Cell and Tissue Research (2019) 378:319–332
https://doi.org/10.1007/s00441-019-03073-z

REGULAR ARTICLE

Protective role of exogenous recombinant peroxiredoxin 6 under ischemia-reperfusion injury of kidney

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Received: 16 February 2019 / Accepted: 8 July 2019 / Published online: 31 July 2019

Abstract

Peroxiredoxin 6 (Prx6) is an important antioxidant enzyme with various functions in the cell. Prx6 reduces a wide range of peroxide substrates, playing a leading role in maintaining the redox homeostasis of mammalian cells. In addition to the peroxidase activity, a phospholipase A2-like activity was demonstrated for Prx6, which plays an important role in the metabolism of membrane phospholipids. Besides that, due to its peroxidase and phospholipase activities, Prx6 participates in intracellular and intercellular signal transduction, thus triggering regenerative processes in the cell, suppressing apoptosis caused by various factors, including ischemia-reperfusion injuries. A nephroprotective effect of exogenous recombinant Prx6 administered before ischemia-reperfusion injury was demonstrated on an animal model. Exogenous Prx6 effectively alleviates the severeness of renal ischemia-reperfusion injuries and facilitates normalization of their structural and functional conditions. Infusion of exogenous Prx6 increases the survival rate of experimental animals by almost 3 times. Application of exogenous Prx6 can be an effective approach in the prevention and treatment of renal ischemia-reperfusion kidney lesions and in preserving isolated kidneys during transplantation.

Keywords Peroxiredoxin 6 · Oxidative stress · Kidney · Ischemia-reperfusion injury

Introduction

A constant supply of oxygen and nutrients and efflux of metabolism products is required for maintaining the normal functioning of the living tissue. Reduced blood flow (ischemia) leads to rapid progression of pathological processes including tissue hypoxia, tissue acidification (due to the accumulation of lactate), impaired membrane permeability, decreased function of ion channels and reduced ATP levels, which ultimately leads to damage of metabolically active tissues. Restoration of the flow of blood saturated with oxygen (reperfusion) in tissues affected by ischemia leads to an even more dramatic increase of the ROS level and a development of oxidative stress (Kalogeris et al. 2012; Granger and Kvietys 2015). Currently, ischemia-reperfusion (I/R) injury is well-known as a key factor of development of many pathological conditions (Eltzschig and Eckle 2011). Particularly, these pathologies include acute kidney injuries that occur due to abnormalities of the blood vessels (renal artery stenosis, thrombosis, etc.), abdominal trauma (mechanical, chemical, etc.) and after abdominal surgery (Uchino et al. 2005; Waikar et al. 2008). Despite advances in the development of preventive and therapeutic methods, the risk of progression of complications negatively affecting the functional state of I/R-injured organs and thus decreasing the life quality and lifetime of the patients still remains high (Hobson et al. 2009; Wald et al. 2009). As the pathogenesis of I/R injuries is related in part to oxidative stress, the main direction of the therapy of such pathologies is suppression of elevation of the ROS level in the injured tissues by antioxidant preparations. One of the promising approaches in the prevention and treatment of I/R injuries is the use of antioxidant enzymes, which display higher efficacy when compared to low-molecular antioxidants of both natural and synthetic origin

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(Maksimenko and Vavaev 2012; Maksimenko 2016). Among the large diversity of enzymes with antioxidant activity, the family of peroxiredoxins (Prxs) is of the greatest interest, because they can play other important roles, acting like chaperones, signaling and regulatory molecules, in addition to their ability to neutralize a wide range of ROS (Ishii 2015; Rhee 2016). So far, 6 types of Prxs have been identified in mammals, which could be divided by the number of conservative cysteine residues in the active site and catalytic mechanism into typical 2-Cys (Prx1–4), atypical 2-Cys (Prx5) and 1-Cys (Prx6) (Wood et al. 2003; Sharapov et al. 2014). Prx1–6 play an important role in maintaining the redox homeostasis in mammalian organism, their level being typically elevated during progression of pathologies accompanied by oxidative stress development, which facilitates normalization of the ROS level in damaged tissues (Hanschmann et al. 2013). Among the peroxiredoxins, Prx6 is of the highest interest due to its ability to neutralize the widest spectrum of peroxide substrates of both inorganic and organic nature, including alkylhydroperoxides, phospholipid peroxides, long-lived protein radicals, peroxynitrite, etc. (Perkins et al. 2014; Fisher et al. 2018; Sharapov et al. 2019). Mice with a knockout for the *PRDX6* gene have high sensitivity to the action of oxidative stress, which results in an elevation of oxidative damage in their organs and tissues, despite a normal expression level of the genes encoding other antioxidant enzymes (Wang et al. 2003). Besides the peroxidase activity, Prx6 displays an activity of Ca^{2+}-independent phospholipase A2 (aiPLA2), which is most active in acidic conditions (pH 4–5) and plays an important role in the metabolism of phospholipids and transduction of intracellular and intercellular signals (Kim et al. 2008; Fisher 2018). Thus, Prx6 is a unique bifunctional enzyme participating in many cellular processes and performing a key protective antioxidant function in the cell (Fisher 2017). Particularly, the protective role of endogenous Prx6 has been shown in pathologies of different organs and tissues, such as the skin (Kümin et al. 2006; Zhang et al. 2014), lungs (Manevich and Fisher 2005; Wang et al. 2008) and upper airway (Novoselov et al. 1999), liver (Eismann et al. 2009), kidneys (Sorrell et al. 2015), eyes and neural system (Tulsawani et al. 2010). Moreover, high therapeutic activity of exogenous recombinant Prx6 has been demonstrated earlier in several animal models, in particular, a model of whole-body X-ray irradiation (Sharapov et al. 2017b), chemical burn of the upper airway (Volkova et al. 2014), I/R injuries of the intestine and mesenteric vessels of rats (Gordeeva et al. 2015; Sharapov et al. 2017a). In this work, the protective effect of exogenous Prx6 was studied in a mouse model of renal ischemia-reperfusion injuries; the possible mechanisms are discussed.

Materials and methods

Isolation of the enzymes

Genetic constructions encoding Prx6 and Prx6-C47S enzymes were obtained and expressed earlier in *E.coli BL21(DE3)* cells (Sharapov et al. 2009). Recombinant proteins harbored His-tag, so the enzymes were purified by affinity chromatography on Ni-NTA-agarose (Thermo Fisher Scientific, USA), according to the manufacturer's recommendations. The technique of protein isolation was described earlier (Sharapov et al. 2009). According to electrophoresis in 10% SDS-PAAG, the purity of the obtained enzymes was at least 95%.

Determination of peroxidase activity of the enzymes

The ability of the enzymes to cleave hydrogen peroxide (H_2O_2) and tert-butyl-hydroperoxide (t-BOOH) was determined by Kang's method (Kang et al. 1998), with minor modifications. Peroxidase activity of recombinant Prx6 was 220 nmol/min/mg of protein (measured with H_2O_2) and 95 nmol/min/mg of protein (measured with t-BOOH). In a similar way, an assessment was performed for the mutant protein Prx6-C47S, which does not possess peroxidase activity.

Animals

The studies were carried out in *BALB/c* mice (8 weeks old, weight 25–30 g) (vivarium of ICB RAS, Pushchino). The animals had ad libitum access to food and water but the access to food was restricted 24 h before surgery.

