OTKA NNF 78846

Abstracts-presentations (all abstracts were presented as oral or poster presentation):

1. Prokai, A. Fekete, K. Rusai, N. Banki, V. Muller, L. Wagner, A. Ver, T. Tulassay, A. Szabo: ***Th eeffect of erythropoietin (EPO) on hypoxia inducible factor (HIF) system during renal ischemic reperfusion (IR) injury: gender differences***, 43rd Annual Scientific Meeting of the European Society for Paediatric Nephrology, Birmingham, UK, 2-5 September, 2009, 2009 (abstract)
2. Pásti Krisztina, Prókai Ágnes, Pekó Nóra, Reusz György, Sallay Péter, Tulassay Tivadar, Szabó J Attila: ***Folyamatos szöveti glukózmonitorizálás (CGMS) vesetranszplantált (NTx) gyermekekben***, Magyar Gyermekorvosok Társasága 53. Nagygyűlése, Eger, 2009. június 18-20, 2009 (abstract)
3. Pásti Krisztina, Prókai Ágnes, Tóth Réka, Pekó Nóra, Reusz György, Sallay Péter, Tulassay Tivadar, Szabó J Attila: ***Poszttranszplantációs szénhidrát anyagcserezavar felmérése és követése vesetranszplantált gyermekekben***, Magyar Gyermekorvosok Társasága 53. Nagygyűlése, Eger, 2009. június 18-20, 2009 (abstract)
4. Prókai Á., Fekete a., Bánki N., Rusai K., Müller V., Vér Á., Wagner L., Degrell P., Tulassay T., Szabó A.J: ***Erythropoietin (EPO) hatása a hipoxia indukálta faktor (HIF) rendszerre a vese iszkémiás-reperfúziós (I/R) károsodása során: Nemi különbségek***, Magyar Gyermekorvosok Társasága 53. Nagygyűlése, Eger, 2009. június 18-20, 2009 (abstract)
5. Prókai Á., Fekete A., Bánki N., Rusai K., Müller V., Vér Á., Wagner L., Reusz Gy., Tulassay T., Szabó J. Attila: ***Erythropoietin (EPO) hatása a hipoxia indukált faktor (HIF) rendszerre a vese iszkémiás-reperfúziós (I/R) károsodása során: nemi különbségek***, Hypertonia és Nephrologia 2009; 13 (S1): 48, 2009 (abstract)
6. Rusai Krisztina, Prókai Á., Szebeni B., Fekete A., Treszl A., Vannay Á., Müller V., Reusz Gy., Heemann U., Lutz J., Tulassay T., Szabó A.J: ***Az erythropoietin (EPO) protektív hatása és a szérum és glükokortikoid regulálta kináz-1 (SGK-1) a vese ischémia/reperfúziós (I/R) károsodásában***, Hypertonia és Nephrologia 2009; 13 (S1): 50, 2009 (abstract)
7. Szabó Attila: ***Az iszkémiás szervkárosodás sejtszintű mechanizmusai***, Magyar Élettani Társaság LXXIII. Vándorgyűlése Budapest, 2009. augusztus 27-29, 2009 (abstract)
8. A.Fekete,K.Rusai,N.F.Bánki,E.Károly,G.S.Reusz, T.Tulassay,A.J.Szabo: ***Heat Shock Protein (HSP) Polymorphism Predisposes to Urinary Tract Malformations and Renal Transplantation in Children?***, The 15th Congress of the International Pediatric Nephrology Assosiation, New York,August 29-September 2, 2010, 2010 (abstract)
9. L.Wagner,N.F.Bánki,A.Prókai,A.J.Szabó,A.Vér, A.Fekete: ***The Effect of Losartan on Na/K-ATPase(NKA) and Heat Shock Protein (HSP) 72 in Rats with Diabetic Nephrolpathy.***, The 15th Congress of the International Pediatric Nephrology Assosiation, New York,August 29-September 2, 2010, 2010 (abstract)

Publications:

1. Kis E, Cseprekál O, Bíró E, Kelen K, Ferenczi D, Kerti A, Szabó AJ, Szabó A, Reusz GS: ***Effects of bone and mineral metabolism on arterial elasticity in chronic renal failure***, Pediatr Nephrol. 2009 Sep 10. [Epub ahead of print], 2009 IF: 1,936
2. Reusz GS, Kis E, Cseprekál O, Szabó AJ, Kis E: ***Captopril-enhanced renal scintigraphy in the diagnosis of pediatric hypertension***, Pediatr Nephrol. 2010 Feb;25(2):185-9, 2010 IF: 1,936
3. Rusai K, Prókai A, Szebeni B, Fekete A, Treszl A, Vannay A, Müller V, Reusz G, Heemann U, Lutz J, Tulassay T, Szabó AJ: ***Role of serum and glucocorticoid-regulated kinase-1 in the protective effects of erythropoietin during renal ischemia/reperfusion injury.***, Biochem Pharmacol. 2010 Apr 15;79(8):1173-81, 2010 IF: 4,838
4. Rusai K,Banki NF,prokai A,Podracka L,Szebeni B,Tulassay T,Reusz GS,Sallay P, Körmendi R,Szabó AJ,Fekete A: ***Heat shock protein polymorphism predisposes to urinary tract malformations and renal transplantation in children.***, Transplant Proc.2010 Jul-Aug; 42(6):2309-11, 2010 IF: 1,055
5. Ágnes Prókai, Andrea Fekete, Nóra Fanni Bánki, Veronika Müller, Ágota Vér, Péter Degrell, Krisztina Rusai, László Wagner, Ádám Vannay, Rosta Máté, Uwe Heemann, Róbert M. Langer, Tivadar Tulassay, György Reusz, Attila J. Szabó: ***RENOPROTECTIVE EFFECT OF***

ERYTHROPOIETIN IN RATS SUBJECTED TO ISCHEMIA/REPERFUSION INJURY: GENDER DIFFERENCES., Surgery 2011 in press IF: 3,603

6. A Prokai, A Fekete, K Pasti, K Rusai, NF Banki, G Reusz, AJ Szabo: ***The importance of different immunosuppressive regimens in the development of post-transplant diabetes mellitus.,*** Pediatric Diabetes 2011 in press IF: 2,628
7. K. Kelen, D. Ferenczi, I. Jankovics, M. Varga, M.Z. Molnar, P. Sallay, G. Reusz, R.M. Langer, K. Pasti, Z. Gerlei, and A.J. Szabo: ***H1N1 Vaccination in Pediatric Renal Transplant Patient.,*** Transplantation Proceedingsdoi:10.1016/j.transproceed.2011.03.072 2011 in press IF: 0,994

Other results:

"Renal ischemia/reperfusion injury: the role of serum and glucocorticoide regulated kinaze-1" Krisztina Rusai won the Petényi Prize from the Hungarian Pediatric Association.

