Gender Differences in Serum and Glucocorticoid Regulated Kinase-1 (SGK-1) Expression during Renal Ischemia/Reperfusion Injury

Krisztina Rusai^{1,2}, Ágnes Prókai^{1,2}, Beáta Szebeni², Krisztina Mészáros¹, Andrea Fekete^{1,2}, Balázs Szalay^{1,2}, Ádám Vannay², Peter Degrell³, Veronika Müller⁴, Tivadar Tulassay^{1,2} and Attila J. Szabó^{1,2}

¹First Department of Pediatrics, ²Research Laboratory of Pediatrics and Nephrology of the Hungarian Academy of Sciences, ³Second Department of Medicine and Nephrology, Faculty of Medicine, University of Pecs, ⁴Department of Pulmology, Semmelweis University, Budapest

Key Words

SGK-1 • Renal ischemia • Gender differences • Testosterone

Abstract

Several studies reported sexual dimorphism in the signaling mechanisms of renal ischemia/reperfusion (I/R). The anti-apoptotic serum and glucocorticoid-regulated kinase-1 (SGK-1) is up-regulated and has a significant protective role in renal I/R. SGK-1 has several target molecules, and inhibition of the inducible nitric oxide synthase (iNOS) transcription is one of its effector mechanisms. The objective of the present study was to examine if there is a gender-specific expression and activation of SGK-1 during renal I/R injury. *In vitro*, treatment of HK-2 kidney proximal tubular cells with different concentrations of 17-beta estradiol had no effect, whereas testosterone increased SGK-1 abundance in a dose-dependent manner. *In vivo*, in a rat model of unilateral renal I/R injury, there was a higher SGK-1 expression and phosphorylation in males 2 and 24 h after ischemia paralleled by reduction in the mRNA expression of iNOS compared to females. Deprivation of testosterone by castration of males resulted in

decreased SGK-1 protein level at all time-points and reduced phosphorylation 2 and 24 h after reperfusion. Our results suggest that testosterone up-regulates SGK-1 in the kidney contributing to sexual dimorphisms in the cell signalling machinery. The significance of the testosterone-regulated SGK-1 level and activity in the kidney needs further investigations.

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Introduction

Renal ischemia/reperfusion (I/R) injury is one of the most common causes of acute renal failure. Moreover, it plays a central role in the development of delayed graft function after kidney transplantation as well [1].

Several studies have reported that females are relatively protected from renal I/R when compared to males. Female rats have longer survival [2] and have decreased elevation in renal function parameters with ameliorated tissue damage after kidney ischemia [3]. Our

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Fax +41 61 306 12 34
E-Mail karger@karger.ch
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Krisztina Rusai MD, PhD
First Department of Pediatrics, Semmelweis University
Budapest, Bókay u. 53-54. 1083 (Hungary)
Tel./Fax +36-1-3343186/52697
E-Mail rusai@gyer1.sote.hu

group has previously demonstrated that males show delayed early postischemic recovery of renal blood flow and lower basal and postischemic levels of heat shock proteins paralleled by decreased function of the Na-K-ATPase [2-4]. Furthermore, both inflammation and apoptosis that are key pathophysiological cellular events in I/R are influenced by sex hormones [3] implicating the potential necessity of gender-based therapy for ischemic kidney injury.

The differences in ischemic susceptibility can be eliminated by castration of males or by treatment with testosterone or dihydrotestosterone, as well as by administration of 17-beta estradiol [2, 5] suggesting that the ratio of testosterone/estrogen may be the important determinant of sex differences in renal I/R injury. However, the exact signaling mechanisms and the effect of gender hormones in ischemic injury are still not well described, and results are, in part inconsistent.

The phosphatidylinositol-3 kinase (PI-3 kinase) pathway plays a crucial role in ischemia induced apoptosis [6]. The PI-3 kinase exerts its protective effects mainly through the phosphorylation, and thus, the activation of Akt and the serum- and glucocorticoid kinase-1 (SGK-1). Within the kinase superfamily, SGK-1 is closely related to Akt, and both act as serin/threonine kinases sharing a 50-55% of structure homology and a number of common target molecules [7-9].

We have recently demonstrated that after renal I/R, mice lacking *sgk-1* present more apoptotic tubular cells than wildtype mice indicating SGK-1 to have a role in renal I/R [10, 11]. SGK-1 is furthermore activated by other cell stress stimuli such as hyperosmotic stress, ultraviolet radiation, heat shock [12], oxidative stress [13], and different growth factors and hormones [8].