Mouse model of renal ischemia-reperfusion injury

The animals were anesthetized by intramuscular injection of Zoletil-100 (Virbac Sante Animale, France) and Rometar-20 (Bioveta, Czech Republic) in 0.9% NaCl solution (40 μg and 7 μg per 1 g of body weight, respectively). The duration of anesthesia was 1.5–2 h. Operations in animals were carried out according to the procedure described in the work (Wei and Dong 2012), with minor modifications. After the beginning of anesthesia, small lateral incisions of skin and muscular layers on both sides were made, opening the access to renal arteries and veins. Then, the left and right renal arteries and veins were clamped simultaneously, which caused the blockage of blood flow to and from the renal tissues, i.e., ischemia. A visible sign of ischemia onset was a change in the kidney color from pale pink to deep purple. Duration of ischemia was 30 min; after that, the clamps were removed for restoration of circulation in the tissue (reperfusion stage). The beginning of reperfusion is accompanied by a change of the kidney color from deep purple to pale red. The lateral incisions were sutured and washed with an antiseptic liquid. After the operation, the animals were

provided with food and water ad libitum. Twenty-four hours and 72 h after the operation, the animals were killed by decapitation and kidneys were excised and divided into three parts according to the method (Rosenblum 2013).

To test the therapeutic effect of exogenous Prx6 and Prx6-C47S proteins, the solution of the corresponding recombinant protein was injected into the tail vein to a final concentration of 20 μg/g of bodyweight 15 min before the beginning of ischemia. Selection of the injection method and the concentrations of protein solutions were carried out according to previous studies (Sharapov et al. 2016).

Histological analysis of renal tissue

The kidney tissues were fixed in 10% formaldehyde solutions, followed by dehydration of samples in an increasing gradient of ethanol concentration and enclosure into paraffin. Three-micron paraffin sections were prepared on a microtome (Thermo Electron Corparation, USA). The obtained sections were stained with hematoxylin and eosin (Biovitrum, Russia). Histological analysis was carried out on a Leica DM6000 microscope (Leica, Germany). Typically, 15–20 fields were inspected for each section of 3 different slides, at 200–500-fold magnification.

Electrophoresis and immunoblotting

To estimate the circulation time of exogenous Prx6 in the animal blood, 1 mg of Prx6 was introduced intravenously into three male Kv-SHK mice and the blood was subsequently sampled (~ 50 μl) after 10, 60, 120, 240 and 360 min for analysis of changes in the Prx6 content. Exogenous recombinant Prx6 contained a His-tag on the C-terminus, which enabled to specifically trace the presence of the recombinant protein. To determine the changes in caspase-3 levels in kidney tissues, about 40 mg of tissue was collected. Kidney and serum protein samples were separated by electrophoresis in 10% PAAG using a Mini Vertical Unit (Amersham, Great Britain) and transferred onto PVDF Hybond-P membrane (Amersham, Great Britain) using a TRANS-BLOT SD semi-dry transfer unit (Bio-Rad, USA). The following primary antibodies were used: monoclonal rabbit anti-His antibodies (1:1000, #12698, Cell Signaling technology, USA); rabbit monoclonal antibody for caspase 3 (1:1000, 9H19L2, Thermo Fisher Scientific, USA); and rabbit antibody for β-actin (1:1000, #4967, Cell Signaling, USA). Secondary goat antibodies against rabbit immunoglobulins, conjugated with horseradish peroxidase (1:1000, P-GAR Iss, IMTEK, Russia), were used for immunoblotting in accordance with the manufacturer's recommendations. The detection was carried out using diaminobenzidine (Amresco, USA). Densitometry was implemented using ImageJ software (www.imagej.nih.gov). Data were normalized to β-actin.

Gene expression level analysis in renal tissue

Gene expression level was determined by reverse transcription and real-time PCR. Total RNA was isolated from tissue samples with ExtractRNA reagent (Evrogen, Russia). RNA quality was estimated electrophoretically in 2% agarose gel. RNA concentration was determined using a NanoDrop 1000c spectrophotometer (USA). Two micrograms of total RNA was used per reverse transcription reaction with MMLV reverse transcriptase and standard dT_{15} oligonucleotide (Evrogen, Russia). Synthesized cDNA was used for PCR with gene-specific primers (Table 1). Real-time PCR was carried out using DNA amplifier DTlite (DNA-technology, Russia) with qPCRmix-HS SYBR kit (Evrogen, Russia), where SYBR Green II is used as an intercalating dye. The PCR cycling regime was as follows: (1) "hot-start" 95 °C, 5 min; (2) denaturation 95 °C, 15 s; and (3) primer annealing and DNA synthesis at 60 °C, 30 s. Stages (2) and (3) were repeated 40 times. The threshold cycle (Ct) value was determined using DTmaster software (DNA-technology, Russia). The signal was normalized to that obtained for the gene of cytoskeletal protein beta-actin (Actb). ΔΔCt was calculated by the formula ΔΔCt = ΔCt (control) − ΔCt (experiment); every ΔCt value was calculated by the formula ΔCt = Ct (gene of interest) − Ct (Actb) (Schmittgen and Livak 2008).

Determination of MDA level in kidney tissues

The level of malonic dialdehyde (MDA) was determined by a standard technique with thiobarbituric acid (TBA). To a 20–30 mg tissue sample, 450 μl of 1% H_3PO_4 and 150 μl of 0.8% TBA were added and the mass was homogenized with a Teflon pestle. Then, the mixture was heated on a boiling water bath for 45 min. After cooling, 380 μl of n-butanol was added and mixed thoroughly. The layer of n-butanol was separated by centrifugation. The optical density of water phase was measured at 546 nm with a Multiskan instrument (Labsystem Plus, Finland).

Biochemical blood analysis

Blood samples were collected from the animals of control and experimental groups before I/R injury, 24 h and 72 h after I/R injury of the kidneys. Biochemical blood analysis was performed using a biochemical express analyzer Reflotron Plus (Roche Diagnostics, Switzerland) according to the manufacturer's instructions.

Statistical data analysis

Statistical data analysis was carried out in a SigmaPlot 11 software package (Systat Software Inc.). Intragroup statistical differences were determined with one-way ANOVA analysis

Table 1 Oligonucleotides used for qRT-PCR. The design of oligonucleotides was carried out in Primer-BLAST (www.ncbi.nlm.nih.gov). Calculated T_m for all primers was 61–63 °C. Real-time PCR was performed at T_m = 60 °C. PCR products were melted from 60 to 90 °C to assess the specificity of the reaction. In addition, amplicon sizes were checked by electrophoresis in 10% PAAG

Genes	GenBank accession number	Oligonucleotides 5′-3′ (F + R)	Amplicon size (bp)
bAct	NM_007393.4	CCTTCCTTCTTGGGTATGGAATCC CACCAGACAGCACTGTGTTG GCA	115
CASP3	NM_009810	AAGGAGCAGCTTTGTGTGTG GAAGAGTTTCGGCTTTCCAG	145
eNOS	NM_021838.2	GAACCTGAGGGTGCCCAG TCCGATTCAACAGTGTCTCCT	71
iNOS	NM_012611.3	GCTACACTTCCAACGCAACA CATGGTGAACACGTTCTTGG	115
IL-6	NM_031168	TAGTCCTTCCTACCCCAATTTCC TTGGTCCTTAGCCACTCCTTC	76
IL-18	NM_008360.1	GTGTTCCAGGACACAACAAG CTTCCTTTTGGCAAGCAAGA	74
NF-κB	NM_008689	CCACGCTCAGCTTGTGAGGGAT GGCCAAGTGCAGAGGTGTCTGAT	106
NRF2	NM_010902	CTCGCTGGAAAAAGAAGTG CCGTCCAGGAGTTCAGAGG	240
AP-1	NM_010591	TGGGCACATCACCACTACAC TCTGGCTATGCAGTTCAGCC	119
KIM-1	NM_001166632.1	TTGCCTTCCGTGTCTCTAAG AGATGTTGTCTTCAGCTCGG	225

and statistical significance between individual experimental groups was determined using unpaired Student's t test. $p < 0.05$ was considered statistically significant. The results were presented as mean value ± standard deviation (SD).