Rusai K, Prókai A, Szebeni B, Fekete A, Treszl A, Vannay A, Müller V, Reusz G, Heemann U, Lutz J, Tulassay T, Szabó AJ: ***Role of serum and glucocorticoid-regulated kinase-1 in the protective effects of erythropoietin during renal ischemia/reperfusion injury.*** won the best scientific publication in 2010 award from the Hungarian Nephrology Association.

Due to her scientific achievements partially supported by OTKA NNF 78846, Krisztina Rusai won the Prima Junior Award in 2010.

Some of our results were also presented at the Semmelweis Scientific Student Conference (TDK) and in 2010 and 2011 three I. prizes and II. second prizes were achieved.

SCIENTIFIC SUMMARY

INTRODUCTION

Renal ischemia/reperfusion (I/R)-induced acute renal failure still has high rates of morbidity and mortality. In renal transplantation, I/R has a major role in delayed graft function and chronic allograft injury. The exact pathomechanism of I/R is still unclear, but growing evidence indicates that sex differences exist in kidney response to renal ischemic injury (*1-3*).

Our previous studies have revealed the importance of a nitric oxide (NO) pathway showing the pivotal role of NO and endothelin in the gender-dependent renal response to ischemic injury and aging (*4*). Moreover, we also demonstrated the importance of Na+/K+ATPase-a1 and heat shock protein (HSP)72 in this gender dependent injury (1-3).

Erythropoietin (EPO) is an essential growth factor of hemopoietic progenitor cells (*5*), but its extra-hemopoietic effects imply additional therapeutic possibilities. Indeed, a wealth of experimental data is being generated with respect to the protective effect of EPO against the ischemic myocardium (*6,7*) liver (*8,9*) and renal injury (*10-12*). Moreover, several clinical trials also are being processed. According to the trial of the Hannover Medical School (Germany), rHuEPO alpha significantly increased the glomerular filtration rate in transplant patients (ClinicalTrials.gov number: NCT00425698).

The anti-apoptotic, anti-oxidative, and anti-inflammatory effects of EPO have been investigated in several studies (*12-16*), but the complete molecular mechanisms involved in the prevention of renal I/R injury are not yet fully understood.

A growing body of evidence supports the connection between EPO and the HSP70 family. It has been shown that EPO attenuates myocardial infarct size by enhancing the HSP70 protein level (*17)*. In addition, the induction of HSP70 by EPO administration inhibits apoptotic cell death in rat ischemic kidney (*18*).

Furthermore, EPO exerts its immediate anti-apoptotic effects via an activation of multiple signalling pathways of which phosphorylation and activation of the phosphatidylinositol 3-kinase (PI3-kinase) (*19*) seems to play a pivotal role. SGK1 is an anti-apoptotic down-stream kinase of the PI3-kinase pathway. The expression and activation of the serum and glucocorticoid-regulated kinase 1 (SGK1) is increased by various cell stress stimuli such as hyperosmotic stress, ultraviolet radiation, heat shock (*21*), oxidative stress (*22*) and hypoxia (*23*). In a previous study, we could show that SGK1 is markedly induced after I/R in the kidney and upregulation of SGK1 directly protects kidney tubulus cells from hypoxia (*26*). Gender-specific aspects were not investigated.

Furthermore, we demonstrated that renal I/R injury induces a gender-dependent HSP72 expression (*3*), which maintains basolateral membrane localization of an essential tubular sodium transporter - the Na+/K+ATPase. Under ischemic conditions, its enzyme activity decreases in a gender- and time-dependent manner and because of the disruption of the actin cytoskeleton, Na+/K+ATPase-a1 internalizes or translocates from the basolateral to the apical membrane of renal tubular cells.

Under stress conditions, the heat shock factor 1 (HSF-1) is the major transcription factor that is responsible for induction of HSPs (*24*). Furthermore, hypoxia inducible factor-1 is another transcription factor that gained more attention in ischemic models since it triggers the expression of several factors which play a central role in the defense mechanisms against ischemia (*25*).

There is not much known about gender specific differences of HSF-1 and HIF-1 pathways, and although the PI3-kinase pathway has long been known to be regulated by estrogen, sex differences in SGK1 was not investigated under in vivo stress conditions either.

AIMS

Regarding the proven beneficial effects of EPO in renal ischemic injury and our results showing major effects of HSP72 and SGK-1 during renal I/R injury, the aims of our project was to investigate:

1. whether EPO treatment is protective against serious, unilateral renal I/R damage;
2. whether there is a difference in EPO effects between female and male rats;
3. the role of SGK-1, HSP72 and Na+/K+ATPase in EPO effects;
4. whether there are gender specific differences in SGK-1 expression after I/R in the kidney;
5. the two transcriptional factor pathways (HSF-1 and HIF-1) in serious, unilateral renal I/R damage;
6. possible gender differences in these two signaling pathways.

RESULTS

1. EPO treatment and SGK1 in renal ischemia/reperfusion (I/R) injury

In line with earlier observations of other experimental studies, we could also show that erythropoietin (EPO) has markedly protected the kidney from ischemic injury as reflected by decreased post-ischemic serum creatinine and blood urea nitrogen levels. This was partly mediated by upregulation of SGK1.

In vitro, cultures of kidney cells were exposed to hypoxia. Incubation with EPO at a dosis of 400 U/ml exerted a marked protective effect on cell death. This was paralleled by up-regulation of SGK1 expression as well as phosphorylation, thus activation. Moreover, down-regulation of SGK1 expression through small interfering RNA technique ameliorated the anti-apoptotic effect of EPO treatment suggesting that upregulation of SGK1 is not an epiphenomenon, but rather a significant contributor to the protective EPO effects in this model.

In the *in vivo* rat model of unilateral renal I/R injury, rats were treated with 500 U/kg EPO 24 h prior to ischemia. EPO resulted in less severe tissue injury and ameliorated the elevation in creatinine and urea nitrogen levels 24 h after reperfusion. Furthermore, SGK1 expression and phosphorylation were higher in EPO compared to vehicle treated rats suggesting that SGK1 might be involved also in the in vivo effects of EPO (**Figure 1A-B**.).

