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Mechanisms of nephroprotective effect of mitochondria-targeted antioxidants under rhabdomyolysis and ischemia/reperfusion

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ABSTRACT

Oxidative stress-related renal pathologies apparently include rhabdomyolysis and ischemia/reperfusion phenomenon. These two pathologies were chosen for study in order to develop a proper strategy for protection of the kidney. Mitochondria were found to be a key player in these pathologies, being both the source and the target for excessive production of reactive oxygen species (ROS). A mitochondria-targeted compound which is a conjugate of a positively charged rhodamine molecule with plastoquinone (SkQR1) was found to rescue the kidney from the deleterious effect of both pathologies. Intraperitoneal injection of SkQR1 before the onset of pathology not only normalized the level of ROS and lipid peroxidized products in kidney mitochondria but also decreased the level of cytochrome c in the blood, restored normal renal excretory function and significantly lowered mortality among animals having a single kidney exposed to ischemia/reperfusion. The SkQR1-derivative missing plastoquinone (C12R1) possessed some, although limited nephroprotective properties and enhanced animal survival after ischemia/reperfusion. SkQR1 was found to induce some elements of nephroprotective pathways providing ischemic tolerance such as an increase in erythropoietin levels and phosphorylation of glycogen synthase kinase 3β in the kidney. SkQR1 also normalized renal erythropoietin level lowered after kidney ischemia/reperfusion and injection of a well-known nephrotoxic agent gentamicin.

1. Introduction

Acute renal injury (ARI) in critically ill patients is highly associated with poor prognosis, and despite the increasing efforts to alleviate fatal consequences of ARI, the mortality rate among these patients remains a severe problem [1–3]. Moreover, not only ARI itself but also extrarenal complications accompanying or predisposing to ARI often result in multiorgan failure and greatly contribute to the fatality of a kidney malfunctioning [4,5]. In general, current strategies to treat ARI or its consequences include four principal methods. The most common and widespread method is hemodialysis, which is based on the artificial, external removal of harmful wastes and excess salt and fluids from the blood. Another approach includes the induction of natural mechanisms of cell protection such as modulation of the immune system or ischemic preconditioning. The third approach uses pharmacologic intervention to prevent or alleviate the deadly effects of ARI. And finally, when all three listed approaches fail, the only option remains a kidney transplant. Without going into the details of these four lines of treatment for ARI, we conclude that all four are still very costly, complicated, and inconvenient while often resulting in an imperfect outcome. However, pharmacologic treatment of ARI and associated pathologies has demonstrated appreciable progress and has potential that has yet to be exhausted. For example, where certain kidney pathologies can be attributed to the consequences of oxidative stress, antioxidant treatment is an attractive approach. Thus, in a great number of cases, patients with kidney failure due to these pathologies may potentially benefit from exposure to antioxidants when carefully and wisely used [6–11], reviewed in Koyner et al. [12].

Mitochondria-targeted antioxidants have been developed to provide specific delivery of antioxidant molecules to the interior of the mitochondrion, which potentially suffers from oxidative stress more than other cellular compartments. The chimeric molecule of such an antioxidant in principle contains a cation, bearing delocalized charge to allow movement into the mitochondrial matrix conjugated with an antioxidant moiety (e.g., coenzyme Q10 [13] or plastoquinone [14,15]). The proton motive force existing in the inner mitochondrial membrane becomes the driving machinery for a transport of these cationic antioxidants into mitochondria, thus achieving a drug concentration 10,000 times higher in the mitochondrial matrix than in the cytosol [13–16]. The beneficial effect of these compounds has been demonstrated in a number of cell pathologies, although the mechanism of cell protection remains poorly understood [13,16–21].

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In this study, we evaluated a mitochondria-targeted chimeric compounds either carrying an antioxidant moiety or without it as a potential agent to efficiently alleviate the deleterious consequences of ARI arising from two distinct pathologies, both of which apparently involve oxidative stress: kidney ischemia/reperfusion (I/R) and rhabdomyolysis (also called myoglobinuria or crush syndrome as a specific case of rhabdomyolysis). Spontaneous myoglobinuria is caused by necrotic degradation of striated muscles, resulting in the appearance of the muscle protein myoglobin in the bloodstream with subsequent kidney dysfunction. In earlier studies we [22,23] and others [24] demonstrated the key role of mitochondria as a source and a target of oxidative stress and apparent involvement of the mitochondrial permeability transition in both pathologies. Particularly, we demonstrated that myoglobin supplementation to kidney tubules caused apparent oxidative stress evidenced by a rise in ROS level in the tubules and significant loss of the mitochondrial transmembrane potential [23]. For kidney I/R, we have also identified some features of endogenous protective pathways against tissue damage caused by this intervention involving the beneficial role of the inhibition of glycogen synthase kinase-3 (GSK-3). To facilitate the future design of directed pharmacologic interventions to normalize renal function subsequent to ARI, we explored the underlying mechanisms of nephroprotection and the role of mitochondria in these two pathologies using a positively charged membrane-permeable, mitochondrial-targeted compounds carrying an antioxidative moiety [15] or without it [25]. These compounds, named SkQR1 and C12R1, have structures presented in Fig. 1. In an earlier study [26], we have shown that injection of SkQR1 results in a drop of blood creatinine level elevated under I/R and experimental rhabdomyolysis. These findings demanded to run a comprehensive study to explore in detail the nephroprotective potential of mitochondria-targeted drugs under ARI.

2. Materials and methods

2.1. Modeling of glycerol-induced rhabdomyolysis in rats

Experiments were performed on outbred white male rats (180–200 g) fed ad libitum. Animal protocols were approved by the institutional review boards. Rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal). Rhabdomyolysis was induced by a standard method by injection of 10 ml/kg of 50% water solution of glycerol (ICN, USA) into the leg muscles of rats as described earlier [27]. Control animals were untreated. The therapeutic protocol of SkQR1 used to treat this pathology was next: i/p injection of 100 nmol/kg SkQR1 1 hr after induction of rhabdomyolysis with subsequent injections at 13, 25, and 37 hrs; in total, each rhabdomyolytic animal received 400 nmol/kg SkQR1. On the second day after the injection, blood samples were taken and kidneys were excised for the mitochondria isolation with further determination of malondialdehyde in the tissue and mitochondria. Blood creatinine and urea concentrations were determined using the CellTac blood analyzer. The rhabdomyolysis model experiments were performed with at least eight animals in each group.