Results

Estimation of Prx6 circulation time in the blood flow of animals

To prove that the possible Prx6 effects during I/R injury were caused by its presence in the animal organs, blood serum immunoblotting was carried out to estimate the changes in the amount of the recombinant enzyme in the blood flow in the initial period (10 min), as well as a long time after intravenous administration (Fig. 1). Figure 1 shows that in the first hour after intravenous injection of Prx6, around 80% of the initial amount of the protein is present in the blood. The content of exogenous Prx6 decreases with time, by about 3 times in 6 h. The mutant form Prx6-C47S displayed similar results (data not shown). Thus, exogenous recombinant proteins Prx6 and Prx6-C47S were present in the animal blood during the whole period of ischemia (30 min) in the amount at least 80–90% from the initial content and during the following reperfusion period, a significant part of the administered protein circulated in the peripheral blood for at least the first 6 h. It has been earlier shown that the initial period of kidney damage during I/R is the first 4–6 h (Williams et al. 1997) and peak damage is reached 24 h after I/R, which is a good criterion for estimating the protective properties of exogenous peroxiredoxins.

Survival of animals after I/R preceded by administration of Prx6 and Prx6-C47S

At the first stage of the study, the survival of animals during 5 days after I/R and I/R preceded by administration of recombinant Prx6 and Prx6-C47S 15 min before ischemia was tested

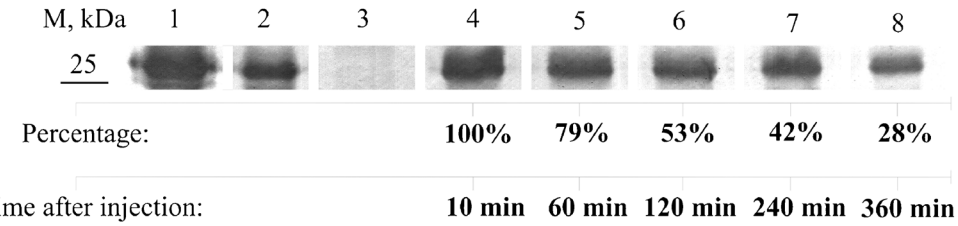

Fig. 1 Immunoblotting of mouse blood serum after intravenous Prx6 injection. 1 and 2 pure preparation of recombinant Prx6 (500 and 100 ng, respectively); 3 blood plasma of control animals which did not receive Prx6; 4–8 blood plasma samples of mice 10, 60, 120, 240 and 360 min after intravenous injection of 1 mg of Prx6

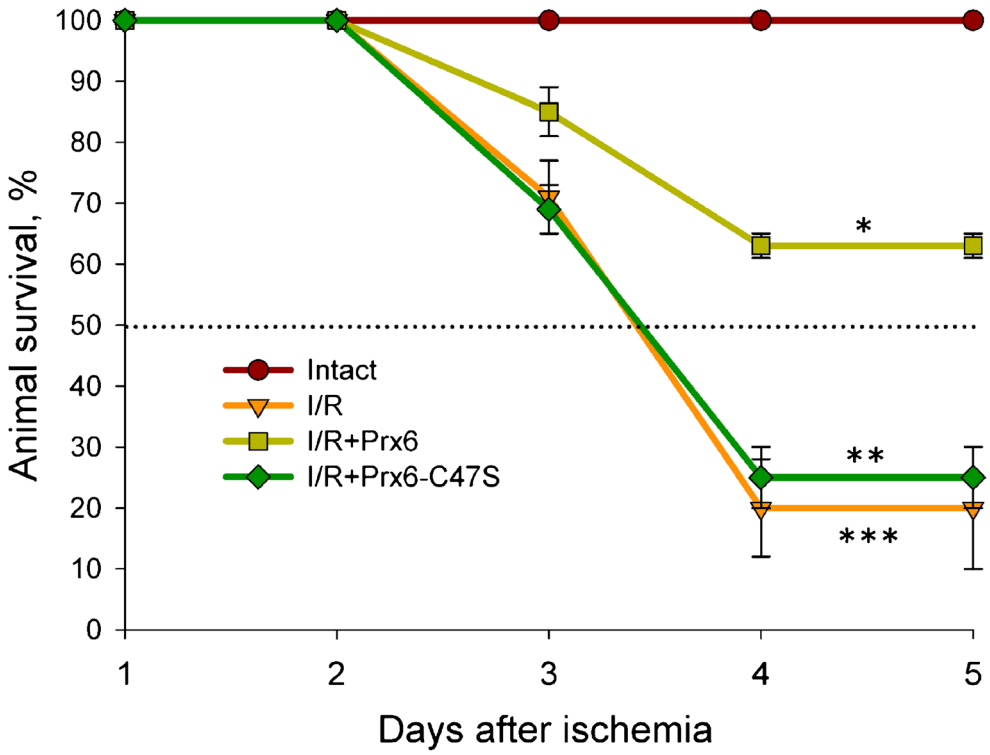

Fig. 2 Survival of mice during 5 days after 30 min ischemia of both kidneys and subsequent reperfusion and after injection of recombinant enzymes, Prx6 and Prx6-C47S, 15 min before ischemia ($n = 30$ for each group). *$p < 0.05$; **$p < 0.05$; ***$p < 0.05$

(Fig. 2). Figure 2 shows that following 72–120 h in the groups with I/R or Prx6-C47S injection before I/R, the survival was 20% and 25%, respectively. In the group where Prx6 was administered before I/R, the survival was more than 60%. Hence, the first 72 h after I/R injury of both kidneys was the critical period for the survival of animals. It is necessary to note that the changes in the kidney mass were observed 24 h and 72 h after I/R preceded by administration of recombinant Prx6 and Prx6-C47S (Table 2). It can be seen from Table 2 that 24 h after ischemia in the I/R group and the I/R group that received Prx6-C47S, an increase of kidney mass by 1.5–1.6 times was observed, which is a symptom of acute renal edema. In the Prx6-treated group, the kidney mass was increased insignificantly, by about 1.1 times compared to the control group. In 72 h, the kidney mass in the I/R group and the I/R group injected with Prx6-C47S before I/R was decreased 1.1- and 1.25-fold, respectively. In the group with Prx6 administration, the kidney mass did not change and remained at the same level, corresponding to normal values for intact animals.