Under these conditions, SGK-1 acts as a strong anti-apoptotic kinase partly by phosphorylating the transcription factor forkhead transcription factor like 1 leading to the regulation of genes involved in cell survival from which the suppression of the inducible nitric oxide synthase (iNOS) has gained more attention [14].

The regulation of the PI-3 kinase pathway shows gender-specific differences; Akt protects from I/R injury [15] and its phosphorylation was demonstrated to be promoted by estradiol [5] and inhibited by testosterone [16] in experimental models of renal I/R injury.

Although SGK-1 has intensively been investigated in stress models, its activation and function with regard to sex-specific differences have not been examined in experimental models of cell survival. Since SGK-1 has a key role in cell stress and its homologue, the PI-3 kinase

regulated Akt was shown to be markedly influenced by sex hormones during I/R, our aim was to explore gender specific differences in SGK-1 together with iNOS in renal I/R injury using both *in vivo* and *in vitro* models.

Materials and Methods

Reagents

Testosterone and 17-beta estradiol were purchased from Sigma Aldrich, Hungary.

Cell culture

The human kidney HK-2 cell line derived from human proximal tubular kidney cells was purchased from American Type Culture Collection (ATCC, Rockville, MD). All cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (Sigma Aldrich, Hungary) and were propagated at 37 °C in a humidified air containing 5% CO₂ incubator.

In vitro treatment of cells

HK-2 cells were seeded out and plated on 6-well plates 24 h prior to commencement of treatment.

For treatment of cells with estrogen and testosterone, the medium was changed to serum-free DMEM. Estrogen and testosterone were added to the culture medium in a final concentration of 10 nM, 50 nM and 100 nM for both treatments. Treatment concentration ranges were chosen based on previous publication using testosterone and estradiol with HK-2 cells [17].

Control cells were incubated with serum-free DMEM without administration of hormones. After 24 h of incubation time, cells were harvested for further analysis.

FACS analysis

SGK-1 expression was assessed using the following protocol: cell pellets were washed with 0.5 ml PBS, incubated with 0.5 ml FACS™ Permeabilizing Solution2 (10x) (Perm2; BD Bioscience Co., Soft Flow, Hungary) for 10 min in the dark, room temperature (RT). After permeabilisation, cells were centrifuged (800xg/ RT/7 min) and incubated with 1 µl anti-SGK-1 rabbit antibody (generous gift from Prof. Florian Lang, Physiology Institute, University of Tübingen, Germany) for 30 min, RT, in the dark. Cells were washed with 0.5 ml Perm2 and centrifuged [800xg/RT/5 min] and incubated with 1 µl Cy5-conjugated secondary antibody (Cy5-conjugated AffiniPure F(ab')₂ Fragment Goat anti-rabbit IgG (H+L) antibody, Jackson ImmunoResearch Laboratories Inc., Izinta Ltd., Budapest, Hungary) for 30 min RT, in the dark.

Negative controls were incubated with the Cy5-conjugated secondary antibody only. Cells were washed with 0.5 ml Perm2, centrifuged (800xg/RT/5 min) and resuspended in 500-µl PBS. The cytometric measurement and data analysis were carried out using a FACSAria cytometer with a BD FACSDiva Software (Becton Dickinson, San Jose, CA, USA).

	primer pairs	annealing temperature (°C)
iNOS	Forward: 5'-GGTTTCCCCAGTTCCTCA-3' Reverse: 5'-TCTCCATTGCCCCAGTTTTT-3'	58
SGK-1	Forward: 5'-TGCTCTATGGCCTG CCTCCGTTCT-3' Reverse: 5'-GTCAGTGGGCCCCGCTCACATTG-3'	59
GAPDH	Forward: 5'-GGTGAAGGTCGGAGTCAACG-3' Reverse: 5'-CAAAGTTGTCATGGATGACC-3'	56

Table 1. Primer pairs for PCR analysis

In vivo renal I/R model

Castration of male rats was performed under anaesthesia one week before induction of unilateral ischemia. Successful castration was detected by shrinkage of the seminal vesicle glands.

