DOCA-induced Phosphorylation of Glycogen Synthase Kinase 3 β

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Key Words

Aldosterone • Mineralocorticoids • Heart • PKB • SGK

Abstract

Mineralocorticoid excess leads to cardiac fibrosis, a leading cause of morbidity and mortality. Cardiac hypertrophy and fibrosis are inhibited by the glycogen synthase kinase GSK3 which itself is a target of protein kinase B (PKB) and the serum and glucocorticoid inducible kinase SGK1. Phosphorylation of GSK3 by PKB or SGK1 inhibits GSK3 activity and should thus favour the development of cardiac hypertrophy and fibrosis. As SGK1 is transcriptionally upregulated by mineralocorticoids and has been recently shown to play an important role in the pathogenesis of mineralocorticoid-induced cardiac fibrosis, the present study explored whether mineralocorticoid excess had any effect on the phosphorylation status of the α and β isoforms of GSK3. Western blotting using an antibody specific for the PKB/SGK1 consensus phosphorylation site in GSK3 α/β (serine 21 and 9 respectively) revealed an increase in GSK3 α/β phosphorylation in human embryonic kidney 293 (HEK293) cells overexpressing wild type SGK1,

constitutively active SGK1, but not catalytically inactive SGK1. The effect of SGK1 was mimicked by PKB and SGK3. Furthermore, DOCA/high salt treatment of wild type mice induced a robust increase in cardiac GSK3 β phosphorylation and, to a much lesser extent, GSK3 α phosphorylation. However, under this treatment GSK3 β phosphorylation was apparent even in mice lacking functional SGK1, indicating that the phosphorylation of GSK3 β was not exclusively mediated by this kinase. Despite similar cardiac GSK3 β phosphorylation cardiac fibrosis following DOCA/high salt treatment was significantly blunted in SGK1 knockout mice. In conclusion, mineralocorticoid excess leads to phosphorylation and thus inactivation of GSK3 β , an effect not only due to upregulation of SGK1 but as well due to activation of additional kinases. The inactivation of GSK3 may play a permissive role in the stimulation of cardiac fibrosis but may by itself not be sufficient to trigger cardiac fibrosis.

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Introduction

Cardiac hypertrophy and fibrosis frequently progress to dilated cardiomyopathy and sudden cardiac death [1-

5]. Compelling evidence points to a role of the serine/threonine kinase glycogen synthase kinase 3 (GSK3) in the regulation of cardiac hypertrophy and fibrosis [1, 6-8]. In humans there are 2 GSK3 isoforms, namely GSK3 α and GSK3 β which share, within their catalytic domain, a 97% sequence homology. Although the 2 isoforms share substrates they differ in expression patterns and functions. Thus, despite the expression of both isoforms in the heart the majority of research has focused on the GSK3 β isoform and its role in cardiac pathophysiology.

GSK3 is under the control of PKB, which phosphorylates and thus inactivates the kinase [9, 10]. The phosphorylation consensus sequence of PKB is shared by the serum- and glucocorticoid-inducible kinase isoforms SGK1, SGK2 and SGK3 [11]. As a matter of fact, SGK3 [12] and SGK1 [13-16] have similarly been shown to phosphorylate GSK3 β . All three isoforms of SGK and PKB [14] are activated via a signaling cascade involving phosphatidylinositol 3 kinase and phosphoinositide dependent kinase PDK1 [14, 15, 17, 18]. The activation of SGK1 requires phosphorylation at serine 422, the activation of PKB phosphorylation at Thr308 and Ser473, replacement of these amino acids by aspartate leads to the respective constitutively active kinases ^{S422D}SGK1 [14] and ^{T308D,S473D}PKB [17]. Destruction of the catalytic subunit by replacement of the lysine at position 127 with asparagine leads to the inactive mutant ^{K127N}SGK1 [14].

There are numerous reports in the literature documenting that phosphorylation of GSK3 β and thus inhibition of the kinase, favours the development of cardiac hypertrophy and fibrosis [1, 7, 8, 13]. On the other hand, increasing GSK3 β activity has been observed in the failing heart [9]. Moreover, the integrin linked kinase 1 (ILK1), which plays a role in the upregulation of extracellular matrix protein deposition [19], can phosphorylate and inhibit GSK3 α/β , either directly or via PKB, thus removing GSK3-induced negative regulation of the AP1 transcription factor, a key transcription factor in the regulation of collagen I and fibronectin genes. Furthermore, ILK1 expression is elevated in diabetic nephropathy [20], another fibrosing disorder associated with excessive stimulation of SGK1 transcription [21-23].