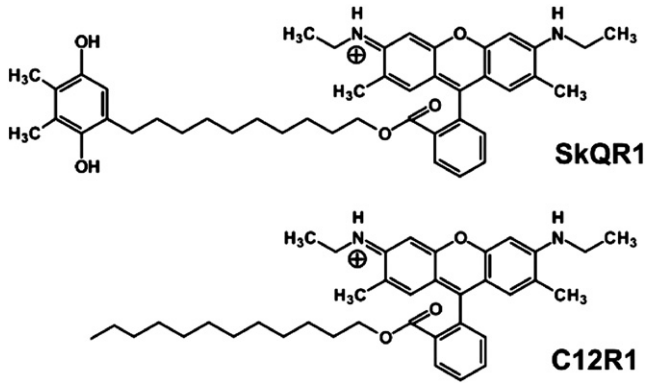


Fig. 1. The chemical structure of SkQR1 (a chimeric molecule combining a mitochondria-targeting rhodamine derivative with plastoquinone, a plant-derived antioxidant) and C12R1 representing SkQR1 without quinone moiety.

2.2. Ischemia/reperfusion protocol

The animals were subjected to 40-min warm ischemia of the left kidney as described in Plotnikov et al. [26]. Briefly, unilateral renal arteries were clamped by a microvascular clip for 40 min, and then circulation was restored by removing the clip. Nephrectomy of the right side was executed together with ischemia. During operation, the body temperature of the rat was maintained at 37 ± 0.5 °C using a thermoregulatory heating unit connected to a rectal probe. On the second day after ischemia blood samples were taken to determine creatinine and urea concentrations using a CellTac blood analyzer (Nihon Kohden Corp., Japan). The therapeutic protocol of SkQR1 treatment: i/p injection of 100 nmol/kg SkQR1 3 hrs before I/R, 1 hr after I/R, and subsequent injections at 13, 25, and 37 hrs; in total, each animal received 500 nmol/kg SkQR1. Sham-operated rats were used as controls. Rats were allocated into the following groups: (1) sham ($n = 12$), (2) I/R + saline ($n = 12$), (3) I/R + SkQR1 ($n = 12$).

2.3. Renal histology

The kidney was isolated immediately after sacrificing the animal and washed with ice-cold phosphate-buffered saline. It was then fixed in a 10% neutral buffered formalin solution, embedded in paraffin and used for histopathological examination. Five micrometer thick sections were cut, deparaffinized, hydrated, and stained with hematoxylin and eosin. The renal sections were examined in blinded fashion for tubular necrosis, hemorrhagic and hyaline casts in the kidneys of all treated animals. A minimum of 10 fields for each kidney slide were examined and scored for pathologic severity. A score from 0 to 4 was given for each pathological sign (necrosis, casts and dilatation): 0, normal histology; 1, from 5% to 25% of tubules have pathology; 2, moderate damage, from 25% to 50% of tubules have pathology; 3, severe, from 50% to 75% have pathology; and 4, almost all tubules in field of view are damaged. The average histological score for each sample was calculated.

2.4. Gentamicin nephrotoxicity protocol

Animals were randomly divided into two groups, each containing 6 animals. The first group (GM-group) of rats received gentamicin intraperitoneally in a single daily dose of 150 mg/kg. The second (GMS-group) of rats received SkQR1 intraperitoneally in a daily dose of 100 nmol/kg 3 h before gentamicin in the same dose as in G-group. Animals in the third (C-group), serving as a negative control, received saline 1 ml/d intraperitoneally. All groups were treated over a period of 6 consecutive days. Following the last application, all animals were sacrificed, and the kidneys were subsequently removed for Western blotting analysis.

2.5. Experiments with renal tubular epithelium cell cultures and kidney slices

Kidneys were excised aseptically from 3- to 7-day-old rats, then homogenized and placed in balanced Hank's solution at pH 7.4. After several washes, the dispensed tissue was placed in 0.1% collagenase and incubated for 20–30 min at 37 °C. Large pieces were removed, and

cells were sedimented by gentle centrifugation (50×*g*) for 3 min. The pellet was resuspended in DMEM/F-12 1:1 containing 10% fetal calf serum (FCS) and seeded in culture plates and glass-bottom dishes. Cells were cultivated in a CO_2 (5%) incubator for 1–2 days before the experiments. The myoglobin solution was added to the cultured epithelial cells to a final concentration of 500 μM and incubated for 1 h at 37 °C in DMEM/F-12 medium containing 10 mM HEPES–NaOH. Next, cells were washed to remove residual myoglobin and incubated with the ROS-sensitive fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA, 10 μM; Invitrogen, USA). The SkQR1, mitochondrial-targeted antioxidant was added to the cultured epithelial cells to a final concentration of 250 nM and incubated for 24 h before myoglobin addition.

Renal cortex tissue slices 200 μm thick were cut with a VibroSlice microtome (World Precision Instruments) and placed into Hank's solution containing 10 mM HEPES–NaOH at pH 7.4 with an appropriate concentration of DCF-DA.

2.6. Confocal microscopy

Experiments were carried out at 37 °C except where otherwise indicated. Kidney slices and renal tubular epithelial cells were washed to remove residual DCF-DA and imaged with an LSM510 inverted confocal microscope (Carl Zeiss Inc., Jena, Germany). Analysis of fluorochrome incorporation was performed in glass-bottom dishes with excitation at 488 nm and emission collected at 500–530 nm. To minimize the contribution of photo-induced mitochondria/cell damage to the relative fluorescence intensities, image analysis was performed on the average of the first four scans only. Images were processed using ImageJ software (NIH, Bethesda, MD, USA).

2.7. Isolation of mitochondria from rat kidney

Kidney mitochondria were isolated by homogenization and differential centrifugation in a medium containing 250 mM sucrose, 20 mM HEPES–KOH, 1 mM EGTA, and 0.1% BSA at pH 7.4. Total mitochondrial protein was determined using a bicinchoninic acid protein assay kit (Sigma Aldrich, USA).

2.8. Measurement of MDA products

Malondialdehyde production in kidney tissue homogenates was explored by a conventional colorimetric method with the use of the malondialdehyde–thiobarbituric acid reaction according to Mihara and Uchiyama [28]. Briefly, each mitochondrial sample was mixed with 0.8% thiobarbituric acid and 1% H_3PO_4 in the ratio by volume 0.9:1.0:3.0. The mixture was boiled for 45 min, cooled to room temperature and then centrifuged at 15,000×*g* for 10 min. Absorbance of the resulting supernatant at 532 nm was measured using a Hitachi 557 spectrophotometer. 1,3,3-tetraetoxipropan (Sigma, USA) was used as a calibration standard. The content of malondialdehyde found was finally normalized by total protein content.