Renal I/R was performed as described previously [18]. Briefly, male and female Wistar rats (weighing between 180-240 g) were subjected to 50 mg/kg pentobarbital sodium intraperitoneal anaesthesia [Nembutal, Abbott Laboratories, Budapest, Hungary] and the abdomen was opened through a midline incision. The left renal artery and vein were clipped by an atraumatic vascular clamp for 50 min. Before the end of ischemia, right nephrectomy was performed. After 50 min of ischemia, the clamp was removed and the animals were allowed to recover. After 2 h and 24 h of reperfusion, rats were re-anaesthetized and the remnant kidney was removed.

The animals (n=6 per each group) were randomized into the following groups: Control male: with sham operation, control female: with sham operation, I/R 2h male: with ischemia and 2 h reperfusion, I/R 2h castrated male: ischemia with 2 h reperfusion, I/R 2h female: ischemia with 2 h reperfusion, I/R 24h male: with ischemia and 24 h reperfusion, I/R 24h castrated male: ischemia with 24 h reperfusion, I/R 24h female: ischemia with 24 h reperfusion.

Animals had free access to standard rat chow and water ad libitum. All animal experiments were approved by the Ethical Committee of the Semmelweis University.

Serum creatinine levels

Serum creatinine level was measured by an automatic laboratory analyzer (Hitachi-712 spectrophotometer).

Renal histopathology

Paraffin sections of kidneys fixed in 4% neutral buffered formalin were stained with hematoxylin and eosin (H&E) and with periodic acid-Schiff (PAS) reagent. Histological changes characterizing I/R injury such as tubular architecture (tubular dilation, swelling and necrosis, luminal congestion with loss of brush border), interstitial edema and infiltration of polymorphonuclear cells were semi-quantitatively evaluated and each scored from 0-4 in a blinded fashion by two independent investigators. Scores were then added and compared.

Real-time polymerase chain reaction

Total RNA was isolated from the cells by RNeasy Total RNA Isolations Kit (Qiagen GmbH, Hilden, Germany) according to the instructions of the manufacturer. The quality and quantity of the RNA were confirmed photometrically [19].

Rat *sgk-1* and *iNOS* mRNA expression in the renal tissues was assessed by SYBR Green real-time PCR on a Light Cycler system [Roche Diagnostics, Mannheim, Germany]. PCRs were performed in glass capillaries, in a final volume of 20 µl containing 3 mM MgCl₂, 0.2 µM of each primer, 2 µl LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany) and 1 µl of cDNA. The conditions of PCRs were as follows: one cycle at 95°C for 8 min, followed by 55 cycles at 95°C for 3 s, 59°C for 5 s and 72 °C for 28 s with a single fluorescence detection point at the end of the extension. Primer sequences are shown in Table 1.

mRNA expression of *GAPDH* was determined by real-time PCR quantification using fluorescence resonance energy transfer (FRET) hybridization probes on a Light Cycler system (Roche Diagnostics, Mannheim, Germany) in a final volume of 20 µl containing 2 mM MgCl₂, 0.17 nM of each hybridization probe, 0.5 nM of each primer, 2 µl of the Light-Cycler FastStart DNA Master HybProbe (Roche Diagnostics) and 1 µl of cDNA. The PCR reaction was carried out under the following conditions: one cycle at 95°C for 8 min, followed by 50 cycles at 95°C for 4 s, 55°C for 8 s and 72°C for 22 s with a single fluorescence detection point at the end of the annealing segment.

Primer pairs for GAPDH were taken from the study of Strehlau et al. [20].

Western blotting

Renal tissues were homogenized by a cell disrupter in cooled lysis buffer containing 1 mg/mL aprotinin, 5 mg/mL leupeptin, 1 M Tris, 0.5 M EGTA, 0.25 M NaF, 0.5 M PMSF, 0.5 M Na₃VO₄ and 0.1% Triton X-100. The lysate was centrifuged at 13.000 g for 15 min to remove insoluble debris. Protein concentrations in the supernatant were determined by the Bradford method using bovine serum albumin (BSA) as standard. All reagents for PAGE and Western blot were purchased from Sigma Aldrich (Hungary). Lysates were solubilized in a buffer of 12.5 mM Tris-HCl, pH 6.7, containing 4.0% SDS, 1 mM EDTA, 15% glycerol and 0.01% bromophenolblue. 50 µg protein of the samples was separated on 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes (Amersham, GE Healthcare Europe GmbH, Munich, Germany). The membranes were blocked with 5% non-fat dry milk (NFDM) for 2 h at room temperature and then probed with rabbit polyclonal anti-SGK-1 antibody [1:400, Upstate, Germany] in 5% NFDM overnight at 4 °C. The membranes were washed with Tween-phosphate-buffered saline, and incubated with anti-rabbit immunoglobuline antibody, a horseradish peroxidase conjugated from goat (1:2000, Amersham) in 5% NFDM for 1.5 h at room temperature. Proteins were detected by enhanced chemiluminescence (ECL,