Table 2 Changes in mass of mice kidneys 24 h and 72 h after I/R injury of both kidneys preceded by administration of recombinant proteins, Prx6 and Prx6-C47S

Group of animals	Change of kidney mass compared to intact group (%)	
	24 h	72 h
Control	100	100
I/R	150 ± 10*	90 ± 10
I/R + Prx6	110 ± 5*	110 ± 9
I/R + Prx6-C47S	140 ± 10*	90 ± 10

*$p < 0.05$ in comparison with control

Histological analysis of renal tissues after I/R injury and I/R preceded by administration of Prx6 and Prx6-C47S

It is known from the literature that renal function remains normal provided that at least 50% of nephrons are preserved (Baum et al. 1975). Histological analysis was carried out to find out what morphological alterations are caused by I/R and preceding administration (15 min before I/R) of recombinant Prx6 and Prx6-C47S before 30 min ischemia and after 24 and 72 h reperfusion (Fig. 3). Morphological analysis of histological sections of the control group showed that 30 min ischemia and subsequent 24 h reperfusion of mice kidneys leads to hyperemia of interstitium and capillary loops of glomerulus. Dystrophic lesions were observed in the epithelium of renal tubules, mainly hydropic dystrophy with disruption of apical segments of epitheliocytes. Necrosis and desquamation of tubular epithelial cells were observed in some convoluted tubules. The lumen of the tubules was filled with protein debris (Fig. 3a). Seventy-two hours after restoration of circulation in the kidneys, moderate hyperemia of capillary loops of glomeruli was still observed. In convoluted tubule epithelium, ballooning degeneration of the cells with destruction of their apical segments was revealed. Clearly visible eosinophilia of the epithelial cytoplasm was probably due to massive protein

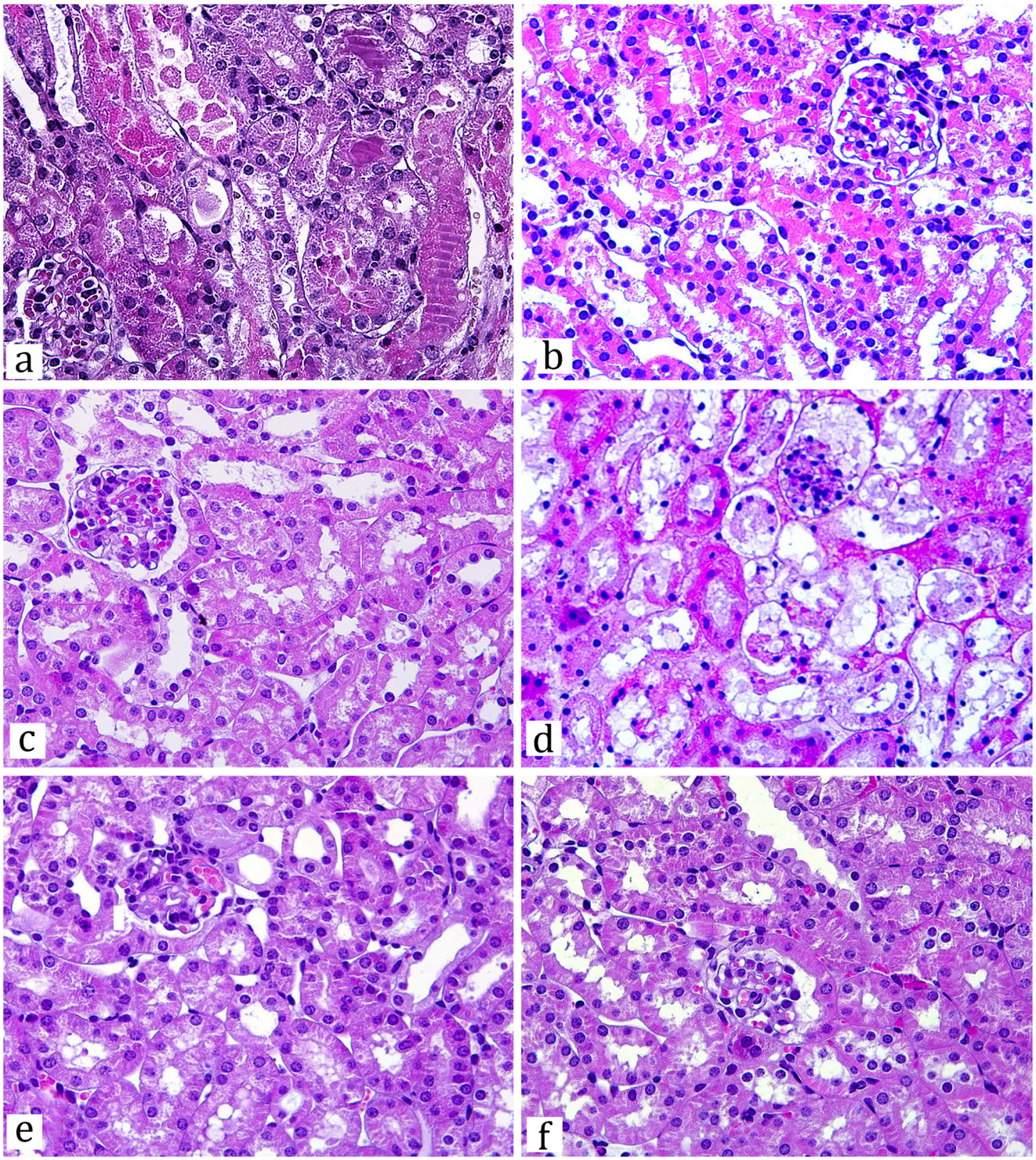

Fig. 3 Structure of the cortical layer of mouse kidney after infusion of recombinant peroxiredoxins followed by ischemia-reperfusion. Duration of ischemia was 30 min, duration of reperfusion was 24 h (**a**) and 72 h (**b**), without treatment. After intravenous injection of Prx6-C47S 15 min before 30 min ischemia, duration of reperfusion was 24 h (**c**) and 72 h (**d**). After intravenous injection of Prx6 15 min followed by 30 min ischemia, duration of reperfusion was 24 h (**e**) and 72 h (**f**). Staining: hematoxylin-eosin. Magnification: × 500

denaturation. Dilated lumens of convoluted tubules of the kidneys were caused by destructive changes in epithelial cells (Fig. 3b). Dystrophic changes without destruction of the cells were observed also in the epithelium of straight tubules.

Administration of Prx6-C47S protein 15 min before 30 min ischemia and subsequent 24 h reperfusion did not prevent hyperemia of glomeruli and interstitium of kidney tissue. Dystrophic alterations in convoluted tubules were clearly

visible (Fig. 3c). After 72 h of reperfusion, venous hyperemia, and dystrophic and degenerative lesions of epitheliocytes with a clear eosinophilia of damaged cells remained in the renal tissue. Accumulation of protein liquid containing tissue debris and desquamated epithelial cells was noted in dilated convoluted tubules. Protein liquid accumulation was also revealed under Bowman's capsule of renal glomeruli (Fig. 3d).

Application of Prx6 enzyme 15 min before 30 min ischemia and subsequent 24 h reperfusion was characterized by milder manifestations of venous stasis in the cortical and medullar layers of kidney parenchyma. Dystrophic changes in the epithelium of convoluted tubules were also revealed but their degree was significantly lower compared to the control I/R group and group pretreated with Prx6-C47S before I/R. Ballooning degeneration and apical destruction of the epithelium of convoluted tubules were noted in single cells, while it was not observed in straight tubules. The lumens of renal tubules were not dilated and they were free of protein liquid and tissue debris (Fig. 3e). After 72 h reperfusion period, the signs of venous stasis were not revealed in the renal interstitium or they were minimal. The epithelium of convoluted tubules was also characterized by minimal manifestations of hydropic and protein dystrophy. Destructive changes of epithelium were not detected (Fig. 3f).