2.9. Western blot analysis

Samples of serum or tissue homogenates were loaded onto 15% Tris–glycine polyacrylamide gels at a concentration of 50 μg total protein per lane. After electrophoresis, gels were blotted onto PVDF membranes (Amersham Pharmacia Biotech, UK). Membranes were blocked with 3% (wt./vol.) non-fat milk in PBS with 0.1% (vol./vol.) Tween 20 and subsequently incubated with primary antibodies: mouse monoclonal anti-cytochrome *c* 1:1000 (BD Biosciences Pharmingen, USA), rabbit polyclonal anti-erythropoietin 1:1000 (Santa Cruz Biotechnology Inc., USA), mouse monoclonal anti-P-GSK-3β 1:1000 (Cell Signaling, USA), mouse monoclonal anti-total-GSK-3β 1:1000 (Cell Signaling, USA), mouse monoclonal anti-porin 1:1000 (BD Biosciences Pharmingen, USA). Membranes were then stained with secondary antibodies: anti-mouse IgG or anti-rabbit IgG conjugated with horseradish peroxidase 1:30000 (Calbiochem, USA). Detection of antibody binding was achieved using the ECL Plus Western blotting kit (Amersham Pharmacia Biotech, UK). After scanning, the density of the resulting staining was measured for each band using ImageJ software (NIH, Bethesda, MD, USA).

2.10. Statistics

All experiments were performed at least in triplicate. All data are presented as mean ± SEM. Comparisons between groups were made using a Student *t* test with a *P* value less than 0.05 taken to indicate statistical significance.

3. Results

3.1. SkQR1 rescues kidney function from the deleterious effects of myoglobinuria

We examined myoglobinuria as the model of oxidative insult affecting kidney function. When myoglobin was administered to cultured renal epithelial cells, these cells displayed the same signs of oxidative stress, namely the mitochondrial membrane potential, as evaluated by uptake of the mitochondrial dye TMRE, decreased (not shown) while the production of ROS in the cells, as indicated by DCF fluorescence, increased (Figs. 2A and B). The exact colocalization of ROS and mitochondrial dye (not shown) demonstrates that as in the case of I/R [22], mitochondria become a source of such induced ROS production. The pretreatment of the cells with SkQR1 (250 nM) 1 day prior to myoglobin administration alleviates oxidative stress induced by myoglobin.

In another model of myoglobin-induced oxidative stress in the kidney, we used intramuscular injection of glycerol to induce muscle degradation and the release of myoglobin into the bloodstream. Exploration of the dynamics of the resulting rhabdomyolysis-induced acute renal failure showed that within 2 days after injection, blood creatinine and BUN reached their maximum levels, indicating critical renal failure [23]. The therapeutic protocol of SkQR1 used to treat this pathology was next: i/p injection of 100 nmol/kg SkQR1 1 hr after induction of rhabdomyolysis with subsequent injections at 13, 25, and 37 hrs; in total, each rhabdomyolytic animal received 400 nmol/kg SkQR1. Earlier we have demonstrated partial normalization of serum creatinine after SkQR1 treatment [26]. Similarly, the level of BUN in the serum of rhabdomyolytic animals demonstrated about a 7-fold increase; after SkQR1 treatment, it was lowered by about 2-fold (Fig. 3A). Simultaneously, the excessive generation of malondialdehyde in the tissue (Fig. 3B) and in isolated kidney mitochondria (not shown) caused by myoglobinuria was almost fully reversed by SkQR1 injection after induction of rhabdomyolysis. C12R1 having no antioxidant moiety possessed some although limited nephroprotective properties (Fig. 3A).

Cytochrome c, probably originating from the breakdown of tissues, has been observed in the blood serum of rhabdomyolitic animals [23,29]. This important marker of rhabdomyolitic degradation of the kidney was used in our study to evaluate the severity of kidney damage during myoglobinuria. Figs. 3C and D demonstrate that rats treated with SkQR1 before myoglobinuria exhibited significantly lower levels of serum cytochrome c than untreated rhabdomyolitic animals, suggesting that SkQR1 pretreatment alleviated rhabdomyolitic damage to the tissues.

3.2. Ischemia/reperfusion of the kidney induces oxidative stress

In our previous study, we demonstrated the key role of mitochondrial dysfunction in kidney damage after I/R [22]. In this study, we

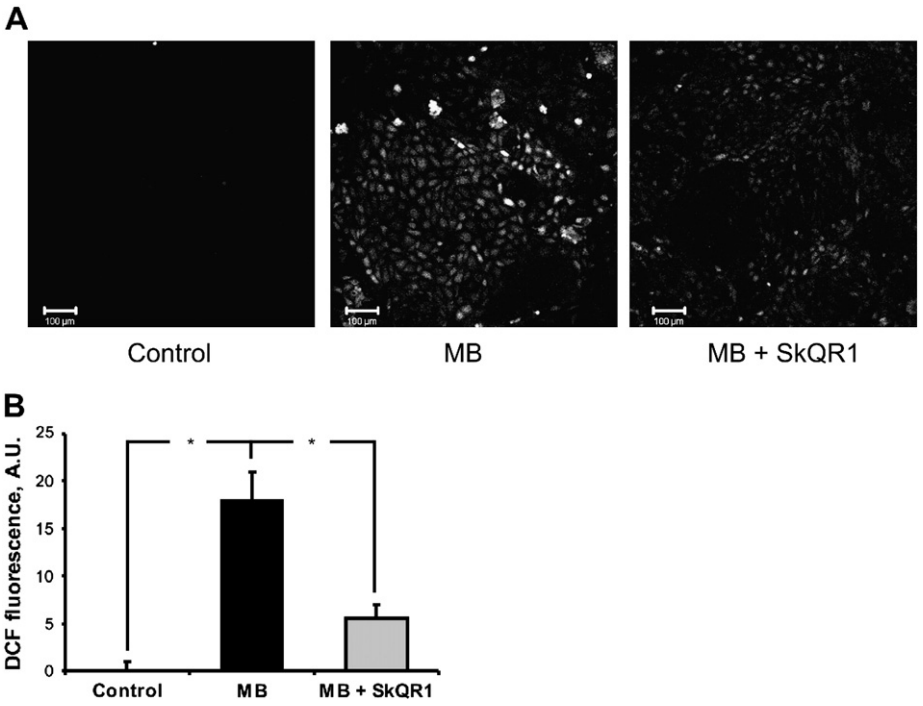


Fig. 2. Evidence of higher ROS production in renal tubular cells exposed to myoglobin. (A) Confocal microscopy of cultured tubular cells stained with DCF-DA. Control cells did not show any visible DCF fluorescence. After incubation with 500 μM myoglobin (MB) for 1 hr, DCF fluorescence increased significantly; quantitative results are shown in B. Preincubation with 250 nM SkQR1 24 hrs prior to myoglobin addition (MB + SkQR1) results in significantly lower DCF fluorescence in response to myoglobin administration.