Triggers of cardiac fibrosis include mineralocorticoid excess [5, 24]. The mineralocorticoids aldosterone and deoxycorticosterone acetate (DOCA) are partially effective through expansion of the extracellular volume and a subsequent increase of blood pressure [25], by enhancing salt appetite [26] and by decreasing renal salt excretion [27]. The effects of mineralocorticoids are

partially mediated by SGK1 [28], which is strongly upregulated by mineralocorticoids [29, 30]. Specifically, SGK1 mediates part of the effects of mineralocorticoids on the renal epithelial Na⁺ channel ENaC [29, 30]. Gene targeted mice lacking SGK1 (*sgk1*^{-/-}) show normal Na⁺ excretion and blood pressure under normal salt intake, but their ability to retain Na⁺ and maintain blood pressure under a salt deficient diet is impaired [31].

Given the regulation of SGK1 by mineralocorticoids on the one hand and the ability of SGK1 to phosphorylate GSK3 on the other, we hypothesised that mineralocorticoid excess could lead to phosphorylation of GSK3 and that this effect may play a permissive role in the development of cardiac hypertrophy and fibrosis.

Materials and Methods

Animal experimentation

Mice deficient in SGK1 (*sgk1*^{-/-}) were generated as previously described [31]. Wild type (*sgk1*^{+/+}) and SGK1 knockout (*sgk1*^{-/-}) mice were implanted with a 21 day release 50 mg DOCA pellet (Innovative Research of America, Sarasota, FL) in the neck area [32] during anesthesia (intraperitoneal medetomidin 0.5 mg/kg + midazolam 5 mg/kg + fentanyl 0.05 mg/kg, which was reversed by subcutaneous atipamezol 2.5 mg/kg + flumazenil 0.5 mg/kg + naloxon 1.2 mg/kg). Mice had free access to a standard mouse diet (Altromin, Heidenau, Germany) and tap water or 1% NaCl. Systolic arterial blood pressure was determined by the tail-cuff method before and on day 16 of DOCA/1% NaCl treatment [33]. For Western blot analysis, whole hearts were removed on day 18 of DOCA/1% NaCl treatment and immediately frozen in liquid nitrogen, the tissue was then homogenised using a glass homogeniser in lysis buffer (see below). In a separate series, animals (n = 5 each) were treated by repeated implantation of 21 day release 50mg-DOCA pellets and 1% NaCl in drinking water. Animals were sacrificed and hearts removed 12 weeks after initiation of the treatment for analysis of morphology (see below).

Cell culture and transfection

Human embryonic kidney (HEK293) cells, (American type culture collection, Manassas, VA, USA), were seeded at a density of 1 x 10⁶ in 60mm cell culture dishes in DMEM medium containing 10% fetal calf serum under standard culture conditions (37°C, 5% CO₂). 6-8 h after seeding cells were transfected with empty vector pIRES2-EGFP or pEGFP-C1 (Invitrogen, Karlsruhe,

Fig.1. *In vitro* phosphorylation of GSK3 β by SGK1.

(a) Western blot demonstrating phosphorylation of GSK3 α/β at serine 21 and 9, respectively, in HEK293 cells expressing wild type SGK1 (SGK1) and constitutively active ^{S422D}SGK1 but not in cells transfected with the empty vector (Control) or with the inactive SGK1 mutant ^{K127N}SGK1. The lower panel demonstrates the abundance of GSK3 β protein. (b) Arithmetic means $\pm$ SEM (n = 6) of the densitometric analysis. * indicates statistically significant (p < 0.05) difference to control values. (c) Western blot showing GSK3 α/β phosphorylation in HEK293 cells overexpressing wild type SGK1 (SGK1) and constitutively active ^{S422D}SGK1 in the absence or presence of staurosporine

(50 nM, 1h). The lower panel demonstrates the abundance of GSK3 β protein. (d) Arithmetic means $\pm$ SEM (n = 6) of the densitometric analysis.

