSK3 β (Ser⁹). Endogenous GSK3 β expression can be discriminated from transfected GSK3 β protein by a shift in its molecular weight. After 72 h transfection, GSK3 β was expressed in all transfected cells (wtGSK, dnGSK, caGSK). Phosphorylation of GSK3 β was seen in dnGSK- and wtGSK-transfected cells. In caGSK-transfected myocytes, Ser⁹ was replaced by Ala, resulting in the absence of any phosphorylation of the transfected protein. All preparations showed only slight phosphorylation of the endogenously expressed GSK3 β .

Validation of GSK3 β phosphorylation

After 24 h, cardiomyocytes were treated for 5 min with 1 μ mol/L [D-Ala²-D-Leu⁵]-enkephalin (DADLE), a Δ -opioid receptor agonist, or 1 μ mol/L SB216763, a GSK3 β inhibitor, and then lysed. Samples were subjected to standard SDS-PAGE and immunoblot techniques using the GSK3 β antibodies described below.

Assessment of mitochondrial membrane potential

To ensure expression of transfected GSK3 β proteins, myocytes were cultured for 72 h. Cells were loaded with 100 nmol/L tetramethylrhodamine ethyl ester (TMRE; Molecular Probes,

Eugene, OR, USA) for 20 min, which causes cells to fluoresce in proportion to their mitochondrial membrane potential ($\Delta\Psi_m$). A reduction in TMRE fluorescence served as an indicator of the loss of $\Delta\Psi_m$, which would occur with mPTP formation. After loading with TMRE, cells were washed twice with TMRE-free medium and then incubated in the presence of 100 $\mu\text{mol/L}$ H_2O_2 , which is known to induce mPTP formation, as seen at reperfusion following myocardial ischaemia.¹ Cells were exposed to H_2O_2 for 40 min, followed by measurement of fluorescence intensity at 582 nm (FL-2 channel) using a flow cytometer (FACSCalibur; Becton Dickinson, Heidelberg, Germany) and CellQuest software (Becton Dickinson, Heidelberg, Germany). Data were gated to exclude debris and the average fluorescence of 5000 cells was computed. Fluorescence measurements provide data in arbitrary units (a.u.).

The following groups of TMRE-loaded myocytes were studied: (i) no H_2O_2 ; (ii) exposure to H_2O_2 (control); and (iii) exposure H_2O_2 in the presence of 1 $\mu\text{mol/L}$ SB216763 or 1 $\mu\text{mol/L}$ DADLE. Non-transfected myocytes served as controls, whereas additional studies were performed in myocytes transfected with wtGSK, dnGSK and caGSK. One group was exposed to the structurally related GSK3 β inhibitor SB415286 (5 $\mu\text{mol/L}$).

Materials

Antibodies to phosphorylated (p-) GSK3 β (Ser⁹) and GSK3 β were obtained from Cell Signalling Technology (Danvers, MA, USA). SB216763 and SB415286 were obtained from Biomol (Hamburg, Germany). All other chemicals were from Sigma-Aldrich Chemical (München, Germany).

Statistics

Data are presented as the mean $\pm$ SEM. Differences in TMRE fluorescence between groups were compared by one-way ANOVA with Fisher's LSD post hoc testing. $P < 0.05$ was considered significant.

Results

Cultured isolated cardiomyocytes

Cultured cardiomyocytes were photographed at different time points throughout the study. At 2 h after isolation, cells were attached to the laminin-coated coverslips and showed a typical rod-shaped striated morphology. Photographs were taken 24, 48 and 72 h after isolation and there were no changes in cell morphology observed (Fig. 1).

Expression of GSK3 β protein after adenoviral transfection

Cell lysates from adenoviral-transfected cardiomyocytes were probed for total GSK3 β and p-GSK3 β to clarify the expression of recombinant proteins. These proteins can be discriminated from endogenously expressed GSK3 β by a shift in molecular size. Figure 2 show representative western blots of non-transfected (control) and transfected cells: 72 h after infection, all three variants (wtGSK, dnGSK, caGSK) were detected. Using a phosphospecific GSK3 β antibody detecting phosphorylation at Ser⁹, corresponding samples of wtGSK and dnGSK showed clear phosphorylation. In contrast, the recombinant caGSK protein was not phosphorylated owing to the replacement of Ser⁹ (Fig. 2a).

Phosphorylation of GSK3 β after treatment

Cardiomyocytes treated with either SB216763 or DADLE clearly showed GSK3 β phosphorylation at Ser⁹ compared with untreated cells (Fig. 2b), indicating a mechanism of GSK3 β inhibition via SB216763 and induction via DADLE.