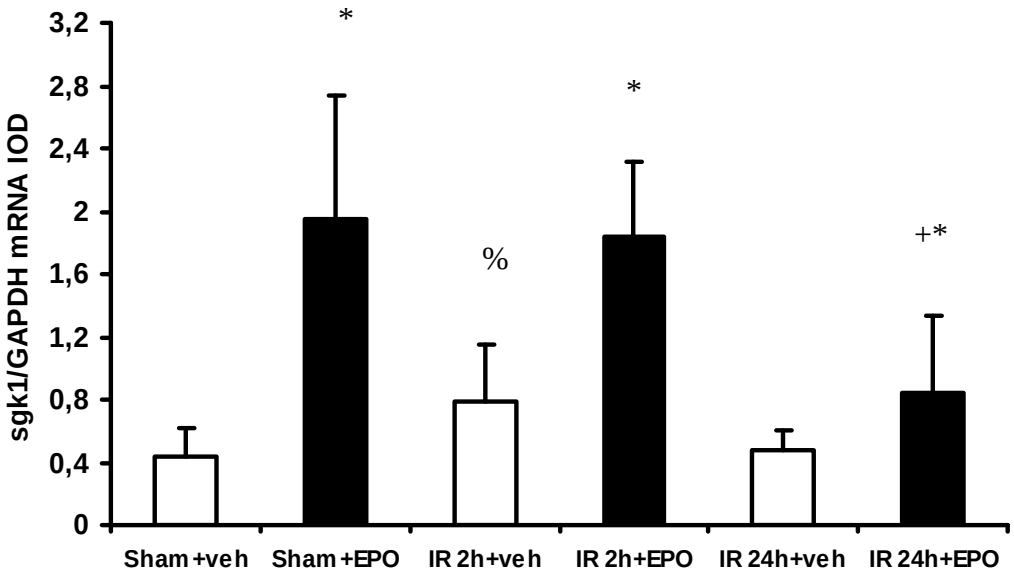


Figure 1A. Erythropoietin (EPO) induced SGK1 mRNA and protein expression. (A) SGK1 mRNA expression was assessed by real-time PCR analysis. I/R caused elevated SGK1 mRNA expression 2 h after reperfusion (%P<0.05 vs. shams). EPO induced SGK1 mRNA expression in all animal groups (*P<0.01 vs. vehicle treated animals). 24 h after reperfusion, SGK1 mRNA expression was decreased compared to the 2 h-animals in the EPO treated animals (+P<0.01 vs. I/R 2h+EPO). (B) SGK1 protein expression was assessed by Western blot analysis. I/R caused elevated SGK1 protein expression 24 h after reperfusion (%P<0.05 vs. Shams).

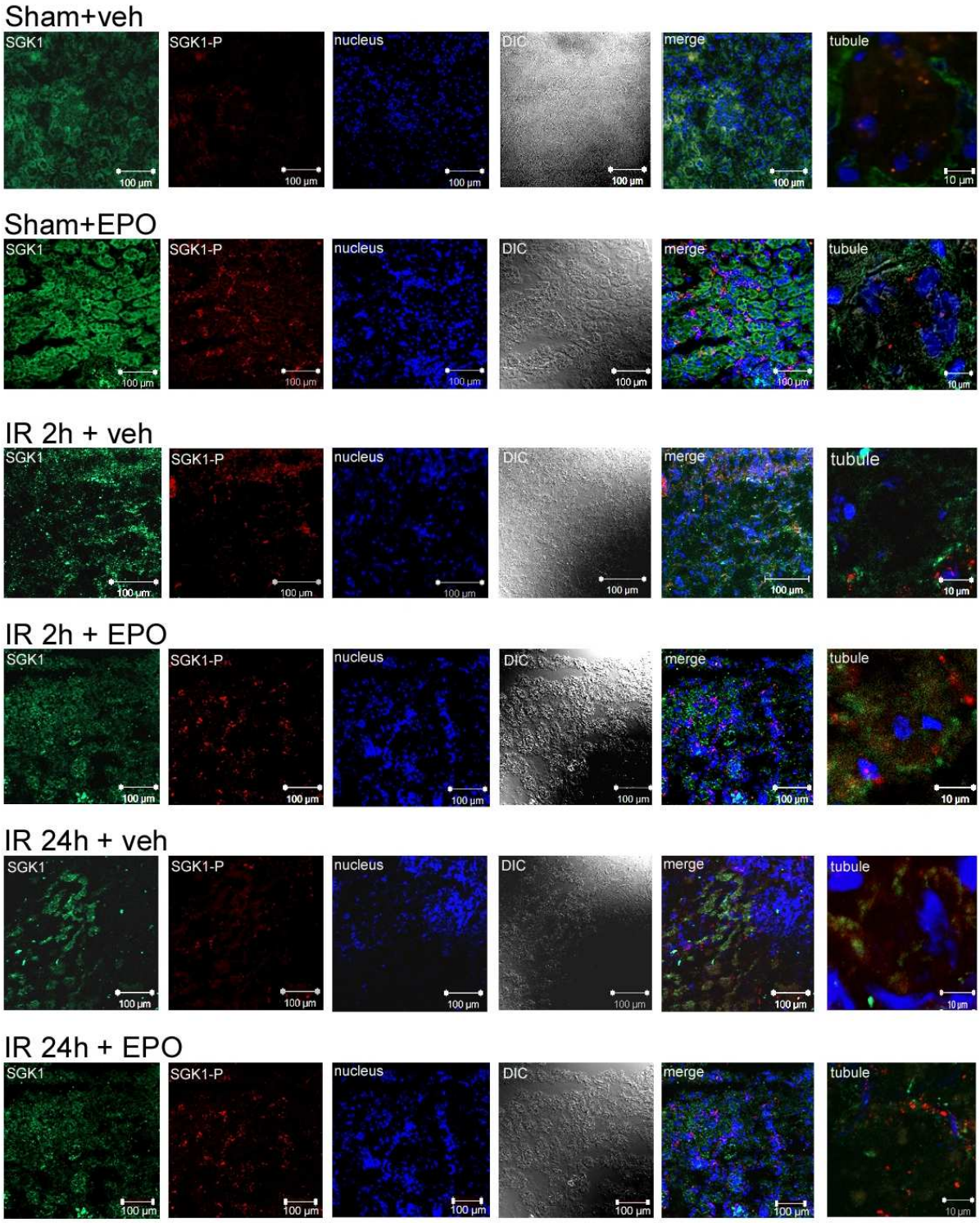


Figure 1B. Immunofluorescent staining of the kidneys showed marked SGK1 expression located in the tubuli. Up-regulated total SGK1 (green fluorescence) (left panel) and phosphorylated-SGK1 (SGK1-P, red fluorescence) were stained in EPO treated animals in the sham-operated group and 2h after ischemia in the tubular cells. 24 h after reperfusion, no marked difference was demonstrated between the vehicle and EPO treated animals. Cell nuclei were labeled with Hoechst (blue staining).