Overall results of histological analysis of renal tissue after I/R and pretreatment with Prx6 and Prx6-C47S enzymes before I/R are summarized in Table 3. Based on histological analysis of the cortical layer of the kidney 24 h and 72 h after I/R, we can conclude that in the group treated with recombinant Prx6 before I/R injury, the degree of renal tissue damage was significantly lowered (compared to the control I/R group and group administered with Prx6-C47S).

Biochemical analysis of animal blood after I/R injury preceded by injection of Prx6 and Prx6-C47S

It is well-known that creatinine and urea content in the blood reflects physiological conditions of the kidney. Urea (Table 4) and creatinine content (Table 5) in the blood of animals after I/R alone and I/R preceded by administration of Prx6 and Prx6-C47S was measured 1, 2 and 3 days after I/R injury, according to the study (Gowda et al. 2010).

Twenty-four hours after I/R, an increase of urea concentration by 5 times and of creatinine concentration by 6 times was observed in the blood compared to the control group (Tables 4 and 5). An analogous result in a similar animal model has been achieved by other groups of investigators (Hesketh et al. 2014; Yu et al. 2016). In groups with animals pretreated with Prx6-C47S, the urea concentration was 4 times higher and the creatinine level was more than 5-fold higher than the physiologically normal level. The lowest values of urea and creatinine concentrations were registered in the group pretreated with Prx6. After 48 h following I/R, a noticeable more than 2-fold decrease in the concentration of urea and creatinine was observed in all the groups. After 72 h, the urea content in all the groups was stabilized at the level slightly above physiological norm. The concentration of creatinine in the I/R group and group pretreated with Prx6-C47S was more than 2 times higher than the physiologically normal value. In the group pretreated with Prx6, this value was 1.7-fold higher than in the normal one. Based on the obtained data, we can conclude that administration of Prx6 before I/R provides significant preservation of normal renal function.

MDA level in kidney tissues after I/R preceded with Prx6 and Prx6-C47S administration

As I/R of organs is accompanied by oxidative stress, elevation of lipid peroxidation is observed in the damaged tissues. One of the finite products of this process is malonic dialdehyde (MDA). MDA content was analyzed in animal renal tissues 24 h after I/R alone or with pre-administration of Prx6 and Prx6-C47S before I/R (Fig. 4).

As seen from the obtained data, application of Prx6 significantly alleviates lipid peroxidation in kidney tissues. It should be emphasized that Prx6-C47S application has no influence

Table 3 Evaluation of morphometric parameters of the kidney tissues after I/R injury preceded by administration of recombinant enzymes Prx6 and Prx6-C47S before I/R. Histological changes were ranked by the following scale: (−) normal, (+) mild changes, (++) intermediate changes and (+++) prominent changes

Parameter	Control		I/R		I/R + Prx6		I/R + C47S	
	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h
Dilation of Bowman's capsule	−	−	+	++	+	+	+	++
Interstitial hyperemia	−	−	++	++	++	++	++	++
Interstitial infiltrate	−	−	+	+	+	+	+	+
Stasis in blood vessels	−	−	++	++	+	−	++	++
Dystrophy of convoluted tubules	−	−	++	+++	+	+	++	++
Dystrophy of straight tubules	−	−	+	+	−	−	+	+
Dilation of convoluted tubules	−	−	+	+	−	−	+	+
Desquamation of epithelial cells	−	−	++	+++	+	−	++	++
Disruption of epithelial cells	−	−	+++	+++	+	−	++	++

Table 4 Urea concentration in blood of mice during 3 days after I/R injury of both kidneys

Group of animals	Urea concentration (mg/dl)		
	24 h	48 h	72 h
Control	57 ± 9	54 ± 7	56 ± 8
I/R	290 ± 40*	125 ± 10*	80 ± 10*
I/R + Prx6	77 ± 15$^{\#}$	65 ± 6$^{\#}$	60 ± 5
I/R + Prx6-C47S	270 ± 50*	120 ± 10*	62 ± 6

*$p < 0.05$, in comparison with control; $^{\#}p < 0.05$, in comparison with I/R

on peroxidation during renal I/R injury, which demonstrates the leading role of peroxidase activity of Prx6 in the protection of kidney tissues from I/R injury.

Estimation of gene expression level in renal tissues after I/R injury or I/R injury preceded by injection of Prx6 and Prx6-C47S

To understand the molecular mechanisms of the protective action of exogenous Prx6, an analysis of changes in the expression of certain marker genes was carried out (Table 1). Table 6 lists data on marker gene expression level in renal tissue 24 h after I/R alone or I/R and pre-administration of Prx6 and Prx6-C47S. It must be noted that Table 6 contains only data on genes that demonstrated a reliable alteration of their expression level. It is also important to note that 48–72 h after I/R, the gene expression values are normalized, being close to values for intact animals (data not shown).

Kidney injury molecule-1 (KIM-1) is an acknowledged sensitive marker of kidney damage (Bonventre 2014). Actually, analysis of KIM-1 expression alteration in the I/R group and I/R group pretreated with Prx6-C47S demonstrated its increase by 60–100 times, which could be evidence for damage of kidney tissue of different degrees. In the group pretreated with Prx6, the KIM-1 level was 26 times higher than in the control but 2–5 times lower than in the previously mentioned groups. Such an effect testifies that pretreatment of

Table 5 Creatinine concentration in blood of mice during 3 days after I/R injury of both kidneys

Group of animals	Creatinine concentration (mg/dl)		
	24 h	48 h	72 h
Control	0.28 ± 0.02	0.28 ± 0.02	0.28 ± 0.02
I/R	1.8 ± 0.2*	1.1 ± 0.3*	0.7 ± 0.2*
I/R + Prx6	0.6 ± 0.1$^{\#}$	0.55 ± 0.1$^{\#}$	0.5 ± 0.1*
I/R + Prx6-C47S	1.6 ± 0.2$^{\#}$	0.8 ± 0.2$^{\#}$	0.6 ± 0.1*

*$p < 0.05$, in comparison with control; $^{\#}p < 0.05$, in comparison with I/R

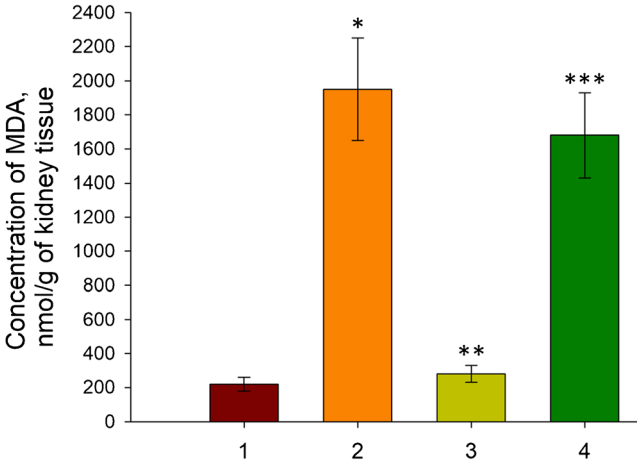

Fig. 4 MDA level in kidney tissues ($n = 5$ for each group). 1 intact mice; 2 24 h after ischemia-reperfusion; 24 h after ischemia-reperfusion injury preceded by infusion of Prx6 (3) and Prx6-C47S (4). *$p < 0.05$; **$p < 0.05$; ***$p < 0.05$

mice with Prx6 before I/R injury can really suppress damage of the kidneys after I/R.