Germany), wild type SGK1 (pIRES2-EGFP-hSGK1), constitutively active SGK1 (pIRES-EGFP ^{S422D}hSGK1), catalytically inactive SGK1 (pIRES-EGFP ^{K127N}hSGK1), wild type SGK3 (pEGFP-C1-hSGK3) or wild type PKB (pEGFP-C1-hPKB) constructs [17, 18, 34], using a modified calcium phosphate method [35]. CaCl₂ (2.5 M; 12.5 μ l) was added to plasmid DNA (3 μ g in 112 μ l of sterile water) and the mixture was diluted by adding 125 μ l of sterile buffer containing (in mM) 50 N,N-bis(2-hydroxyethyl)-2- aminoethanesulfonic acid-HCl, 280 NaCl, and 1.5 Na₂HPO₄ (pH 6.95). The resulting mixture was vortexed (1 min), allowed to stand at room temperature for 20 min, and then added dropwise to HEK293 cells. Transfection efficiency was determined 48h later using fluorescence microscopy to visualise the EGFP protein expression and cells were lysed in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1% Triton X-100, 1% sodium deoxycholate, 1% sodium dodecyl sulfate and protease cocktail inhibitor (Roche, Basel, Switzerland). In some experiments cells were treated with the broad range

Fig. 2. *In vitro* phosphorylation of GSK3 α/β by PKB and SGK3. (a) Western blot demonstrating phosphorylation of GSK3 α/β in cells expressing wild type PKB or SGK3, but not in cells transfected with the empty vector (Control). The lower panel demonstrates the abundance of GSK3 β protein. (b) Arithmetic means $\pm$ SEM (n = 3) of the densitometric analysis. * indicates statistically significant (p < 0.05) difference.

Fig. 3. SGK1 is required for DOCA/high salt-induced development of cardiac fibrosis. H&E (upper panel) and Siriusred staining (lower panel) of cardiac tissue from *sgk1*^{+/+} (left) and *sgk1*^{-/-} (right) mice after a 12 weeks DOCA/high salt treatment. Myocyte hypertrophy, thickening of intramyocardial arteries and myocardial fibrosis after DOCA/high salt treatment is present in *sgk1*^{+/+} (left panel) but not in *sgk1*^{-/-} (right) mice.

serine/threonine kinase inhibitor staurosporine (50 nM, 1 h) prior to cell extraction.

Western Blot Analysis

Whole hearts from untreated and DOCA/high salt treated (18 days) *sgk1*^{+/+} and *sgk1*^{-/-} mice were removed and immediately frozen in liquid nitrogen, the tissue was then homogenised using a glass homogeniser in lysis buffer (see above for the composition), the homogenates were then centrifuged at 10,000 rpm, 4 °C for 15 min, the supernatant was removed and used for Western blotting. Whole cell lysates (50 μg) and heart homogenates (70 μg) were separated by SDS-page (10% Tris-Glycine), transferred to nitrocellulose membranes, blocked for 1 h in blocking buffer (5% fat-free milk in PBS containing 0.1% Tween) and incubated overnight at 4 °C with a rabbit polyclonal primary antibody against phosphorylated GSK3β at serine 21 and 9, respectively, or phospho-serine 473 PKB (diluted 1:1000 in PBS/0.1% Tween containing 5% BSA, New England Biolabs, Cell Signalling Technology). After incubation with a HRP-conjugated anti-rabbit secondary antibody (Santa Cruz, Heidelberg, Germany) visualization with ECL was performed according to manufacture's instructions (Amersham, Freiburg, Germany). Membranes were also probed with either GSK3β or PKB (New England Biolabs, Cell Signalling Technology) antibodies as controls for protein loading. Densitometric analysis of phospho-GSK3β and phospho-serine 473 PKB was performed using Scion Image (Scion, Maryland, USA) and normalized using GSK3β or PKB, respectively.

Morphology

Hearts from untreated or DOCA/high salt treated (12 weeks) *sgk1*^{+/+} and *sgk1*^{-/-} mice were quickly removed under anesthesia, the weight determined and fixed in 4% paraformaldehyde/0.1 M sodium phosphate buffer (pH 7.2) overnight and embedded in paraffin. Dewaxed 5 μm thick transversal heart muscle sections were stained with H&E for visualization of muscle and vessel morphology and Siriusred as a fibrous tissue stain [36]. Sirius stained paraffin sections were quantitatively analysed using a semiautomatic image analyzing system (Optimas, SIS, Münster Germany). Total tissue areas were measured with a 4x objective; red stained fibrotic areas were identified and quantified using a 20x objective. The degree of fibrosis was then calculated as the percentage of total tissue area.

Statistical analysis

Data are provided as means ± SEM, *n* represents the number of independent experiments. All data were tested for significance using the Student t-test. Only results with *P* < 0.05 were considered statistically significant.

Results

Unstimulated HEK293 cells did not show any appreciable staining with the antibody specific for the phosphorylation of the consensus sequence (Arg-Xaa-Arg-Xaa-Xaa-Ser/Thr) for protein kinase B (PKB) and the serum- and glucocorticoid-inducible kinase (SGK)

isoforms ($P_{PKB/SGK}GSK3\alpha/\beta$) (Figure 1a). Overexpression of wild type SGK1 and constitutively active SGK1 (S^{422D} SGK1) led to enhanced phosphorylation of GSK3 α/β whereas the catalytically inactive SGK1 mutant K^{127N} SGK1 was without appreciable effect on the phosphorylation status of GSK3 α/β (Figure 1a & b).