2. Gender differences in the expression pattern of SGK1 in renal I/R injury

We could show that there is a marked sex-specific expression pattern of SGK1 expression. In male animals, there was higher expression and higher activation of SGK1 after I/R when compared to female rats (**Figure 2.**). This higher expression was demonstrated both at the level of mRNA and protein, moreover using immunofluorescent staining localization of total and phosphorylated SGK1 was determined.

In order to determine which sex hormone could be involved in the regulation of SGK1, cells were treated with different concentrations of either testosterone or estrogen, whereas in vivo, animals were gonadectomized.

I*n vitro*, treatment of kidney cells with testosterone stimulated, whereas *in vivo* castration of males decreased SGK1 expression suggesting that SGK1 might be regulated by testosterone.

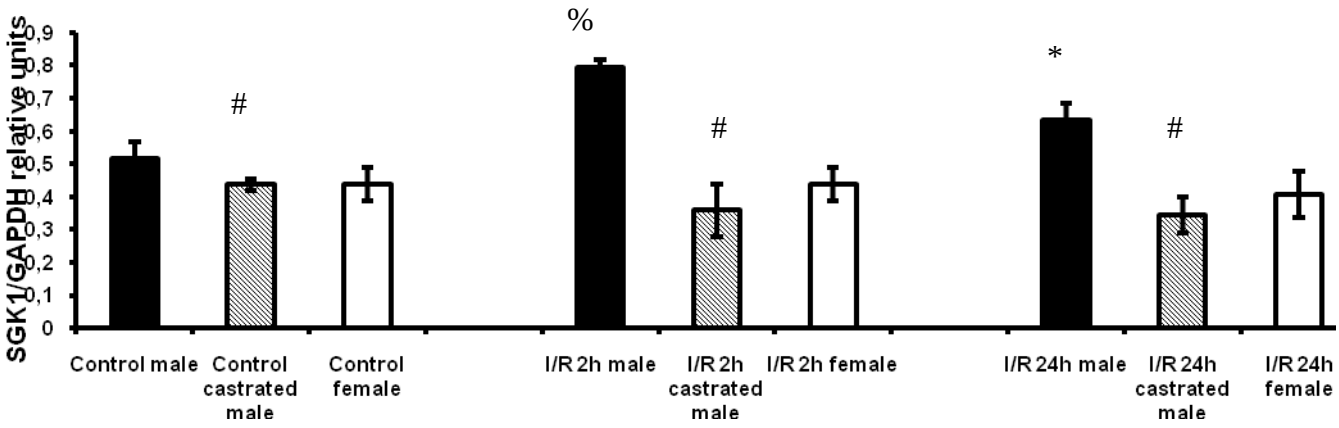


Figure 2. Protein expression of SGK-1 after renal I/R measured by Western blot analysis. I/R caused elevated SGK-1 protein expression 2 h after reperfusion in males compared to control males (%P<0.001 vs. control male). SGK-1 protein abundance was higher both 2 h and 24 h after ischemia in males when 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).

3. EPO treatment and HSP72 and NA^+/K^+ATPase in renal I/R injury

Survival. The EPO administration resulted in a remarkable amelioration of male's postischemic survival. Although all untreated males died as a result of ARF by the third day, those treated with EPO survived until the sixth day, almost twice as long as those untreated. EPO resulted in a slight improvement in females as well. To note, however, irrespective of the EPO administration, pared with their male counterparts (**Figure 3**.).

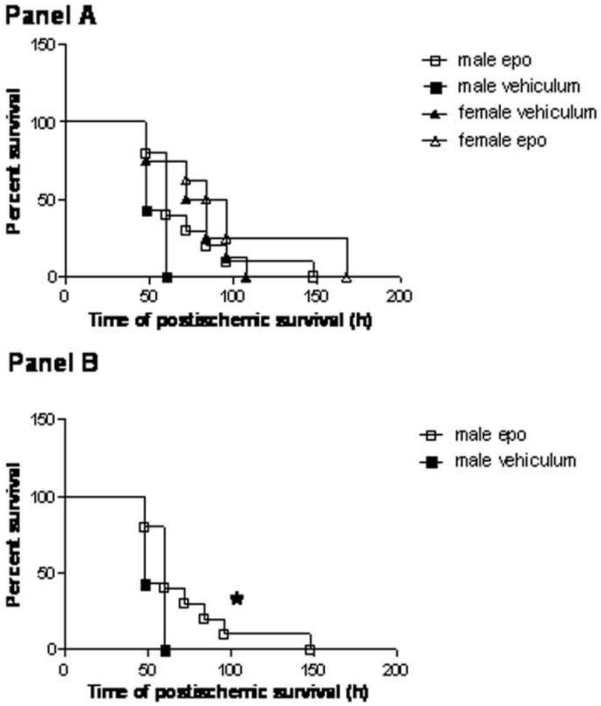


Figure 3. Effect of EPO treatment on the postischemic survival (A); in male + vehicle (-) and male + EPO (,) (B); in female + vehicle (:) and female + EPO (⌂) (C) (n = 8). The cumulative proportion surviving analysis of significance was performed by Kaplan–Mayer analysis (log-rank test). *P # .05 versus male + vehicle.

Serum EPO level. The serum level of endogenous EPO was higher in males versus females not only in controls but also in both postischemic time points. EPO administration increased control and T2 EPO

levels in both genders, whereas at T24, the effect of exogenous EPO treatment disappeared. The dynamics of postischemic changes in serum EPO levels followed a remarkably different manner between males and females; in males, the already higher EPO decreased only at T24, whereas in females, the EPO level dropped to a third of the control value already at T2 (**Table I.**).