confirmed earlier observation that ROS production, as detected by DCF fluorescence in vital cortex sections, was significantly higher in kidneys exposed to I/R as compared to controls (Figs. 4A and B). Digital analysis of fluorescence intensity in confocal images showed that DCF fluorescence profiles were colocalized with mitochondria stained with mitochondrial dye [22], demonstrating that mitochondria are responsible for the ROS burst caused by I/R. This rise in ROS level apparently provoked dramatic changes in lipid structure. Fig. 4C demonstrates that I/R resulted in a significant elevation of the kidney tissue content of malondialdehyde, suggesting the induction of lipid peroxidation. As we demonstrated previously [18], 1 μmol/kg SkQR1 injected 24 hrs before ischemia prevented elevation of DCF signal in a renal tissue. In the current study, both ROS burst and higher level of tissue malondialdehyde were significantly attenuated by pretreatment of rats with much

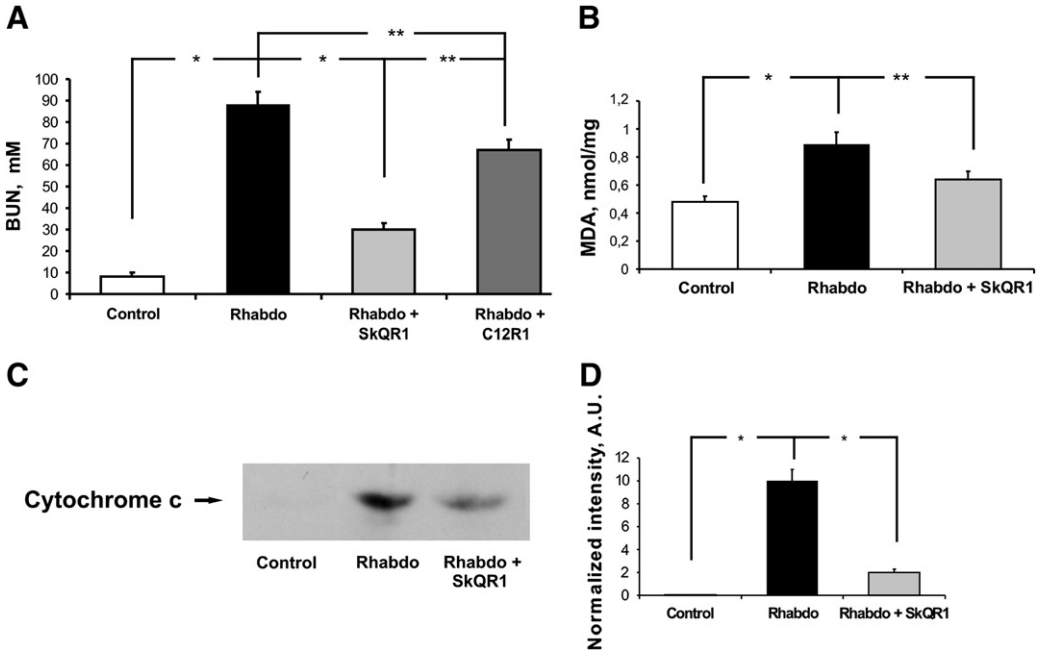


Fig. 3. Indices of kidney dysfunction during rhabdomyolysis and the rescue effect of SkQR1. Concentrations of (A) blood urea nitrogen (BUN) and (C) cytochrome c 24 hrs after induction of rhabdomyolysis in the plasma of control, rhabdomyolitic (Rhabdo), SkQR1-treated (Rhabdo + SkQR1) and C12R1-treated (Rhabdo + C12R1) rats. (B) Malondialdehyde (MDA) in the kidney homogenates of the same groups. (D) Average intensity of the spots shown in C for all experimental groups; 6 blots for 6 different rats were evaluated. SkQR1 or C12R1 injections (100 nmol/kg each) were made 1, 18, 30, and 42 hrs after induction of rhabdomyolysis. *$p<0.005$ and **$p<0.01$.

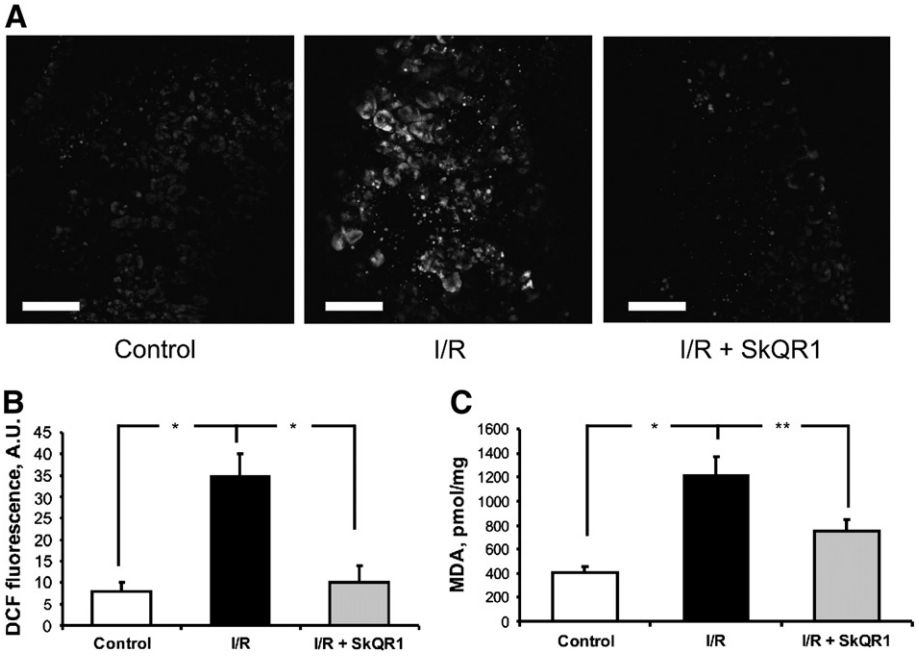


Fig. 4. Confocal microscopy of vital sections from rat kidney cortex stained with DCF-DA (A). Kidneys were taken from untreated rats (Control) and rats subjected to 40-min ischemia and 10-min reperfusion (I/R) with or without 100 nmol/kg SkQR1 injected 3 hr prior to ischemia. (B) Quantitation of DCF fluorescence in A. Five different sections for each experimental group were evaluated along 10 different fields counted in each section. (C) Malondialdehyde (MDA) content in kidney tissue measured 2 days after ischemia. SkQR1 injections (100 nmol/kg each) were made 3 hrs prior to ischemia and 1, 18, 30, and 42 hrs after ischemia. $^{*}p<0.005$ and $^{**}p<0.01$.

lower mitochondria-targeted antioxidant compound SkQR1 with lesser exposure time (100 nmol/kg injected once 3 hrs before ischemia, Figs. 4B and C).