Fig. 1. Effect of 17-beta estradiol and testosterone treatment on the rate of SGK-1-positive HK-2 cells determined by FACS analysis. Testosterone applied in 100 nM dose elevated the rate of SGK-1-positive HK-2 cells compared to control cells (* $P<0.05$ vs. control).

Amersham, UK) by Kodak Image Station 400 D. Prestained standards were used as molecular weight markers (Biorad Laboratories, Munich, Germany). Signals on Western blots were quantified by densitometry using Gel-Pro Analyser 3.1 Software.

Double labeling using immunofluorescence microscopy

Kidney sections were immediately snap-frozen in 30% sucrose for immunohistochemical analysis. They were embedded in Shandon cryomatrix (ThermoElectron Co. USA),

cut into 5–10 μm thick slices with a cryostat, and stored at -80°C . After rinsing in PBS, they were treated in methanolic H_2O_2 for 30 min. Tissue sections were permeabilized in 0.2% Triton X-100 in PBS and then blocked in 0.5% NFDm for 30 min at room temperature. The samples were incubated overnight at 4°C with primary antibodies (anti-total SGK 1:100, Santa Cruz, anti-phospho-SGK1 1:100, Cell Signaling, USA), washed with PBS, and then incubated with secondary antibodies (Alexa Fluor 488 1:100, Alexa Fluor 546 1:100, Invitrogen, Budapest, Hungary) for 30 min at room temperature. DNA was stained

Fig. 2. Serum creatinine levels were measured as a respective marker of renal dysfunction. Data represent mean $\pm$ SD. Renal ischemia/reperfusion (I/R) led to significant elevation in serum creatinine levels 24 h after reperfusion ($+P<0.001$ vs. control male and female). The increase in serum creatinine was ameliorated in females compared to males ($*P<0.05$ vs. I/R 24h male). There was no difference either between control males and females or between the animals 2 h after reperfusion.

with Hoechst 33342 (1:1000 Sigma, Hungary) for 10 min at room temperature. Appropriate controls were performed omitting the primary antibodies to ensure their specificity and to avoid auto-fluorescence.

Stained slides were analysed in a blinded fashion, localisation and expression of SGK1 was detected.

Statistical analysis

Data are expressed as mean $\pm$ standard deviation (SD). Data were tested using the Chi-Square- or Mann-Whitney U test using the SPSS statistical software package (v. 13.0, SPSS GmbH, Munich, Germany). Data were analyzed using one-way ANOVA followed by Dunnett *post hoc* test or Kruskal-Wallis ANOVA for nonparametric data. $P<0.05$ was considered significant.

Results

The effect of estrogen and testosterone on SGK-1 expression in vitro

First, we tested if there is an effect of sex hormones on SGK-1 abundance in proximal kidney tubular cells under *in vitro* circumstances. Incubation of HK-2 cells with three different concentrations of estrogen (10 nM – 50 nM – 100 nM) did not change the percentage of cells positive for SGK-1 expression ($P>0.05$ vs. control) (Fig. 1). On the other hand, testosterone in the highest applied concentration (100 nM) significantly increased the percentage of SGK-1 positive cells ($P<0.01$ vs. control), whereas 10 nM and 50 nM testosterone did not change the rate of SGK-1 positive cells ($P>0.05$ vs. control) (Fig. 1).

Gender differences in renal function and morphology after I/R of the kidney

Renal I/R injury increased post-ischemic serum creatinine level 24 h after reperfusion ($P<0.001$ vs. control) (Fig. 2). Female rats had a reduced elevation in serum creatinine level when compared to males ($P<0.05$ vs. I/R 24h male). At the earlier time-point, 2 h after reperfusion, no significant changes in serum renal function parameter were detected among the groups.