Serum EPO (mIU/mL)	control	T2	T24
Female+vehicle	7.7 ± 4.1	2.5 ± 0.8 *	0.3 ± 0.6 *#
Male+vehicle	11.9 ± 4.2 §	10.0 ± 2.4 §	1.4 ± 1.7 § *#
Female+EPO	15.2 ± 4.7 &	7.3 ± 3.1 &*	1.4 ± 0.6 &*#
Male+EPO	24.7 ± 15.0 &	25.7 ± 6.6 §&	1.17 ± 0.3 *#

Table I. Serum levels of endogenous and exogenous EPO after renal ischemia-reperfusion injury in female and male rats (n = 8/group) Values are means ± SD. yP < .05 versus female. zP < .05 versus vehicle-treated. xP < .05 versus control. {P < .05 versus T2.

HSP72 protein levels. The HSP72 level was higher in untreated females than in males at every time point. After the ischemic insult, the HSP72 protein levels increased in a gender-dependent manner. In females, HSP72 reached its maximum at T2, whereas in males, the rate of increase was slower both with and without EPO treatment. EPO increased the HSP72 protein level in males at T24, whereas in females, the already higher HSP72 level was not elevated.

Na+/K+ATPase-α1 subunit protein levels. Similar to HSP72, the postischemic changes in Na+/K+ ATPase-a1 protein levels were different between the sexes. Na+/K+ATPase-a1 protein levels were higher in untreated females than in males at every time point (Figure 4). EPO treatment was effective only in males by increasing the protein level at T24 to a level even higher than the EPO-treated females.

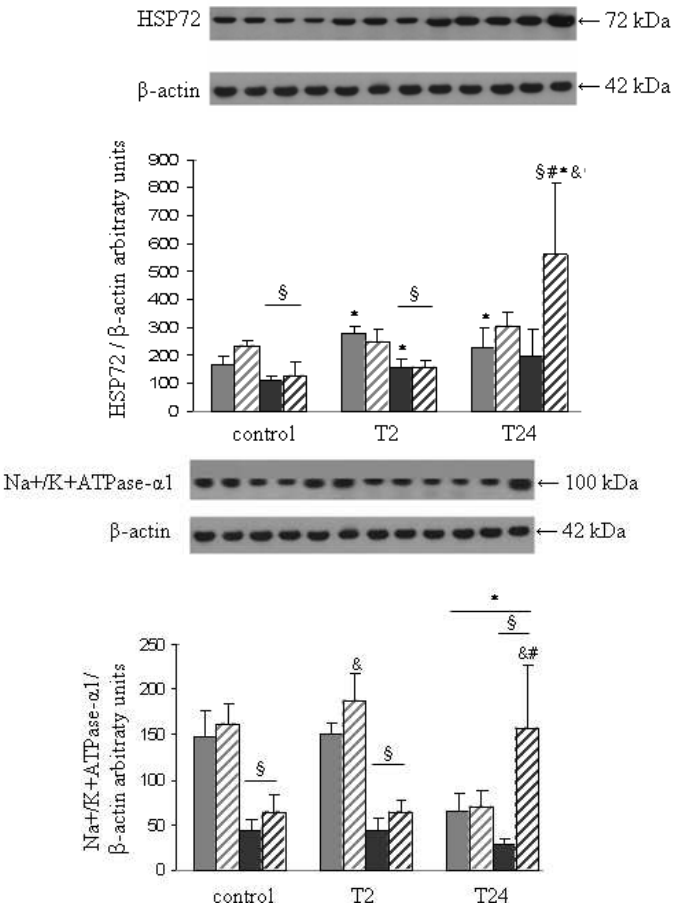


Figure 4. Effect of renal I/R injury on protein expression of HSP72 (Panel A) and Na+/K+ATPase- a1 in female + vehicle, female + EPO, male + vehicle, and male + EPO rat kidney. Protein expression was determined in kidney samples from control and at T2 and T24 of reperfusion after 50 minutes of renal ischemia (n = 8/group). Top: representative examples of Western blot analysis of HSP72 and beta-actin in kidney. Female + vehicle (gray); female + EPO (striped gray); male + vehicle (black); male + EPO (striped black). Values are mean ± SD. xP < .05 versus female, &P < .05 versus vehicletreated, *P < .05 versus T0, #P < .05 versus T2.

Immunolocalization of HSP72 and Na+/K+ATPase- a1 subunit. Immunofluorescent staining was used to investigate the potential relationship between HSP72 and Na+/K+ATPase-a1. In the tubules of control rats, Na+/K+ATPase-a1 was localized on the basolateral membrane domain of tubular cells, with minimal staining in the cytosol or the apical domain. No gender differences were observed at this time point. In contrast, HSP72 staining was virtually undetectable in the tubular cells of control rats. After ischemic injury, Na+/K+ATPase-a1 became more prominent in the cytosol compared with the controls, but this internalization was less pronounced in untreated females versus males. After EPO treatment, however the Na+/K+ATPase-a1 localization remained more pronounced on the basolateral membrane in males as well.

4. EPO treatment and HIF1α / HSF1 in renal I/R injury

Immunolocalization of HIF1α. Immunofluorescent staining was used to describe the source of the HIF1α in renal cortex (**Figure 5**.). In the peritubular fibroblasts of T2 rats, HIF1α became stabile and transported toward the nucleus giving the opportunity to bind to hypoxia responsible element resulting increased EPO expression.

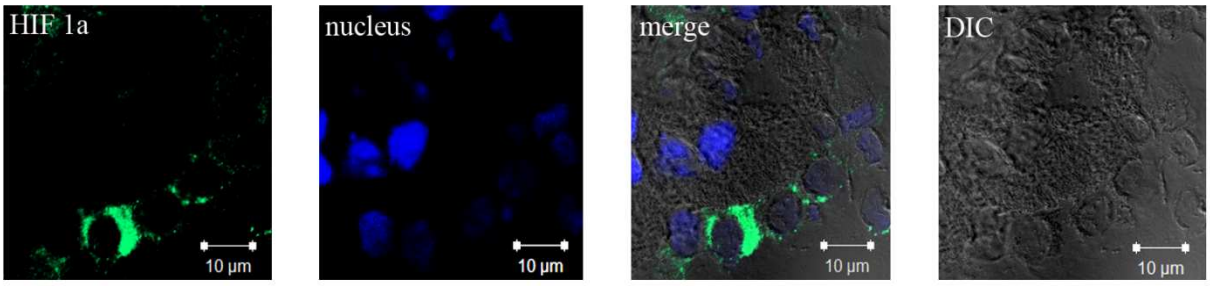


Figure 5. Localization of HIF 1a (green) in the peritubular fibroblasts of representative samples using immunofluorescent staining followed by confocal images.