3.3. SkQR1 lowers the mortality of single-kidney rats exposed to I/R

In the experiments on survival of animals subjected to renal insult, we applied a more severe model of ischemic insult by removing one kidney as was suggested by Dobashi et al. [30] and further extended by Serviddio et al. [31]. In this model, kidney I/R in animals that underwent right nephrectomy caused a dramatic rise in blood creatine [26] and urea nitrogen (BUN) and induced the death of the majority of experimental animals (Figs. 5A and B). The injection of either SkQR1 or C12R1 prior to ischemia and during the reperfusion phase significantly lowered creatinine [26] and BUN and drastically increased the survival of animals (Figs. 5A and B). When SkQR1 was injected only during the reperfusion period, normalization of the kidney function following ischemia did not occur (see BUN level in

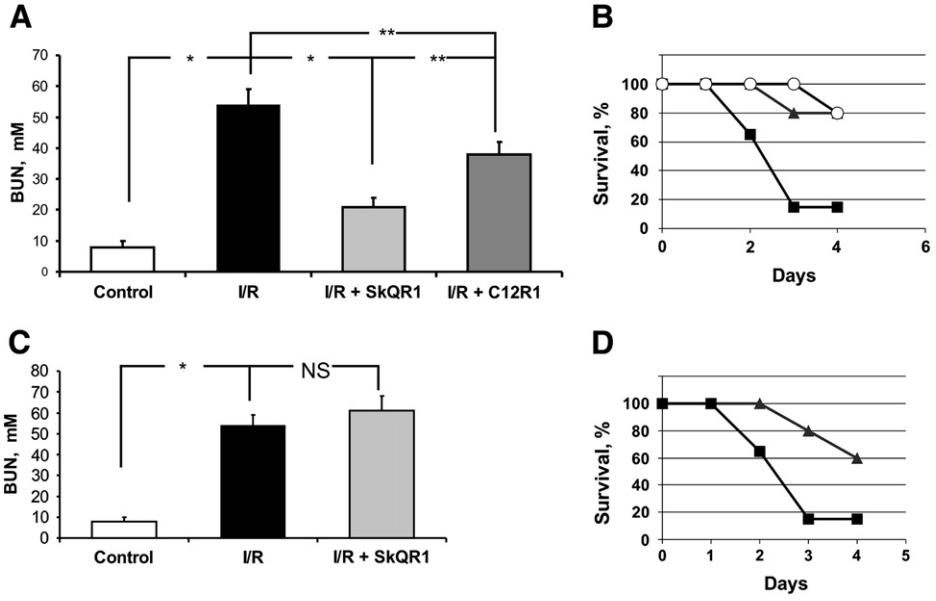


Fig. 5. Blood urea nitrogen (BUN) in control plasma (Control in A and C) and in plasma from rats subjected to 40-min ischemia and 48 hrs of reperfusion (I/R) with or without SkQR1 treatment (I/R + SkQR1). (A) SkQR1 and C12R1 injections (100 nmol/kg each) were made 3 hrs prior to ischemia and 1, 13, 25, and 37 hrs after ischemia; (C) SkQR1 injections (100 nmol/kg each) were made 1, 13, 25, and 37 hrs after ischemia. (B and D) Mortality among rats having a single kidney and exposed to unilateral ischemia and reperfusion without (squares) and with SkQR1 treatment (triangles): (B) SkQR1 (triangles) and C12R1 (circles) injections (100 nmol/kg each) were made 3 hrs prior to ischemia and 1, 18, 30, and 42 hrs after ischemia; (D) SkQR1 injections (100 nmol/kg each) were made 1, 18, 30, and 42 hrs after ischemia. $^{*}p<0.005$ and $^{**}p<0.01$.