SGK1-induced phosphorylation was inhibited by incubation of the cells with the broad spectrum protein kinase inhibitor staurosporine (50 nM, 1 h) (Figure 1c & d).

Similar to overexpression of SGK1, overexpression of wild type SGK3 or PKB in HEK293 cells enhanced GSK3 α/β phosphorylation (Figure 2a & b).

To explore whether DOCA/high salt administration modified GSK3 α/β phosphorylation, SGK1-knockout mice (*sgk1*^{-/-}) and their wild type littermates (*sgk1*^{+/+}) were exposed to an 18 day treatment with DOCA/high salt diet.

Heart weight increased from a value of 206 ± 11 mg ($n = 4$) to a value of 280 ± 39 mg ($n = 9$), an effect, however, not reaching statistical significance ($p < 0.07$). Heart weight in *sgk1*^{+/+} mice was similar in *sgk1*^{-/-} mice prior to (207 ± 12 mg, $n = 4$) and following (208 ± 12 mg, $n = 9$). DOCA/high salt administration. Lack of myocyte hypertrophy in *sgk1*^{-/-} mice was similarly apparent on histological sections taken from a separate set of mice after 12 weeks of DOCA/high salt treatment (Fig. 3 upper panel). In addition, thickening of intramyocardial arteries which was present in *sgk1*^{+/+} mice was not seen in *sgk1*^{-/-} mice (Fig. 3 upper panel). As has been described before [36] and shown in Fig. 3 (lower panel), the DOCA/high salt treatment led to cardiac fibrosis and local myocardial necrosis in *sgk1*^{+/+} mice (28 ± 6 % of tissue area, $n = 5$) an effect significantly blunted in *sgk1*^{-/-} mice (17 ± 6 % of tissue area, $n = 5$).

According to Western blotting, little GSK3 β phosphorylation was observed in cardiac tissue from untreated wild type mice. An 18 day treatment with DOCA/high salt diet, however, resulted in marked GSK3 β phosphorylation of cardiac tissue of wild type mice, which was significantly stronger than the respective staining of cardiac tissue of untreated mice (Figure 4a & b).

Following treatment with DOCA/high salt diet, enhanced GSK3 β phosphorylation was also observed in SGK1 deficient mice (Figure 5) indicating that a lack of SGK1 does not prevent GSK3 phosphorylation. Thus, another kinase contributes or accounts for cardiac GSK3 β phosphorylation following DOCA/high salt treatment. Thus, we explored, whether DOCA/high salt treatment

Fig.4. *In vivo* phosphorylation of GSK3 β before and following treatment of wild type mice with DOCA/high salt diet. (a) Western blot demonstrating phosphorylation of GSK3 α/β at serine 21 and 9, respectively in cardiac tissue from wild type mice left untreated or treated with DOCA/high salt diet for 18 days. The lower panel demonstrates the abundance of GSK3 β protein. (b) Arithmetic means $\pm$ SEM ($n = 5-6$) of the densitometric analysis. * indicates statistically significant ($p < 0.05$) difference to control.

Fig.5. *In vivo* phosphorylation of GSK3 β before and following treatment of SGK1 knockout mice with DOCA/high salt diet. (a) Western blot demonstrating phosphorylation of GSK3 β at serine 9 and 21 in cardiac tissue from SGK1 knockout mice left untreated or treated with DOCA/high salt diet for 18 days. The lower panel demonstrates the abundance of GSK3 β protein. (b) Arithmetic means $\pm$ SEM ($n = 5-6$) of the densitometric analysis. * indicates statistically significant ($p < 0.05$) difference in unpaired t-test.

Fig. 6. *In vivo* phosphorylation of protein kinase B (PKB) before and after DOCA/high salt diet. (a) Western blot demonstrating phosphorylation of PKB in cardiac tissue from *sgk1*^{+/+} and *sgk1*^{-/-} mice left untreated or treated with DOCA/high salt diet for 18 days. The lower panel demonstrates the abundance of PKB protein. (b) Arithmetic means $\pm$ SEM (n = 5-6) of the densitometric analysis.

enhanced the phosphorylation and thus activation of PKB. As illustrated in Figure 6, serine 473 phosphorylation of cardiac PKB was unaffected by treatment of *sgk1*^{+/+} and *sgk1*^{-/-} mice. Thus, the DOCA/high salt treatment did not significantly modify phosphorylation of PKB.