Assessment of $\Delta\Psi_m$ in cardiomyocytes

At least three independent cell isolations of ventricular myocytes from adult rats were preloaded with the $\Delta\Psi_m$ probe TMRE before being exposed to H_2O_2 , which promotes mPTP formation.¹⁶ Mean fluorescence in cells incubated for 40 min in medium without H_2O_2 (no peroxide group; $n = 22$) was 293 ± 22 a.u. (Fig. 3). Mean fluorescence was significantly lower in myocytes exposed to $100 \mu\text{mol/L}$ H_2O_2 for 40 min (control; $n = 16$) at 103 ± 12 a.u. ($P < 0.001$), indicating a loss of $\Delta\Psi_m$, presumably related to mPTP formation. Treatment of cells with SB216763, the structurally related SB415286 or the Δ -opioid receptor agonist DADLE preserved TMRE fluorescence, despite exposure of cells to H_2O_2 (208 ± 29 , 213 ± 35 and 223 ± 50 a.u., respectively; each $P < 0.05$ vs control; $n = 23$, 13 and 6, respectively), indicating a protective role of GSK inhibition and opioid receptor activation on $\Delta\Psi_m$ depolarization and, thus, mPTP formation.

Protection similar to that seen with SB216763 or DADLE was observed in myocytes expressing wtGSK (209 ± 24 and 211 ± 44 a.u., respectively; each $P < 0.05$ vs control; $n = 3$ and 8, respectively), whereas wtGSK transfection alone resulted in a similar H_2O_2 -induced fall in mean fluorescence ($n = 11$), indicating that the viral infection procedure *per se* had no protective effect (Fig. 3).

To confirm the involvement of GSK3 β in the protective signalling, we inhibited GSK3 β with the adenoviral transfer of a dn plasmid (Fig. 4). The dnGSK-transfected myocytes were similarly protected against peroxide stress compared with SB216763- or DADLE-treated cells (217 ± 26 a.u.; $P < 0.001$ vs control; $n = 22$). Further exposure of the dnGSK-transfected cells to either SB216763 or DADLE showed no significant additive effects of GSK3 β inhibition or Δ -opioid receptor activation ($n = 17$ and 6, respectively).

It is assumed that GSK3 β plays a role between the surface receptor and the mitochondria. In order to further support this signalling with a highly specific approach, myocytes were transfected with caGSK in which Ser⁹ was replaced with Ala, making phosphorylation at that site impossible. Because GSK3 β is active until Ser⁹ is phosphorylated, the mutant is permanently active. Figure 4 shows that the response of transfected cells to H_2O_2 was no different from that on the non-transfected (control) cells, but that DADLE was no longer able to preserve fluorescence in these cells (128 ± 17 and 141 ± 29 a.u., respectively; NS vs control; $n = 18$ and 8, respectively; Fig. 4).

Mitochondrial membrane potential was further measured in the absence of H_2O_2 . There were no significant differences in mean TMRE fluorescence in SB216763- and DADLE-treated or transfected cells under these conditions ($n = 6$ each; data not shown).

Discussion

It is believed that opening of mPTPs kills a large number of cardiomyocytes in the first minutes of reperfusion and that IPC protects largely by inhibition of mPTP formation.¹ In the present study, we demonstrated that GSK3 β inhibition delayed the oxidant-induced depolarization of $\Delta\Psi_m$, presumably caused by mPTP opening. Several studies have shown that interventions that protect in this model also protect against infarction in the reperfused heart.⁴ We used SB216763, a cell-permeable, small-molecule inhibitor of GSK3 β that is potent and highly selective.¹⁷

SB216763 inhibits GSK3 β activity with reported IC₅₀ values of 34 nmol/L and shows similar potency towards purified GSK3 β .¹⁷ There has been no significant activity of SB216763 detected towards a panel of 24 other protein kinases, including Akt, pyruvate dehydrogenase kinase 1 and casein kinase-2.^{17,18} Furthermore, maximal neuroprotection is

observed at concentrations of 3 $\mu\text{mol/L}$ SB216763.^{17–19} Recently, we⁵ and others¹¹ have shown that GSK3 β inhibition with SB216763 leads to marked reduction in infarct size. In addition, in the present study we used the Δ -opioid receptor agonist DADLE, which proved to be equally protective in our model. It is well established that treating hearts with Δ -opioid receptor agonists at the time of reperfusion results in marked protection and reduced infarct size compared with preconditioning alone.¹¹ The question is does preconditioning or its mimetic DADLE protect by inhibiting GSK3 β ? This has been difficult to answer; unlike most kinases, phosphorylation of GSK3 β inhibits rather than activates its kinase activity and it is the inhibited state that is thought to be protective. Thus, pharmacological inhibition of GSK3 β mimics the protection afforded by DADLE rather than blocking it and there is no pharmacological activator available. However, the constitutively active mutant solves that problem. Because the protection afforded by DADLE is blocked in caGSK cells, the experiment supports the hypothesis that the opioid affords protection by inhibiting GSK3 β .

We further supported our pharmacological studies by using highly specific GSK3 β inhibition with adenoviral transfer of the dnGSK plasmid. The amino acid replacement at position 85 from lysine to arginine within the ATP-binding cassette causes catalytic inactivity.²⁰ Cells transfected with dnGSK were just as protected against oxidative stress as were cells treated with SB216763 or DADLE.