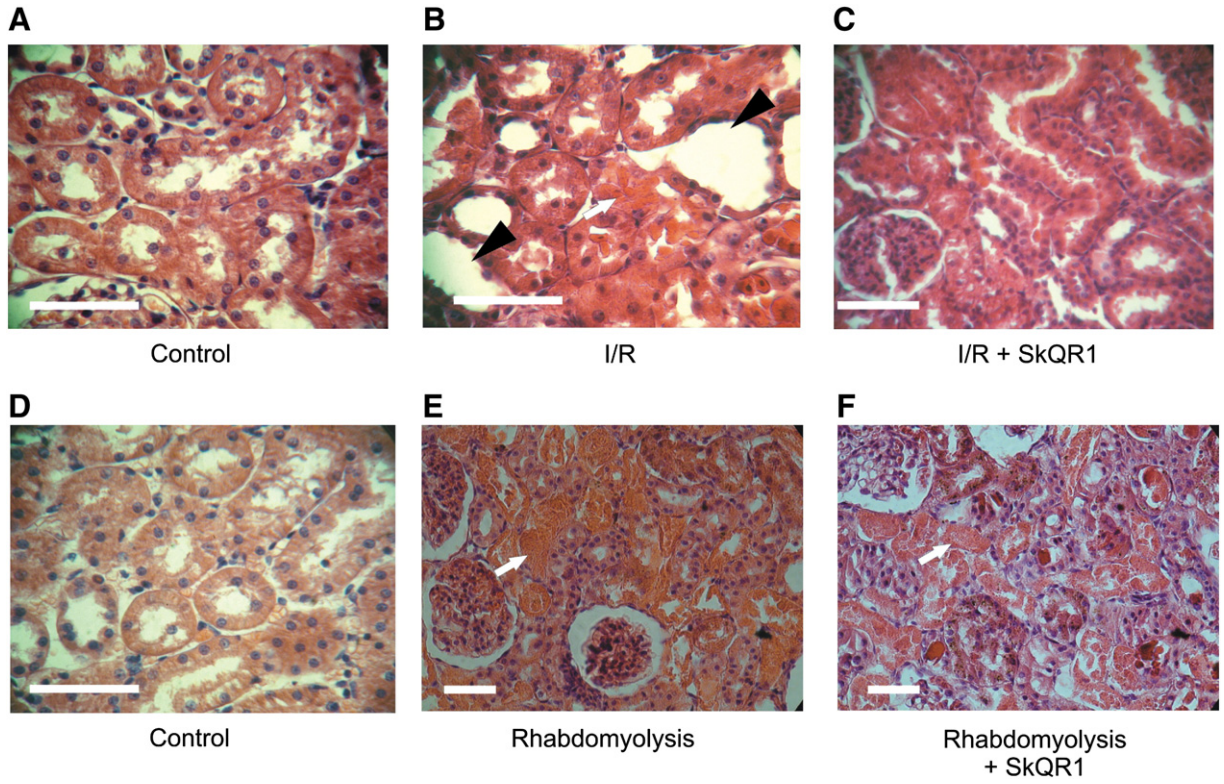


Fig. 6. Effects of SkQR1 on ischemia- and glycerol-induced rhabdomyolysis-induced pathological features in renal morphology. Images show kidney sections stained with hematoxylin and eosin. (A) Control kidney. (B) Kidney excised 2 days after 40-min warm ischemia. Kidney tubules are observed with apparent focal necrosis of epithelial cells (shown by arrows) and noticeable dilatation (black arrowheads). (C) SkQR-treated kidney excised 2 days after 40-min warm ischemia. (D–F) Kidney of control rats and excised 2 days after induction of rhabdomyolysis. Necrotic kidney tubules (white arrows) are shown. Bar, 50 μm.

Table 1
Effects of SkQR1 on ischemia- and glycerol-induced rhabdomyolysis-induced renal pathological features.

	Ischemia/reperfusion			Rhabdomyolysis		
	Control	IR	IR + SkQR1	Control	Rhabdo	Rhabdo + SkQR1
Necrosis	0	2.21 ± 0.08	0.60 ± 0.02	0	3.8 ± 0.3	2.8 ± 0.25
Protein casts	0.05 ± 0.02	1.00 ± 0.15	1.27 ± 0.2	0.05 ± 0.02	1.2 ± 0.1	0.8 ± 0.05
Dilatation	0	2.21 ± 0.2	1.47 ± 0.15	N/A	N/A	N/A

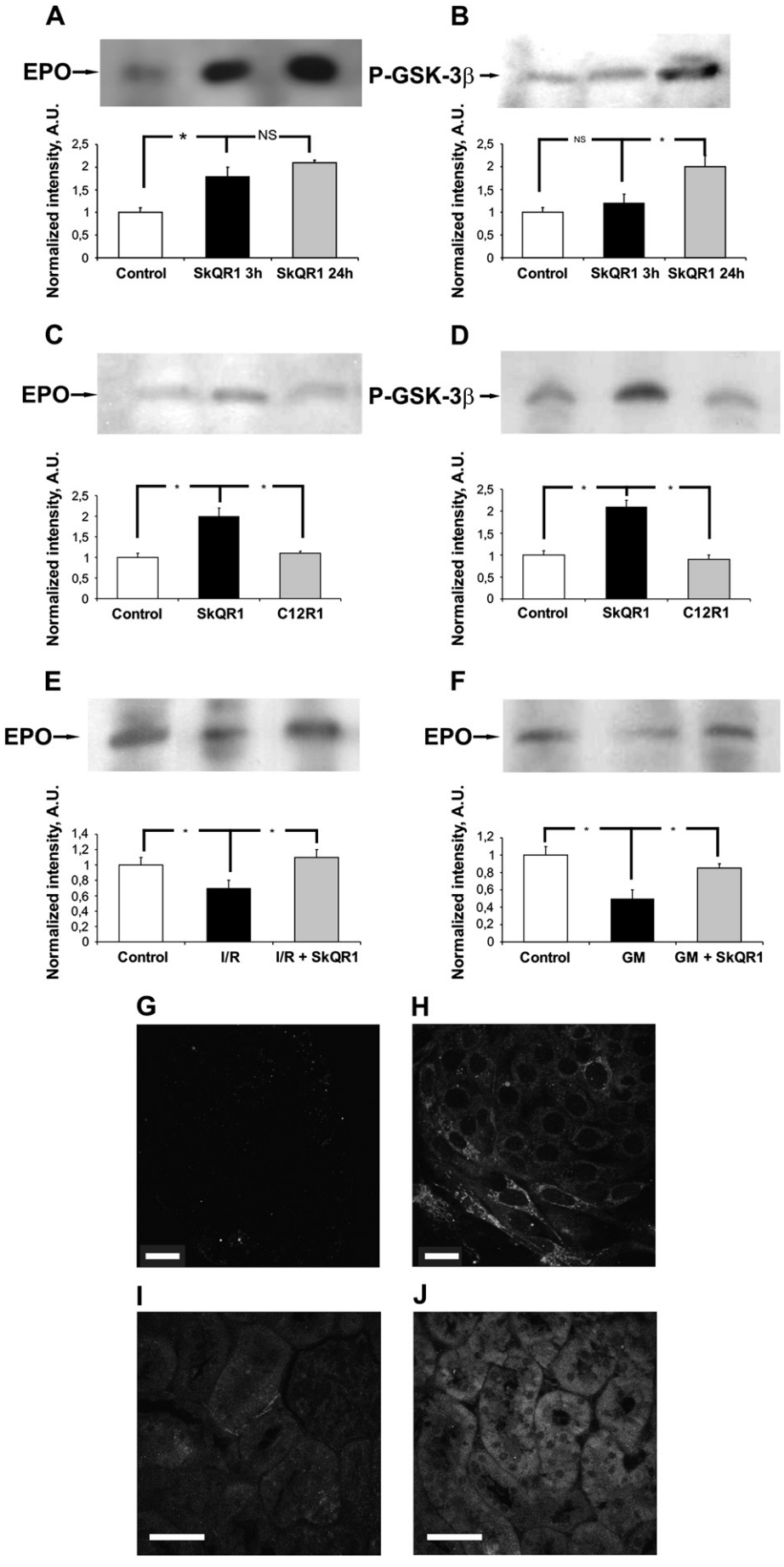
Fig. 5 C); however, the survival of animals is increased (versus untreated ones) within 3–4 days, critical for animals with right nephrectomy exposed to I/R (Fig. 5D).

It is worth mentioning here that SkQR1 under the concentration used (100–1000 nmol/kg) did not have any influence on renal functioning of intact animals: diuresis, the amount of products of nitrogen metabolism, reabsorption ability of tubules, and glomerular filtration rate were not changed neither on 1st nor on 4th day after SkQR1 injection. In addition, moderate hepatotoxic effect of SkQR1 in a dose of 1 μmol/kg has been found on day 1 after injection expressed in slight elevation of blood AST and ALT.