Discussion

The present observations demonstrate an enhanced phosphorylation of GSK3 α/β by overexpression of SGK1 and SGK3 and increased cardiac GSK3 β phosphorylation during mineralocorticoid and salt excess. Phosphorylation of GSK3 on these residues is expected to inhibit enzyme activity [10]. Given the putative inhibitory effect of GSK3 β on the development of cardiac hypertrophy and fibrosis [1, 7, 8, 13], the phosphorylation of GSK3 β may thus play a permissive role in cardiac fibrosis.

The most obvious candidate kinase accounting for the phosphorylation of GSK3 β during mineralocorticoid excess is SGK1 which is strongly upregulated by mineralocorticoids [29, 30] and indeed phosphorylates GSK3 β [13, 15, 18]. Moreover, abundant SGK1 transcript levels have been found in a variety of fibrosing tissues including diabetic nephropathy [11, 21-23, 37], glomerulonephritis [38], Crohn's disease [39], lung fibrosis [40], liver cirrhosis [41] and fibrosing pancreatitis [42]. Most recently, SGK1 has been shown to be required for the stimulation of connective tissue growth factor (CTGF) formation and subsequent stimulation of matrix protein formation in cardiac tissue of DOCA/high salt treated mice [36]. Both, CTGF formation and fibrosis were virtually absent in SGK1-knockout mice.

As shown in the present study, SGK1 can, *in vitro*, induce GSK3 α/β phosphorylation. To our surprise, however, GSK3 β phosphorylation was not abrogated by the lack of SGK1 in the *sgk1*^{-/-} mouse. These data do not rule out *in vivo* phosphorylation of GSK3 β by SGK1 but indicate that some other kinase serves this function at least in the *sgk1*^{-/-} mouse. It should be kept in mind that the Western blots reflect the abundance of GSK3 β in the complete tissue and do not allow conclusions on localized SGK1 expression and GSK3 β phosphorylation.

Studies with a PDK1 mutant, which activates PKB but not SGK [43] suggested that GSK3 is phosphorylated by protein kinase B (PKB) rather than SGK. Thus, an obvious candidate would be PKB, which has earlier been shown to phosphorylate GSK3 α/β [44]. However, we have no evidence of enhanced serine phosphorylation of PKB in response to DOCA/high salt treatment in neither *sgk1*^{-/-} nor *sgk1*^{+/+} hearts. Again, altered PKB phosphorylation in specific cells may escape detection in Western blots from total tissue.

Similar to SGK1, PKB is activated through a signaling cascade involving the phosphatidyl-inositol 3 (PI3) kinase and phosphoinositide-dependent kinase PDK1 [14, 15, 17, 45-48]. Surprisingly, although cardiac PI3 kinase activity is reportedly enhanced by mineralocorticoids [49], no PKB activation was observed in the present study. Notably, it has been previously reported that DOCA-induced PI3 kinase activity has no stimulatory effect on PKB/Akt phosphorylation [50, 51].

Whatever the mechanism, mineralocorticoid excess leads to GSK3 β phosphorylation in cardiac tissue, a finding which to our knowledge, has not been previously demonstrated. In contrast to our findings in cardiac tissue,

mineralocorticoids have been reported to decrease GSK3 β phosphorylation in rat aorta [50], a rather surprising finding considering that PI3-kinase activity is increased by DOCA treatment [49].

The inhibition of GSK3 β through PI3-kinase, PDK1 and PKB is expected to play a permissive role in the development of cardiac hypertrophy and fibrosis. However, inhibition of GSK3 β alone may not be sufficient for the triggering of both, as *sgkl*^{-/-} mice are significantly less sensitive to DOCA/high salt induced cardiac hypertrophy and fibrosis (Figure 3) [36] despite phosphorylation of GSK3 β (Figure 5). Those observations do suggest that inhibition of GSK3 β may at best be permissive for the stimulation of cardiac hypertrophy and fibrosis. However, we did not determine GSK3 β activity and we did not knockout GSK3 β or prevent its inactivation. Thus, our observations do not allow any safe conclusions

about the functional role of GSK3 β in cardiac hypertrophy and fibrosis.

In conclusion, DOCA/high salt treatment leads to strong phosphorylation of GSK3 β in cardiac tissue from both *sgkl*^{+/+} and *sgkl*^{-/-} mice. This effect may play a permissive role but may not be sufficient for the development of cardiac hypertrophy and fibrosis.

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