Transcription factor NRF2 is the main transcription factor regulating the expression level of the genes of antioxidant response, thus playing a crucial role in maintaining the redox homeostasis of the tissues. During I/R injuries of mouse kidneys, a significant elevation of NRF2 expression was observed in the control group (by 7.5 times), being slightly weaker in the group with pre-administration of Prx6-C47S before I/R (by 5.5 times). Surprisingly, despite the significant elevation of NRF2 expression, a significant (by more than 2 times) expression change of the genes encoding antioxidant enzymes could not be detected in these groups (Table 1). Administration of Prx6 before I/R injury normalized the NRF2 level to almost the same values as in intact animals.

Table 6 Change of gene expression level 24 h after I/R injury compared to intact animals, $n = 10$ for each gene. The statistical significance of changes in the level of gene expression was additionally determined using Rest 2005 (v. 1.9.12) software (Pfaffl et al. 2002).

Gene/group	Intact	I/R	I/R + Prx6	I/R + Prx6-C47S
KIM-1	1.0	106 ± 30*	26 ± 10*$^{\#}$	90 ± 20*
NRF-2	1.0	7.5 ± 0.8*	0.8 ± 0.2*$^{\#}$	5.5 ± 0.5*
NF-κB	1.0	5.5 ± 0.7*	2.0 ± 0.4$^{\#}$	8.0 ± 0.6*
IL-6	1.0	2.5 ± 0.8*	8.0 ± 1.0*$^{\#}$	6.0 ± 1.5*
IL-18	1.0	7.0 ± 1*	1.4 ± 0.6$^{\#}$	3.0 ± 1.0*
iNOS	1.0	14 ± 3*	2.6 ± 1.0*$^{\#}$	5.8 ± 1.5*
eNOS	1.0	22.0 ± 4*	2.1 ± 0.8*$^{\#}$	5.0 ± 1.0*
AP-1	1.0	7.5 ± 0.8*	3.0 ± 0.2*$^{\#}$	6.5 ± 1.0*
Caspase-3	1.0	4.5 ± 0.7*	0.4 ± 0.1$^{\#}$	3.5 ± 0.5*

*$p < 0.05$, in comparison with intact control; $^{\#}p < 0.05$, in comparison with I/R

The expression level of transcription factor NF-κB after I/R injury was elevated by 5–8 times, which could be evidence for its activation aimed to restore normal homeostasis in kidney cells and to provide their better survival. In this context, a noticeable increase of the expression level of IL-6 (2.5–6 times) and IL-18 (3–7 times) genes was observed, which probably relates to stimulation of the immune response. Pre-administration of Prx6 decreased the NF-κB and IL-18 level by 2–3 times compared to the first two groups. This can be related to scavenging of ROS and normalization of cell homeostasis caused by Prx6 injection. It is interesting to note that Prx6 injection before I/R caused a slight activation of IL-18 level and, in contrast, a noticeable elevation of IL-6 expression (by 2–8 times) compared to the control group. Besides its pro-inflammatory effect, IL-6 is known to stimulate regenerative processes in the cell (Scheller et al. 2011).

The expression level of the genes of nitrogen oxide synthases (inducible iNOS and endothelial eNOS) was strongly elevated during I/R injury, iNOS by 14 times and eNOS by 22 times, which led to an increase of NO content in the blood, probably as an adaptive response. Such a drastic elevation of iNOS and eNOS expression was not observed when Prx6 was pre-administered, though their level was higher than in the control (by 2–5.5 times).

The obtained data testify an elevation of transcription factor AP-1 (apoptosis regulator) expression level by 6–8 times in the I/R group and I/R group pretreated with Prx6-C47S. In the same groups, the expression level of effectory caspase-3 (Casp-3) was increased by 3–5 times. Such elevation can be related to the observable increase of apoptotic cell death in kidney tissue. In the group pre-treated with Prx6, AP-1 expression was increased by 3 times, whereas the Casp-3 level was even 2 times lower than the control values, which evidences an alleviation of apoptotic cell death. To confirm that increased Casp-3 expression was indeed related to its induction in renal tissue and apoptosis triggering, immunoblotting of kidney tissues was carried out 24 h after I/R alone and I/R preceded by injection of Prx6 and Prx6-C47S enzymes (Fig. 5).

Figure 5 demonstrates that caspase-3 was activated by almost 2 times in the I/R group and I/R group with pre-administered Prx6-C47S compared to the control group. This fact can be indicative of elevated apoptotic death of the cells in kidneys of these experimental groups. Injection of Prx6 before I/R suppressed activation of caspase-3 and its quantity in the cells was similar to that in the control group.

Discussion

The presented data give evidence on the ability of preparatory injection of antioxidant enzyme Prx6 before I/R injury to significantly decrease the degree of kidney damage. This is expressed in decreased animal mortality, prominent preservation of tissue morphology in both kidneys and filtration capacity of the kidneys by creatinine and urea (Fig. 2, Tables 4 and 5). It should be specially noted that these Prx6 effects are mostly related to its peroxidase activity allowing exogenous Prx6 to neutralize oxidative stress developed during I/R injuries of organs. This is confirmed by noticeable alleviation of MDA level in renal tissue in the presence of exogenous Prx6. At the same time, mutant Prx6-C47S lacking peroxidase activity displayed nearly no protective properties. These results are in concordance with the data on the protective role of Prx6 during I/R injuries of the intestine (Gordeeva et al. 2015; Sharapov et al. 2017a).

In the control groups of animals, after the first day of I/R injury of kidneys, venous hyperemia, dystrophic changes in epithelial cells, mainly in convoluted tubules and necrosis of separate epithelial cells were observed in the damaged tissues. Desquamated cells and tissue debris in the lumens of the tubules altered urodynamics. Venous hyperemia persisted at the third day after ischemia; the severity of damage of the epithelium in convoluted tubules was increasing. More and more epithelial cells displayed denaturation of cytoplasmic proteins (clear eosinophilia of the cytoplasm). Protein liquid is accumulated in the lumen of the tubules and under Bowman capsule. Morphological studies including quantitative analysis showed that pre-administration of recombinant Prx6-C47S did not significantly affect the state of the renal tissue after 24-h and 72-h periods of reperfusion following ischemia, the conditions being quite similar to I/R injury without treatment. Administration of Prx6 enzyme was notably effective with regard to correction of I/R injuries. Under the action of this preparation, there was no apparent hyperemia in the kidney interstitium and capillary loops of glomeruli 24 h after ischemia. Dystrophic changes of the epithelium of convoluted tubules were less expressed; no destructive changes were detected in epithelial cells, as well as no protein mass accumulation in the lumens of renal tubules. By the end of the third day, no destructive changes of epithelial cells could be revealed. A decrease of protein and hydropic dystrophy of epithelial cells, which returned to their normal size, was noted. Normalization of the size of the convoluted tubule epithelium was reflected also in the lumen properties: neither dilation of tubules (caused mainly by destruction of the epithelium) nor their strong constriction (due to dystrophic "swelling" of the cells) was observed. Thus, Prx6 administration provides preservation of normal renal tissue morphology under I/R injury.