3.4. SkQR1 alleviates histopathological changes in a renal tissue

Histological study demonstrated changes in kidney morphology induced by both I/R and rhabdomyolysis consistent with those described elsewhere [8,10]. The changes observed in the kidney exposed to 40 min of warm ischemia in a single-kidney rat appear to indicate severe tubular necrosis (Fig. 6B versus control in A) and mild casts formation (Table 1). In the cortex, the tubules with fully necrotic cells are evident. Outside of these areas, the tubules did not show visible morphological effects. The morphological findings of dilatation of proximal tubules are suggestive of an obstructive lesion. Rats

Fig. 7. Changes in renal erythropoietin (EPO) and phosphorylated glycogen synthase kinase-3β (P-GSK-3β) levels. (A–F) Detection of EPO (A, C, E, F) and P-GSK-3β (B, D) in the total kidney tissue. Representative Western blots with corresponding densitometry below the blots are shown. The densitometry results represent an average over 6 blots obtained from the kidneys of 4 different rats. Band densities were normalized to the density of the α-tubulin band. 1 μmol/kg SkQR1 was injected i/p 3 and 24 hrs before excising the kidney; 1 μmol/kg C12R1 was injected 24 hrs before excising the kidney; kidneys after I/R were analyzed 2 days after I/R with or without SkQR1 treatment. Kidneys after gentamicin (GM) or gentamicin + SkQR1 treatment were analyzed after 6 consecutive days of the treatment. $^{*}p<0.01$. (G and H) Immune reactivity to anti-EPO Ab of cultured kidney epithelial cells treated with 250 nM SkQR1 for 24 hrs (H). (G) Untreated cells. (I and J) Confocal micrographs of rat kidney slices stained with anti-EPO Ab: (I) control kidney and (J) kidney treated with 1 μmol/kg SkQR1 for 24 hrs.


A
EPO
Normalized intensity, A.U.
2,5
2
1,5
1
0,5
0
*
NS
Control
SkQR1 3h
SkQR1 24h
B
P-GSK-3β
Normalized intensity, A.U.
NS
*
Control
SkQR1 3h
SkQR1 24h
C
EPO
Normalized intensity, A.U.
Control
SkQR1
C12R1
D
P-GSK-3β
Normalized intensity, A.U.
Control
SkQR1
C12R1
E
EPO
Normalized intensity, A.U.
1,4
1,2
1
0,8
0,6
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0
Control
I/R
I/R + SkQR1
F
EPO
Normalized intensity, A.U.
1,2
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0,4
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Control
GM
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subjected to SkQR1 therapy exhibited decreased kidney tissue damage (Fig. 6C). SkQR1 treatment lowered the number of necrotic tubules and dilatation of tubules and resulted in practically normal histological appearance but did not affect the casts' formation (Table 1).

Rhabdomyolytic rat kidneys demonstrated marked pathological features in the cortex and outer medulla. The renal sections showed severe tubular necrosis (Fig. 6E) as well as hyaline and hemorrhagic casts (Table 1). Rats treated with SkQR1 yielded kidney sections showing much less pronounced tubular necrosis (Fig. 6F), but the number of hyaline and hemorrhagic casts was almost the same as in untreated rats (Table 1).

3.5. SkQR1 provides some elements of ischemic tolerance

In the experiments presented above, we observed the significant nephroprotective properties of a mitochondria-targeted compound, SkQR1. To gain insight into the mechanisms of nephroprotection afforded by SkQR1, we measured the concentrations of two components of anti-ischemic defense signaling systems, specifically erythropoietin (EPO) and GSK-3β. Single i/p injection of SkQR1 (1 μmol/kg) caused 80% rise of EPO level in the kidney after 3 hrs and about 100% elevation after 24 hrs (Figs. 7A and J) while the level of phosphorylated GSK-3β (enzymatically inactive form) reached 120% and 200% of baseline 3 and 24 hrs after injection of SkQR1, respectively (Fig. 7B). Remarkably, we detected the induction of EPO production in primary culture of rat kidney cells 24 hrs after incubation with 250 nM SkQR1 (Figs. 7G and H). Single i/p injection of C12R1 (1 μmol/kg) did not change EPO and phospho-GSK-3 level in a renal tissue after 24 hrs (Figs. 7C and D). When elements of intrinsic protective pathways were analyzed, we found that 2 days after I/R, EPO production in kidney was significantly decreased versus control animals, whereas after I/R with SkQR1 treatment, renal EPO content was similar to control (Fig. 7E).

In addition, we ran experiments with antibiotic gentamicin known for its nephrotoxicity as a side effect when applied. We observed a significant drop of EPO level in a total kidney tissue after 6 days of gentamicin treatment, with restoration of EPO level after 100 nmol/kg SkQR1 was injected daily 3 hrs before gentamicin (Fig. 7F).

4. Discussion

The unique ability of the mitochondrion to build H^+ transmembrane potential for supporting ATP synthesis was recently exploited to create drugs targeted to mitochondria. The design of these drugs is based on the use of a construct containing a hydrophobic part carrying positive charge delocalized over a number of coupled double bonds which can be driven into mitochondria and considered as a locomotive [13–16]. The linker locomotive part of the molecule is bound to another component carrying the function, which is in demand. In the SkQR1 molecule shown in Fig. 1, the locomotive moiety, a derivative of rhodamine 19, is bound via a linker to a natural antioxidant of plant origin, plastoquinone [14]. This chimeric compound by design was intended to quench the excessive production of ROS in mitochondria. Experiments showed that this compound is easily permeable through the inner mitochondrial membrane and must be distributed in mitochondrial matrix considering the presence of the transmembrane potential over the inner mitochondrial membrane (about 150–180 mV, negative inside) and the potential existing over cellular membrane (about 50–60 mV, negative inside) [14,15,21]. Taken together, these potentials result in a driving force for cation electrophoresis leading to concentrations of the cationic substance about 10,000 times higher than in the extracellular milieu. If one accounts for the high hydrophobicity of this compound, this concentration gradient will be even higher, implying that intramitochondrial content of this compound will be on the order of millimolars when it is administered to the blood or extracellular fluid at submicromolar concentrations.

By design, a potential mitochondrial-targeted drug should demonstrate very powerful antioxidative properties inside the mitochondria under conditions of oxidative stress. Since mitochondria are known to be one of the major players in oxidative stress of any origin, the treatment of oxidative stress-related pathologies with compounds like this may be highly beneficial. Specifically, since a great number of renal pathologies involve oxidative stress, mitochondria-targeted antioxidant drugs should ameliorate renal damage in these pathologies.