Renal I/R injury caused marked alterations in renal histology compared with kidneys of sham-operated animals (Fig. 3A). At 2 h of reperfusion, renal tissue was oedemic, swelling and blabbing of tubular cells were already detected, but no difference was demonstrated between male and female animals.

Parallel to the significant elevation in serum creatinine, 24 h after reperfusion, widespread degeneration of tubular structure (tubular atrophy and loss of brush borders) was observed (Fig. 3A at this time-point, renal tissues of female rats demonstrated less severe histological features of injury such as significantly ameliorated atrophy and infiltration of leukocytes when compared to male rats ($P<0.05$ vs. I/R 24h male) (Fig. 3A-B).

The expression and phosphorylation of SGK-1

Control male rats had significantly higher *sgk-1* mRNA expression than females ($P<0.05$ vs. control female) (Fig. 4A). In the post-ischemic period, there was a significant elevation in both genders 2 h after reperfusion which was higher in males than in females ($P<0.01$ vs. I/R 2h female). 24 h after reperfusion, *sgk-1* decreased

Fig. 3. Female animals show attenuated morphologic changes of ischemia/reperfusion (I/R) injury. (A) PAS-stained kidney sections are shown from each animal group at a magnification of 200x. I/R led to widespread degeneration of tubular architecture, tubular dilation, swelling, necrosis and apoptosis, loss of brush borders especially 24 h after reperfusion. Figures are representative of $n = 6$ for all groups. (B) I/R led to morphologic changes in the kidney tissue ($+P<0.001$ vs. control males and females). In female animals, there were ameliorated I/R histological changes 24 h after reperfusion ($*P<0.01$ vs. I/R 24h male).

in males ($P<0.05$ vs. I/R 2h male), but remained unchanged in females. At this time-point, no difference between the genders was detected.

In parallel, SGK-1 protein level tended to be higher in control males compared to females, however the difference did not reach the level of significance ($P=0.062$ vs. control female) (Fig. 4B). After ischemia, marked elevation of protein level was detected in males 2 h after reperfusion ($P<0.05$ vs. control male), and the difference between males and females was more pronounced ($P<0.001$ vs. I/R 2h female). At 24 h

time-point, SGK-1 protein level returned approximately to the control level, but the difference between the two genders remained ($P<0.05$ vs. I/R 24h female).

Using Western blot analysis, the phosphorylated, thus activated form of SGK-1 was detectable only 2 and 24 h after reperfusion. In males, the level of phospho-SGK-1 was significantly higher at both time-points vs. females ($P<0.05$ vs. female respectively) (Fig. 4C).

Immunofluorescent staining revealed that both total and the phosphorylated isoforms can be detected in all groups (Fig. 5). In the controls, no difference

Fig. 4. (A) mRNA expression of *sgk-1* after renal ischemia/reperfusion (I/R) injury assessed by real-time PCR analysis. I/R caused elevated *sgk-1* mRNA expression 2 h after reperfusion in males compared to controls (% P <0.001 vs. control). *Sgk-1* mRNA expression was higher in control males and in males 2 h after reperfusion versus respective females (* P <0.05 vs. female, respectively). Castration of males decreased *sgk-1* mRNA level compared to males 2 h after reperfusion (# P <0.01 vs. I/R 2h male). (B) Protein expression of SGK-1 after renal I/R measured by Western blot analysis. I/R caused elevated SGK-1 protein expression 2 h after reperfusion in males compared to control males (% P <0.001 vs. control male). SGK-1 protein abundance was higher both 2 h and 24 h after ischemia in males when compared to females (* P <0.05 vs. female, respectively). Castration of males decreased SGK-1 level compared to males at all examined time-points (# P <0.01 vs. male, respectively). (C) Phosphorylation of SGK-1 was detected per Western blot analysis 2 and 24 h after reperfusion. Phosphorylation of SGK-1 was higher in males (* P <0.01 vs. female, respectively). Castration of males significantly reduced phosphorylation (# P <0.01 vs. I/R 2h male).

of SGK-1 localization was demonstrated between the genders. In the I/R 2h groups, a more pronounced total and phospho-SGK-1 tubular staining were observed in males compared to females which persisted in the 24 h group without marked differences in the localization.

Considering the *in vitro* data, to investigate the relevance of testosterone in SGK-1 expression *in vivo*, male rats underwent castration and were then subjected to renal I/R.