HIF1α protein levels. The HIF1α level was higher in untreated males than in females at every time point (**Figure 6**.). After the ischemic insult, the HIF1α protein levels increased in a gender-dependent manner. In females, HIF1α increased by T2 and even further by T24. In males, the rate of the increase was more robust in untreated rats compared to females, whereas in those receiving EPO this raise completely diminished.

HSF1 protein levels. The postischemic changes in HSF1 protein levels were different between the sexes. HSF1 was higher in untreated females than in males at every time point (**Figure 6**.). However, EPO treatment lowered the HSF1 in both postischemic female groups to a level comparable to that of controls.

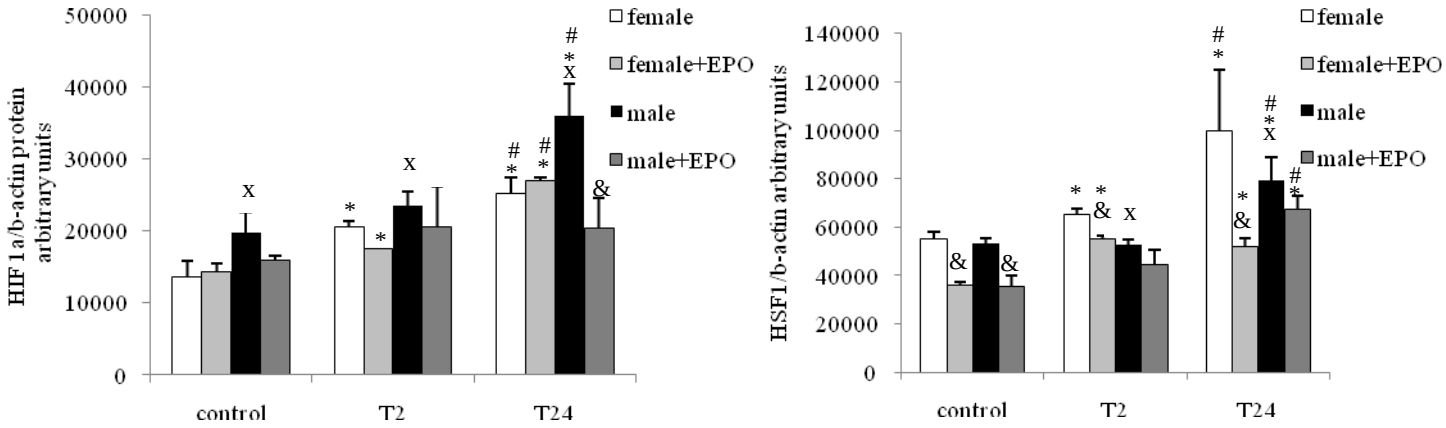


Figure 6. Effect of renal I/R injury on protein expression of HIF 1 α and HSF1 in female + vehicle, female + EPO, male + vehicle, and male + EPO rat kidney. Protein expression was determined in kidney samples from control and at T2 and T24 of reperfusion after 50 minutes of renal ischemia (n = 8/group). Female + vehicle (white); female + EPO (light gray); male + vehicle (black); male + EPO (dark gray). Values are mean ± SD. x P < .05 versus female, &P < .05 versus vehicle treated, *P < .05 versus T0, #P < .05 versus T2.

CONCLUSIONS

Our results based on both survival and molecular studies suggest that EPO protects against severe, unilateral renal I/R injury, especially in male rats. Furthermore, we believe this beneficial effect might be partly the result of EPO's HSP72-mediated impact on Na^+/K^+ATPase-α1.

Moreover, our data report a new signaling molecule to be involved in EPO cytoprotective actions, since the present study identified for the first time an important role of SGK1 in the anti-apoptotic and possibly, renoprotective effect of EPO during renal I/R injury.

The EPO induced up-regulation of SGK1 was observed both under *in vitro* and *in vivo* circumstances. Moreover, *in vitro*, inhibition of SGK1 blocked the protective effect of EPO under hypoxia confirming a direct role of SGK1 in the EPO effect.

These observations provide insights into a novel signaling mechanism by which EPO partly exerts its potent tissue protective actions. Given our results along with previous reports, the clinical use of EPO possibly leading to reduced cellular damage due to ischemic events should be considered, however, further investigations are needed to establish the feasibility and efficacy of EPO in clinical settings.

Our study has revealed a gender-dependent protective mechanism during renal I/R injury. In males mostly the HIF 1α, while in females the HSF is the dominant transcriptional pathway. The EPO treatment results in disappearance of the characteristic signal pathway activation in both genders. The explanation for that could be either a direct negative feedback effect on the transcription factors or an indirect renal protective effect of EPO by which these transcriptional factors do not need to be activated.

We could also demonstrate that the anti-apoptotic SGK1 shows a gender-specific expression pattern after renal I/R with higher levels in male rats. These results confirm previous contradictory reports showing that SGK1 might be up-regulated and activated by testosterone.

OUTLOOK

With respect to the future, the effective and safe administration of EPO and EPO mimetic drugs may also have therapeutic potential in preventing ischemic kidney injury in clinical settings such as open-heart, aorta surgery and renal transplantations. Finally, the individualized EPO administration between males and females might also be considered in every-day clinical practice.

OTHER RESULTS:

Since we found that HSP72 and steroids play a key role in ischemic renal injury we focused on another field too. Smoking is the leading risk factor of chronic obstructive pulmonary disease (COPD) and lung cancer. Corticosteroids are abundantly used in these patients; however, the interaction of smoking and steroid treatment is not fully understood. Heat shock proteins (Hsps) play a central role in the maintenance of cell integrity, apoptosis and cellular steroid action. To better understand cigarette smoke-steroid interaction, we examined the effect of cigarette smoke extract (CSE) and/or dexamethasone (DEX) on changes of intracellular heat shock protein-72 (Hsp72) in lung cells. Alveolar epithelial cells (A549) were exposed to increasing doses (0; 0.1; 1; and 10 μM/μl) of DEX in the medium in the absence(C) and presence of CSE. Apoptosis, necrosis, Hsp72 messenger-ribonucleic acid (mRNA) and protein expression of cells were measured, and the role of Hsp72 on steroid effect examined.