Gross *et al.*¹¹ showed that GSK3 β was phosphorylated at Ser⁹ (and hence inactivated) when rat ischaemic hearts were protected with opioids such as morphine and the δ -selective opioid receptor agonist BW373U86 administered just prior to reperfusion. Furthermore, infarct size in rat isolated hearts was reduced when GSK3 β inhibitors such as SB216763 or SB4152865286 were administered at the time of reperfusion. The results of the present study support these findings: both the GSK3 β inhibitor SB216763 and the Δ -opioid receptor agonist DADLE were able to protect isolated cardiomyocytes against oxidative stress. Furthermore, we confirmed these findings in a highly selective manner using viral transfection of the dnGSK plasmid.

In the present study we used a cell model of peroxide-induced mPTP formation. Oxidative stress has been proposed to cause cell death in many experimental systems, including ischaemia–reperfusion. In the present study, we monitored changes in $\Delta\Psi_m$ in rat cardiomyocytes exposed to 100 $\mu\text{mol/L}$ H₂O₂, the latter being a well-established oxidative stressor.^{21–23} Opening of the mPTP is known to cause a collapse of $\Delta\Psi$.¹⁶ m. Hausenloy *et al.*¹ proposed that mPTP opening is involved in cell death during reperfusion of the ischaemic cell and that inhibition of mPTP is the pivotal end-effector in cardioprotection through IPC. In the present study, cardiomyocytes treated with SB216763 or DADLE, or cells expressing dnGSK, retained their TMRE fluorescence (and hence $\Delta\Psi_m$) during H₂O₂ stress much longer than untreated cells. It is very likely that the loss of TMRE fluorescence is due to mPTP opening because, in a previous study, we showed that cyclosporine A, an mPTP blocker, delayed the loss of fluorescence by an amount comparable to the present interventions.⁵

Why then were Nishino *et al.*¹² unable to precondition a transgenic knockin mouse in which the heart was expressing a caGSK? The most likely answer is that during their months of development they recruited another pathway to take over the function of this important kinase. However, if the genetic alteration is rapid, then the preconditioning response appears to be blocked.

In summary, these experiments do support a critical role for GSK3 β in the signalling pathway for the preconditioning mimetic DADLE. They also support inhibition of GSK3 β at

the time of reperfusion as a safe and effective strategy for treating acute myocardial infarction in humans.

Acknowledgments

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Figure 1.
Phase contrast microscopy images demonstrating the integrity of cultured cardiomyocytes.
The photographs were taken at different time-points during culture, as indicated.

Figure 2.

a) Western blots of total glycogen synthase kinase (GSK) 3 β and phosphorylated (p-) GSK3 β to verify expression of recombinant GSK3 β proteins. Expression of transfected (transf.) GSK3 β was differentiated from that of endogenous (End.) GSK3 β by a shift in molecular size. Non-transfected myocytes served as controls. Recombinant proteins of wild-type (wtGSK), constitutively active (caGSK) or dominant-negative (dnGSK) GSK3 β plasmids were detected 72 h after adenoviral infection. Corresponding samples were incubated with phosphospecific GSK3 β antibody. Myocytes transfected with wtGSK and dnGSK showed clear phosphorylation. In contrast, myocytes transfected with caGSK showed no phosphorylation of the recombinant protein. (b) Cardiomyocytes stimulated with 1 μ mol/L SB216763 (SB21) or [d-Ala²-d-Leu⁵]-enkephalin (DADLE) exhibited GSK3 β phosphorylation compared with untreated control cells, suggesting a role for GSK3 β within the protective signalling of DADLE.

Figure 3.

Rat isolated cardiomyocytes stained with tetramethylrhodamine ethyl ester (TMRE) and stressed for 40 min by exposure to 100 $\mu\text{mol/L}$ H_2O_2 (control) exhibited a significant reduction in mean fluorescence compared with untreated cells (no peroxide). Treatment of cells with SB216763 (SB) and the other GSK3 β inhibitor SB415286 (SB41) significantly increased mean fluorescence, indicating that glycogen synthase kinase (GSK) 3 β inhibition preserves mitochondrial membrane potential in the face of oxidative stress. A similar protective effect was seen after treatment of cells with the Δ -opioid receptor agonist [d-Ala²-d-Leu⁵]-enkephalin (DADLE). Myocytes transfected with wild-type (wtGSK) GSK3 β plasmid showed no changes in response to either H_2O_2 , SB216763 or DADLE compared with non-transfected cells. Data are the mean $\pm$ SEM. * P < 0.05 compared with control; § P < 0.05 compared with no peroxide.

Figure 4.

After adenoviral transfection of myocytes with a dominant-negative (dnGSK) glycogen synthase kinase (GSK) 3 β plasmid, cells were protected against the H₂O₂ load, confirming a role for GSK3 β in a highly specific approach. Further treatment of cells with 1 μ mol/L SB216763 (SB) or 1 μ mol/L [d-Ala²-d-Leu⁵]-enkephalin (DADLE) had no additive effect. Myocytes transfected with the constitutively active (caGSK) plasmid could no longer be protected by DADLE, indicating a role for GSK3 β between the surface receptor and the mitochondria. Data are the mean $\pm$ SEM. * P < 0.05 compared with control; § P < 0.05 compared with no peroxide.