Castration did not change the level of *sgk-1* mRNA in control animals (P >0.05 vs. control male) (Fig. 4A).

Fig. 5. Immuno-fluorescent staining of the kidneys showed marked SGK1 expression located in the tubuli. Up-regulated total SGK1 (green fluorescence) (left panel) and phosphorylated-SGK1 (SGK1-P, red fluorescence) were stained in all groups: in male animals there was a more pronounced total SGK-1 staining 2 h after reperfusion compared to females. Furthermore, phosphorylated SGK-1 showed a marked staining 2h and 24 h after reperfusion than in females respectively. Cell nuclei were labeled with Hoechst (blue staining).

However, 2 h after reperfusion, where the most pronounced difference was detected between males and females, castration markedly decreased the

mRNA expression about to the level in females ($P<0.01$ vs. I/R 2h males) (Fig. 4A). 24 h after reperfusion, no difference was demonstrated between the groups.

Fig. 6. mRNA expression of *iNOS* in renal ischemia/reperfusion (I/R) injury assessed by real-time PCR analysis. *iNOS* mRNA level was significantly higher in control males and 2 h after reperfusion compared to females (* $P < 0.05$ vs. female, respectively). 2 h after reperfusion, *iNOS* mRNA increased in both genders compared to controls (# $P < 0.01$ vs. control, respectively).

SGK-1 protein expression was decreased by castration at all time-points approximately to the level detected in female rats ($P < 0.05$ vs. control males, $P < 0.01$ vs. I/R 2h males and vs. I/R 24h males) (Fig. 4B).

The expression of iNOS

The mRNA expression of *iNOS* was significantly lower in control males than in females ($P < 0.01$ vs. control female) (Fig. 5) ($P < 0.001$ vs. control female). 2 h after reperfusion, *iNOS* was elevated in both genders, however there was still a marked difference in males compared to females ($P < 0.01$ vs. I/R 2h female). 24 h after reperfusion, *iNOS* mRNA level decreased in both genders ($P < 0.01$ vs. I/R 2h) and there was still a tendency towards lower expression in males ($P = 0.079$ vs. I/R 24h females).

Discussion

In our study, we analyzed the gender specific differences in the SGK-1 pathway during the I/R injury of the kidney at different time-points.

SGK-1 is a serine/threonine protein kinase, member of the PI3-kinase pathway, ~50–55% homologous to Akt/PKB. The expression, enzymatic activity of SGK-1 are regulated in a stimulus-dependent manner in a variety of cell types and experimental conditions that have implicated it as a key component of the cellular stress response [21]. Target molecules of SGK-1 include transcription factors which regulate cell survival mechanism such as gene expression of *iNOS* and other target molecules. Furthermore, SGK-1 activates various ion channels such as epithelial sodium channel [eNaC] and NaCl co-transporter which altogether render SGK-1 a central transmitter of cell survival, ion transport and homeostasis regulated mainly through the kidney [8, 22–24].

The expression and activation of the SGK-1 homologue Akt has been reported to show gender specific differences in various experimental models such as I/R injury of the heart and kidney. Since the ratio of activated and total Akt was higher in females during cardiac I/R injury [25] and the same was demonstrated in the kidney [16], we hypothesized that expression of SGK-1 might be different between male and female animals after renal ischemia and thus, could contribute to the sexual dimorphism in the cell survival machinery.

First, our results confirmed our previous findings showing that kidney injury is attenuated in female animals with decreased elevation of serum creatinine and ameliorated tissue injury when compared to male rats.

Sgk-1 mRNA expression in the kidney were higher at all examined time-points in males paralleled by higher protein levels except in control animals, where only a tendency towards elevation could be demonstrated. In line with the total SGK-1 level, phosphorylation and thus, activation of SGK-1 was much more pronounced in males after ischemia supported by Western blot and immunofluorescent staining.

The expression of *iNOS* which is inhibited by SGK-1 showed a strong gender specific pattern according to changes in SGK-1 levels, i.e. control females and females with 2 h reperfusion had significantly higher expression of *iNOS* mRNA with the same tendency 24 h after reperfusion. Although results on *iNOS* expression after ischemia in males and females are somewhat contradictory, our data are well in accordance with the only kidney results reported by Park et al. [16]. They observed that calcium independent NOS [*iNOS*] activity in the kidney was significantly higher in intact females, and in females after renal ischemia when compared to males, respectively [16]. On the other hand, a study on cerebral ischemia demonstrated that post-ischemic *iNOS*

is lower in females than in males [26]. These opposite results might be explained by the application of different ischemic models with different organs, ischemia and reperfusion times that can markedly impact the highly complex expression pattern of iNOS. Nevertheless, it is important to note that in our study, according to previous assumptions, SGK-1 and its negative target iNOS expression correlated well with each other.