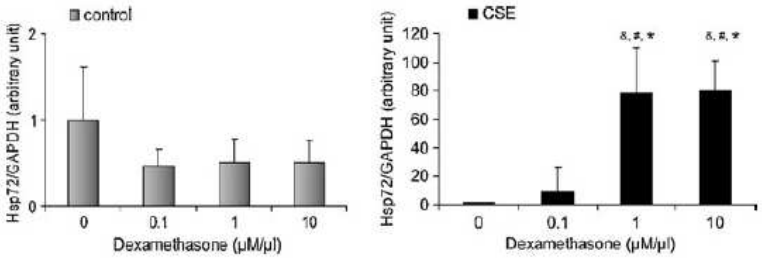
Table 1 Treatment groups

C	DMEM+10% FBS+1% AB
C+DEX (0.1)	DMEM+10% FBS+1% AB+0.1 μM/μl dexamethasone
C+DEX (1)	DMEM+10% FBS+1% AB+1 μM/μl dexamethasone
C+DEX (10)	DMEM+10% FBS+1% AB+10 μM/μl dexamethasone
CSE	Cigarette smoke extract+10% FBS+1%AB
CSE+DEX (0.1)	Cigarette smoke extract+10% FBS+1% AB+0.1 μM/μl dexamethasone
CSE+DEX (1)	Cigarette smoke extract+10%FBS+1% AB+1 μM/μl dexamethasone
CSE+DEX (10)	Cigarette smoke extract+10%FBS+1% AB+10 μM/μl dexamethasone
scr-RNA	DMEM+10% FBS+1% AB+transfected with scrambled silencing RNA (as negative transfection control)
siRNA	DMEM+10% FBS+1% AB+transfected with Hsp72 silencing RNA

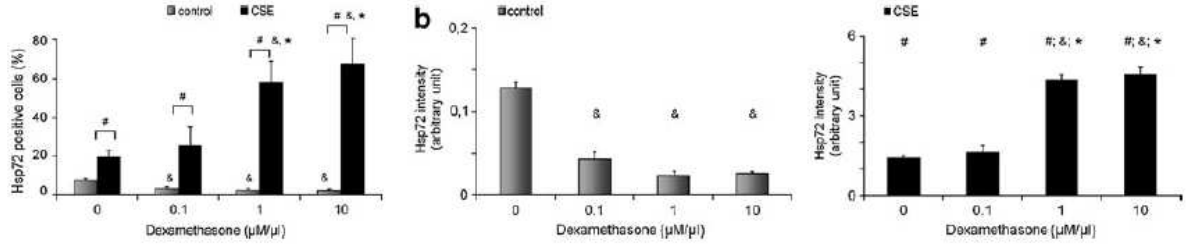
Cell number after the treatments: Under control conditions cells proliferated, reaching about 8.55±1.1×105 final cell number/vial. Following DEX treatment no significant change in cell number was noted. In contrast, CSE treatment significantly reduced the cell number 24 h after incubation as compared to controls. CSE+DEX co-treatment dose-dependently and significantly increased the total cell count as compared to CSE treatment alone, reaching similar number in both DEX (10) groups (C+DEX (10): 10.22±0.77×105 cell/vial; CSE+DEX (10): 8.86±0.49×105 cell/vial).

Apoptosis: DEX slightly decreased the number of the apoptotic cells in controls, reaching statistical significance only in the C+DEX (10) group. In steroid-naïve CSE-treated cells, apoptosis tripled as compared with the steroid naïve controls. DEX treatment significantly reduced apoptosis in all CSE-treated groups, abolishing the difference between CSE-treated and respective control groups.

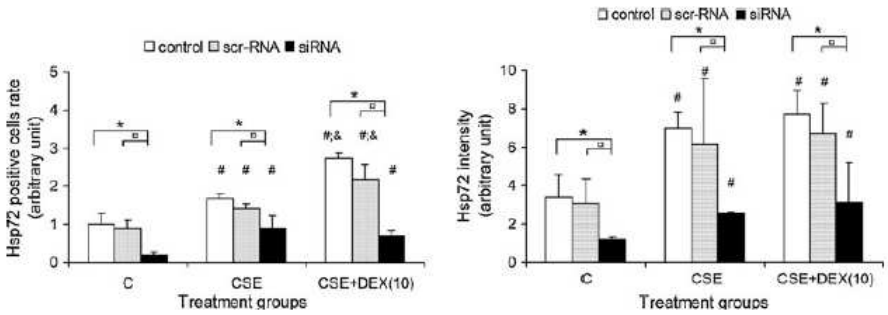
Necrosis: The ratio of necrotic cells did not differ in controls, whether DEX treatment was used or not (C, 3.2±0.63%; C+DEX (0.1), 2.95±0.36%; C+DEX (1), 3.2±1.39%; C+DEX (10), 3.08±1.32%). In contrast, CSE significantly increased the number of necrotic cells. Similarly to controls, DEX had no additional effect on necrosis in CSE-treated cells resulting in significantly higher necrotic cell rate in all CSE groups (CSE, 6.24±1.02%; CSE+DEX (0.1), 6.12±2.11%; CSE+DEX (1), 6.68±1.1%; CSE+DEX (10), 4.7±0.75%; p<0.05 vs. respective control group).