It is well-known that oxidases play an important role in the pathogenesis in ischemia-reperfusion of tissues. In particular, NADPH oxidases (NOX) largely determine tissue damage in ischemia-reperfusion by generating ROS in endothelial and vascular smooth muscle cells (Hilenski et al. 2004). NOX are bound to the cell membrane and generate superoxide into the extracellular space, which is converted into secondary

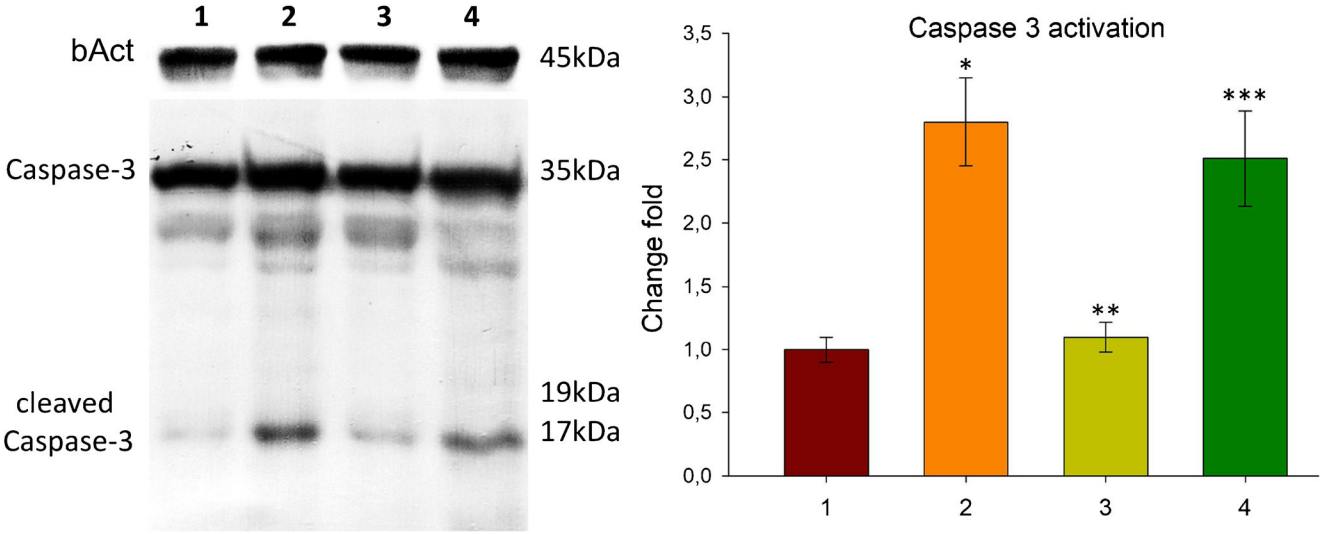

Fig. 5 Immunoblotting of kidney tissue for beta actin and caspase-3 (n = 5 for each group). 1 intact mice; 2 24 h after ischemia-reperfusion; 24 h after ischemia-reperfusion injury preceded by infusion of Prx6 (3) and Prx6-C47S (4). Data normalized to beta actin (45 kDa). The level of activation of caspase-3 was determined by the ratio of procaspase-3 (35 kDa) to cleaved caspase-3 (17–19 kDa). *$p < 0.05$; **$p < 0.05$; ***$p < 0.05$

ROS by enzymatic and non-enzymatic pathways. Xanthine oxidase (XO) is yet another important source of ROS in I/R injury. It is known that during ischemia of organs, a rapid transformation (with the involvement of ROS) of xanthine dehydrogenase into xanthine oxidase is observed (Wu et al. 2018). At the same time, it has been shown that XO is localized not only in the cytoplasm but also on the outer surface of the plasma membrane of endotheliocytes (Vickers et al. 1998) and during ischemia/reperfusion, the enzyme can be released from the liver and intestine into the general circulation and bind with glycosaminoglycans located on the surface of endothelial cells (Granger and Kvietys 2015). In addition, it has recently been shown that XO suppression reduces the severity of I/R-induced damage of the kidney in mice (Haga et al. 2017). Thus, extracellular ROS generated by NOX and OX can be neutralized by exogenous recombinant Prx6 introduced into the blood prior to I/R damage of the kidney.

As mentioned earlier, Prx6 is able to neutralize a wide range of hydroperoxides; thus, its administration before I/R can affect the level of peroxides in the body. At the time of oxidative stress development (the stage of reperfusion of the kidneys), Prx6 is predominantly found in the bloodstream of animals (Fig. 1). This raises a question of how exogenous Prx6 is able to neutralize ROS generated inside the cells into which it does not penetrate. In addition to passive diffusion through the membrane, hydroperoxides are known to be actively transported from cells into the extracellular space using aquaporins (Bienert et al. 2007; Tornroth-Horsefield 2010). Thus, being located in the extracellular space, exogenous Prx6 can participate in the elimination of peroxides formed not only in the intercellular space but also released from the cells.

I/R injury is accompanied by alteration of redox homeostasis in the cells, which leads to activation of redox-sensitive intracellular signaling molecules such as transcription factor NRF2 (Aminzadeh et al. 2013). Activation of NRF2 during renal I/R injury evidences an elevation of the expression level of genes encoding antioxidant enzymes via interaction with cis-regulatory element ARE (antioxidant responsive element). The main function of the NFR2/ARE regulatory system is the maintenance of intracellular redox homeostasis via regulation of expression of antioxidant response genes including peroxiredoxins (Godoy et al. 2011). Besides that, NRF2 interacts with other redox-sensitive transcription factors NF-κB and AP-1, thus influencing their transcription activity. A decrease of NRF2 expression was observed in the group pretreated with Prx6, which could be a sign of normalization of redox homeostasis of the tissues.

One of the key genes providing normal homeostasis in the cell in stress conditions and controlling the balance between their survival and death, when being activated, is transcription factor NF-κB (Pires et al. 2018). The expression level of this gene in the untreated I/R group was significantly elevated, which showed its activation in order to restore the normal homeostasis in kidney cells and provide their survival. For instance, NF-κB was shown to interact with N-terminal kinase c-Jun (JNK), lowering its activity and preventing necrotic and apoptotic death of the cells (Reuther-Madrid et al. 2002; Morgan and Liu 2011). Since NF-κB is a universal transcription factor, its activation can regulate expression of a large number of genes, including various interleukins (IL-6, IL-18, etc.) (Biet et al. 2002; Morgan and Liu 2011). Along with elevated NF-κB expression, a noticeable increase of IL-6 and IL-18 could be observed in the same groups. Elevated values