SGK-1 directly inhibits programmed cell death [6, 27]. Over the past years, data have emerged from *in vivo* and *in vitro* studies demonstrating that ischemic cell death pathways are fundamentally different in males and females. It appears that females have different pro-survival pathways, from which Akt is the one that has intensively been investigated [28]. Based on these data, it was tempting to speculate that female animals who are relatively protected from ischemic injury could have higher levels of SGK-1. Surprisingly, the opposite result could be shown and more interestingly, we detected only a very slight increase in SGK-1 expression in females after the ischemic insult.

In order to study, which sex hormone could be involved in the regulation of SGK-1, we treated kidney cells *in vitro* with different concentrations of either 17-beta estradiol or testosterone. Testosterone had a dose-related effect on SGK-1 expression, whereas 17-beta estradiol did not exert any effect. *In vivo*, castration of males decreased SGK-1 levels in controls, and also after I/R injury. These data together suggest that testosterone could be responsible for the gender-specific differences seen in renal SGK-1 expressions. In control animals, there was no difference in SGK-1 protein expression; however, we detected higher mRNA expression in males. The fact that the difference in protein levels was pronounced only after an ischemic insult suggests that under normal conditions in the kidney, the testosterone effect is overwhelmed by other factors controlling SGK-1.

To the best of our knowledge, this is the first study to investigate the level of SGK-1 *in vivo* in males and females. *In vitro*, there are conflicting results concerning SGK-1 and sex hormones. Previously, Shanmugam et al. reported that in accordance with our results, SGK-1 was up-regulated by a synthetic androgen stimulation in human prostate cancer cells [29]. They also demonstrated that androgen receptor interacts with two distant *sgk-1* promoter regions suggesting *sgk-1* as an androgen-regulated gene. Contradicting these results, in another study, there was no effect of different doses of testosterone or dihydrotestosterone on

sgk-1 mRNA in human renal proximal tubular cell line [30].

Testosterone exerts its effect through binding to intracellular receptors and thus, modulating transcription and protein synthesis, triggering genomic events [31]. Furthermore, like other steroid hormones, testosterone has rapid, non-genomic effects through direct interaction with intracellular kinases [24]. The results of our study implicate that testosterone might have both a genomic and non-genomic effect on SGK-1 regulation, since both mRNA expression and phosphorylation were up-regulated in male rats and were then reduced by castration. Interestingly, a recent study has revealed a testosterone-dependent interaction between the androgen-receptor of testosterone and the p85 α subunit of the PI3-kinase leading e.g. to the phosphorylation of the endothelial nitric oxide synthase [32]. This supplies evidence for a possible mechanism through which testosterone might directly lead to phosphorylation of SGK-1.

Not much are known about gender-specific associations of SGK-1 target molecules other than iNOS. In line with our results, the SGK-1 activated NaCl co-transporter is overactivated in males after angiotensin II infusion [33]; and eNaC is expressed higher in kidneys of testosterone treated rats [34]. These reports suggest that in these investigated SGK-1 downstream pathways, in accordance with our data, there is a shift towards higher expression levels in males. However, if the higher expression / activity is an effect of SGK-1 or different sex hormones have a direct role in their regulation, needs to be further investigated.

In conclusion, our results have demonstrated that the anti-apoptotic SGK-1 shows a gender-specific expression pattern after renal I/R with higher levels in male rats implicating that SGK-1 does not take part in the protective pathways fostering attenuated ischemic injury in females. Nevertheless, these results confirm previous contradictory reports showing that SGK-1 might be up-regulated and activated by testosterone.

In this study, we were able to demonstrate an additional pathway that probably contributes to gender-specific differences during I/R in the kidney. Unfortunately, *in vivo* studies investigating gender-based medication in the setting of kidney transplantation where I/R is a major contributor to delayed graft function are missing; however, the spectrum of experimental data demonstrating sex-specific mechanisms in the I/R-driven signaling highlights the necessity of such studies.

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