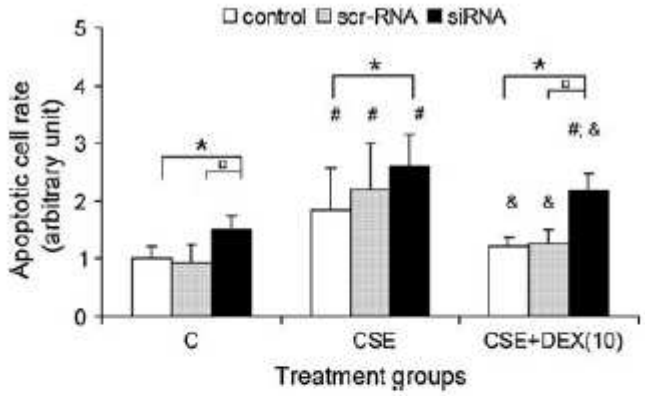
Hsp72 mRNA expression. A549 alveolar epithelial cells were treated with increasing doses of dexamethasone (DEX) in the medium: 0 (steroid naïve), 0.1, 1, and 10 μM/μl in the absence (*controls*) and presence of cigarette smoke extract (*CSE*). Hsp72mRNA expression was determined by real-time PCR. Optical density of Hsp72 was corrected for that of GAPDH. DEX treatment alone did not change Hsp72 mRNA expression, while in the presence of CSE DEX administration was associated with significant increase in Hsp72 mRNA expression. Expression of Hsp72 mRNA in the steroid naïve control group is expressed as 1; relative changes are presented for all other groups (arbitrary unit). (&$p<0.01$ vs 0 μM/μl DEX; #$p<0.05$ vs control; *$p<0.01$ vs 0.1 μM/μl DEX)



Hsp72 protein expression: percentage of Hsp72-positive cell (**a**) and intracellular amount/intensity of Hsp72 (**b**). A549 alveolar epithelial cells were treated with increasing doses of dexamethasone (DEX) in the medium: 0 (steroid naïve), 0.1, 1, and 10 μM/μl in the absence (*controls*) and presence of cigarette smoke extract (*CSE*). Hsp72 protein expression was determined by flow cytometric analysis using rabbit anti-human Hsp72 IgG primary antibody and rabbit Cy5-conjugated secondary antibody. The ratio of Hsp72 expressing cells and the intracellular Hsp72 intensity of cells were measured. DEX treatment decreased Hsp72 protein expression in controls, whereas co-treatment with CSE and DEX increased the Hsp72 protein expression dose dependently. In all CSE-treated groups, Hsp72 protein expression was significantly higher as in respective control groups, with the highest difference measured in DEX (10) cells. Data for protein levels of Hsp72 were obtained by computerized analysis of the FACS. (&$p<0.01$ vs 0 μM/μl DEX; #$p<0.01$ vs control; *$p<0.01$ vs 0.1 μM/μl DEX)



Hsp72 protein expression after siRNA treatment: percentage of Hsp72-positive cells (**a**) and intracellular amount/intensity of Hsp72 (**b**). Hsp72 silencing RNA (siRNA) was used in control (**c**), cigarette smoke extract (*CSE*)-treated and CSE plus dexamethasone 10 μM/μl (DEX (10) co-treated A549 alveolar epithelial cells. Scrambled (Scr)-RNA was used similarly as negative control for the transfection. After silencing, Hsp72 protein expression was measured by flow cytometry. In all groups, siRNA effectively suppressed Hsp72 expression. In a steroid-naïve control, values (*C*) are expressed as 1; relative changes are presented for all other groups (arbitrary unit). (&$p<0.01$ vs CSE-treated groups; #$p<0.05$ vs steroid-naïve control groups; *$p<0.01$ vs. control and □$p<0.01$ vs. scr groups)



Apoptosis following Hsp72 siRNA treatment. Hsp72 silencing RNA (siRNA) was used in control (*C*), cigarette smoke extract (*CSE*) treated and CSE plus dexamethasone 10 μM/μl (DEX (10) co-treated A549 alveolar epithelial cells. Scrambled (Scr)-RNA was used similarly as negative control for the transfection. After silencing Hsp72 apoptosis was measured by annexin V/propidium iodide staining using flow cytometric analysis. Decrease in Hsp72 protein after siRNA treatment was associated with significant increase of apoptotis following CSE treatment. Apoptosis in the steroid naïve control group (*C*) is expressed as 1; relative changes are presented for all other groups (arbitrary unit). (&$p<0.01$ vs CSE-treated groups; #$p<0.05$ vs steroid naïve control groups; *$p<0.01$ vs. control and □$p<0.01$ vs. scr groups)

In conclusion, our data confirmed that CSE induces apoptosis and necrosis in alveolar epithelial cells. DEX reduces CSE-induced cellular damage, by decreasing apoptosis. This is the first evidence of DEX-CSE interaction showing a key role of Hsp72 in alveolar epithelial cell survival. Our siRNA experiments confirmed that elevated Hsp72 is essential in the observed anti-apoptotic and protective effects of DEX following CSE exposure. Hsp72 might represent a new key molecule and a potential therapeutic target in smoke exposed lung cells. As millions of smokers are treated with glucocorticoids new data on cigarette smoke and glucocorticoid interaction are needed. Future experiments are necessary to evaluate the role of Hsp72 in smoker and non-smoker COPD patients, especially assessing the effects on alveolar destruction.

CLINICAL STUDIES:

Beside our basic science works we also examined some clinical problem. One of them is the role of HSP72 genetic polymorphism in renal transplant children. Our findings suggest an association between the carrier status of *HSPA1B* (1267)G with urinary tract malformations, leading to end-stage renal disease requiring kidney transplantation. This observation raises further questions about the clinical and therapeutic relevance of this polymorphism to pediatric nephrology.

Arterial stiffness (Ast) individually predicts cardiovascular (CV) mortality. Ast increases via vascular calcification and can be characterized by pulse wave velocity (PWV). We assessed the influence of mineral and bone metabolism on Ast in dialyzed (D) and renal transplanted (Tx) children by measuring fetuin-A and bone markers [bone-specific alkaline phosphatase (BALP); beta-CrossLaps (β)]. Normalized PWV/height (PWV/h) of 11 D and 17 Tx patients was measured by applanation tonometry. Levels of calcium (Ca), phosphate (P), fetuin-A, and bone markers were analyzed. Ca × P/fetuin-A ratio was calculated to characterize the balance of calcification and inhibition. Cumulative dose of calcitriol was also assessed. Fetuin-A was lower in D and Tx compared with healthy controls. Bone markers and Ca × P/fetuin-A of D were significantly higher than those of Tx and controls. In D PWV/h correlated with Ca × P/fetuin-A and BALP (r=0.8; p=0.005, r=0.6, p=0.05, respectively); BALP correlated with Ca × P/fetuin-A (r=0.7, p=0.01). In Tx, there was a correlation between calcitriol administered before transplantation and PWV/h (r=0.5, p=0.04). Increased bone turnover was coupled with an increased potential of calcium-phosphate precipitation, as shown by the increased Ca × P/fetuin-A. It might explain the connection between disturbed mineral and bone metabolism and Ast. Tx might be beneficial on Ast, though follow-up studies are needed.

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