of IL-6 and IL-18 expression can be mediated by stimulation of the immune response of the cells and modulation of the activity of Th1 cells, cytotoxic T-lymphocytes, NK-cells, macrophages and dendrite cells (Biet et al. 2002; Scheller et al. 2011). Pretreatment with Prx6 lowers the expression levels of NF-κB and IL-18. This could be related to a decrease of ROS content and normalization of redox homeostasis caused by Prx6. However, some antioxidants, such as aspirin, sodium salicylate, indometacine, L-cysteine, N-acetylcysteine, vitamin E and its derivatives, have been shown to be capable of blocking NF-κB activation (Staal et al. 1990; Yamamoto et al. 1999; Palayoor et al. 1999; Acarin et al. 2000). Prx6 could exert a similar effect. In addition, Prx6 can influence the level of NF-κB via the TLR4/NF-κB signaling pathway. Peroxiredoxins mainly have intracellular localization upon damage of the cell plasma membrane and they enter the extracellular space and function as danger signaling molecules (DAMPs) (damage-associated molecular patterns), promoting activation of the immune system, triggering regeneration processes, stimulating angiogenesis, etc. (Shichita et al. 2012; Riddell et al. 2012; Vénéreau et al. 2015; Feldman et al. 2015). Recently, it has been shown that under ischemic damage of brain cells, Prx6 released due to cell disruption can act as an endogenous ligand of the TLR4 receptor (Kuang et al. 2014). The effect on the TLR4/NF-κB signaling pathway has also been shown for some other members of the peroxiredoxin family (Riddell et al. 2012). The interaction of Prx6 with TLR4 triggers a cascade of processes, in which NF-κB performs the main role, which allows initiating the processes of emergency cell repair and suppressing the development of apoptosis (Hellweg 2015). Moreover, we suggest that intravenous administration of recombinant Prx6 in animals 15 min before I/R injury may lead to a preconditioning effect. By the time of reperfusion and the maximum increase in the level of ROS, the cells have already started the mechanism of repair and antioxidant response under the effect of injected exogenous Prx6 (which is perceived as DAMP), through stimulation of the TLR4/NF-κB signaling pathway. Therefore, the subsequent stimulus, reperfusion, does not result in a synergistic increase in the expression of NF-κB. Thus, stimulation of the NF-κB signaling pathway, which occurs both through Prx6 binding to the TLR4 receptor and through the regulation of intracellular/extracellular ROS generated by I/R, allows protecting the cells from apoptosis, triggering inflammatory and repair processes and activating anti-apoptic factors (Hellweg 2015). Perhaps, the signal-regulatory function of exogenous Prx6 (stimulation of the TLR4/NF-κB signaling pathway) does not play a leading role in the protective action upon I/R injury of the kidney, since the mutant form Prx6-C47S (which has the same spatial structure as the wild-type Prx6 protein but does not possess peroxidase activity) is also able to interact with the TLR4 receptor but does not reduce the level of damage to the renal tissue and increase the survival of animals. We can assume that the activation of Prx6 in DAMP requires over-oxidation of the peroxidatic residue C47 (Cys-SH → SOH → SO_2H → SO_3H). It is known that the oxidation of the active site of Prx6 can affect protein conformation (Kim et al. 2016). In particular, the oxidation of Prx1–6 leads to the unfolding of the α-2 helix (on which the peroxidatic cysteine is located) and the formation of a flexible loop with oxidized C47-SOH (SO_2H, SO_3H) (Karplus and Hall 2007; Perkins et al. 2014). It has been shown that the formed loop with C47-SOH can interact with other proteins, for example with πGST (Zhou et al. 2013). In addition, it has been shown that over-oxidized peroxiredoxins change their tertiary and quaternary structure (Veal et al. 2017). In the oxidized state, Prx6 (in addition to dimers) can form high-molecular oligomeric structures (Sharapov et al. 2018; Chowhan et al. 2019), which can perform a signaling function in the extracellular space. In connection with the foregoing, it can be assumed that the mutant form of Prx6-C47S cannot be oxidized and may not function as a DAMP. At the same time, it should be noted that the role of Prx6 (Prx6-C47S) as a DAMP is currently not well understood and requires further research.

Despite the decrease of NF-κB and IL-18 levels, in groups of animals that received an injection of Prx6 before I/R, strong activation of IL-6 (by 2–8 times when compared to control) was shown. It is well-known that IL-6 has not only a pro-inflammatory effect realized via a trans-signaling mechanism but also an anti-inflammatory effect capable of activating regenerative reactions in the cell (Scheller et al. 2011).

A significant elevation of the expression level of the genes encoding nitrogen oxide synthases (inducible iNOS and endothelial eNOS) was observed in the I/R group. Elevation of iNOS and eNOS expression during I/R injury leads to increase of NO level in the blood via activation of the NF-κB/MAPK signaling pathways (Park et al. 2003). Increase of NOS synthesis, particularly eNOS, affects the severity of I/R injury and the time required for restoration (Shesely et al. 1996). For example, a number of studies have shown the role of nitrogen oxide (NO) in decreasing vessel thrombosis during the reperfusion period. Besides, NO prevents migration and aggregation of monocytes in the vessels and regulates tubuloglomerular feedback in the kidneys, which leads to change of tone ratio of afferent and efferent arterioles of the glomerulus, sodium excretion and regulation of the angiotensin level, which is an important element of the renin-angiotensin-aldosterone system, which regulates the vascular tone (Ishimura et al. 2002; Park et al. 2003). In the case of pre-administration of Prx6, such a dramatic elevation of iNOS and eNOS expression was not observed, though it was higher than in the control. Decreased iNOS and eNOS activation could be explained, as it was mentioned earlier, by the suppression of NF-κB activity by antioxidant enzymes used in the present study (Wang et al. 2002). However, eNOS inhibition has been shown to cause excess vasoconstriction, increased severeness

of post-ischemia conditions, enhancement of microvascular thrombosis and increased mortality (Ishimura et al. 2002).

Elevation of the expression level of transcription factor AP-1 is observed during I/R injury. AP-1 activation begins from the activation of apoptosis-regulating kinase-1 (ASK-1) in response to increasing levels of ROS in the cells via oxidation of thioredoxin (Trx), which inhibits it in normal conditions. The active form of ASK-1 provides activation of N-terminal kinase c-Jun (JNK) by its phosphorylation and this kinase subsequently interacts with transcription factor AP-1. AP-1 transcription factor can regulate various genes, such as FAsL, Bim, or Bcl3 (Shaulian and Karin 2002), which results in stimulation of proliferation and cell survival or their apoptotic death (Zhang et al. 2015). In concordance to that, increase of the effectory caspase-3 (Casp-3) level is observed along with elevation of AP-1 activity, which leads to increase of apoptotic death of the cells. Pre-administration of Prx6 before I/R decreases the ROS level in the cells, thus lowering the activation level of the ASK-1/JNK/AP-1 pathway and decreasing apoptotic cell death.

Thus, it can be concluded that administration of Prx6 before ischemia-reperfusion injury provides significant preservation of morphological and physiological parameters of the tissues. The nephroprotective effect of exogenous Prx6 in I/R conditions is mediated by its antioxidant activity and its participation in different signaling and regulatory pathways in the cell (Sharapov et al. 2019). In addition, we suppose that recombinant Prx6 application can be an effective approach in prevention and treatment of renal ischemia-reperfusion injuries. However, it should be noted that long-term administration of protein preparations can lead to immunological reactions; therefore, short-term use of recombinant Prx6 in the acute phase of I/R kidney injury is the most likely mode of application. The most promising approach may be the use of recombinant Prx6 as a component of perfusion media, to preserve an isolated kidney during transplantation. The possibility of practical application of recombinant Prx6 in ischemic-reperfusion injury requires additional research.

Funding This work was supported by the Russian Foundation for Basic Research (grant nos. 17-04-00356-a and 19-04-00080-a) and the "Molecular and Cell Biology" program of the Presidium of the Russian Academy of Sciences.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving animals The study protocol was approved by the institutional Ethics Committee of Institute of Cell Biophysics RAS and all experiments were carried out according to international regulations listed in the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and ICB RAS Manual for Working with Laboratory Animal no. 57 (30.12.2011).

Informed consent Informed consent was obtained from all individual participants included in the study.

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