In the present study, surgical ischemia/reperfusion of the kidney as well as myoglobin and glycerol-induced experimental rhabdomyolysis were used as models to test whether SkQR1 can prevent or cure ARI. Both pathologies are known to greatly contribute to the onset of acute renal failure with no known effective pharmacologic means of prevention.

Ischemia followed by reperfusion is a common pathological trigger for the kidney damage considering the high vulnerability of this organ to the transitions, which occur during cessation and restoration of blood flow [32]. Renal injury associated with I/R is a general cause of acute renal failure as has been observed after renal transplantation, shock, sepsis, and renal artery stenosis. Several pharmacological therapies have been proposed to treat renal pathologies associated with I/R. The most efficient options at present include the administration of antioxidants and antioxidative systems, nitric oxide and nitric oxide synthase inhibitors, EPO, peroxisome proliferator-activated receptor agonists, inhibitors of poly(ADP-ribose) polymerase, carbon monoxide releasing molecules, statins, adenosine, and others [33].

Mitochondria suffer from I/R as much as other intracellular components. I/R-induced mitochondrial fragmentation, as observed in cultured kidney cells [34] as well as in kidney tissue [22], is a prime example of mitochondrial morphological rearrangement preceded, accompanied or followed by functional changes expressed in an inability to maintain energy-producing and transducing functions. As a result of ischemic insult, mitochondria are transformed from the organelles producing signaling (protecting) levels of ROS to those producing excessive (pathology-inducing) ROS, apparently by the mechanism known as ROS-induced ROS release [35,36].

Another model of a renal mitochondria-mediated oxidative stress is rhabdomyolysis which is also related to I/R [27,37,38]. Nowadays, rhabdomyolysis is considered one of the major causes of acute renal failure. People usually develop rhabdomyolysis after crush injuries produced by events such as earthquakes, mine collapses, traffic or working accidents, wounding, various intoxications, and many other reasons. Usually, patients have vague symptoms such as swollen extremities and muscle weakness but up to one third of them will develop kidney failure [37]. Sometimes to save a life the affected limb must be amputated.

The most widely used experimental model of rhabdomyolytic acute renal failure involves the subcutaneous or intramuscular injection of hypertonic glycerol, which causes myolysis similar to that caused by traumatic muscle compression followed by the release of muscle content into the bloodstream culminating in tubular necrosis and acute renal failure [39].

Heme-containing proteins or their degradation products, including ionic iron released from damaged muscle, have been found to display tubular nephrotoxic properties partially mediated by the generation of reactive oxygen and nitrogen species [23,39,40].

Our findings strongly demonstrate that the attenuation of mitochondria-mediated oxidative stress in the kidney significantly improves renal function. The specific mitochondria-targeted antioxidant SkQR1 was found to offset and alleviate the consequences of myoglobinuria and renal I/R. The severity of renal damage in rhabdomyolysis was higher than in the case of ischemia/reperfusion.

However, the anti-necrotic effect of SkQR1 on kidney tubules under rhabdomyolysis is less expressed and it had almost no effect on cast formation (described in Fig. 6 and Table 1). Apparently, the striated muscle damage and myoglobin release in the bloodstream under rhabdomyolysis are SkQR1-independent especially as its injection has been made after rhabdomyolysis induction. Thus, SkQR1 might not have the effect on cast formation; however, it does have a strong influence on further events, namely on the myoglobin-induced oxidative stress. We conclude that as a result of SkQR1 action, oxidative stress goes down in parallel with a drop of renal cell death. So, although SkQR1 cannot prevent accumulation of Mb in renal tissue, it ameliorates damaging consequences of this accumulation, thus finally lowering AKI.

In the case of ischemic damage, the primary cause of AKI is oxidative stress induced by internal factors, namely by enhanced ROS generation in mitochondria [22]. In this case, SkQR1 acts directly on the level of mitochondrial ROS so it protects kidney cells more effectively, which is expressed in a lower number of necrotic tubules (see Table 1).

Given that myoglobinuria and renal I/R are among the most widespread and damaging renal pathologies, the renoprotective effect of this compound potentially can be used in the therapy of I/R-related pathologies including rhabdomyolysis.

Importantly, SkQR1 was found to be able to exert protective effects not only through direct scavenging of ROS (which seems reasonable considering its very high final intramitochondrial concentration) but also by providing the kidney with elements of ischemic tolerance signaling mechanisms. We detected two key elements induced by administration of SkQR1, namely EPO and the phosphorylated form of GSK-3β. EPO is known to possess pleiotropic properties distinct from its well-known hematopoietic activity [41–44] that afford protection against ischemic damage (reviewed in Sharples and Yaqoob [45]). The activity of GSK-3β is inversely related to the phosphorylation status of serine-9 in this enzyme while its dephosphorylation results in activation of the kinase [46]. Prosurvival signals were shown to correlate with the presence of the phosphorylated form of GSK-3β and protective signaling pathways converge via inhibition of GSK-3β on the end effector, the permeability transition pore complex, preventing the induction of the mitochondrial permeability transition [47]. Remarkably, apart from its direct antioxidative potential, SkQR1 can upregulate protective signaling mechanisms to afford protection not only in the kidney but also possibly in the heart and brain [26].

Remarkably, some, although limited, protective properties were found for C12R1, bearing no antioxidative moiety in the molecule. Such modification of the SkQR1 molecule resulted in a loss of ability to induce synthesis of EPO in the kidney. It gives strong evidence that, namely antioxidative moiety of SkQR1 molecule is responsible for the induction of signaling pathways providing ischemic tolerance. C12R1-afforded limited protection of the kidney we explain by indirect induction of defense pathway independent on that with EPO involved. Proven protonophorous activity of C12R1 in planar phospholipid membranes and liposomes [42] may be a clue to consider the role of this drug in protective mechanisms through mild uncoupling of mitochondria. Mitochondrial mild uncoupling was shown to result in a significant decrease in the rate of ROS formation by mitochondria, providing a tolerance to oxidative stress-induced pathologies [48].

Interestingly, there is a tight link between tissue EPO level and the specific activity of GSK-3β. Specifically, it has been shown that EPO administration stimulates the production of phosphorylated GSK-3β in the heart [49,50] and the kidney [51] (our unpublished data), demonstrating the synergy between these two signals. It is noteworthy that 3 hrs after SkQR1 administration, we could observe a rise in EPO concentration in the kidney cells while the level of phosphorylated GSK-3β was almost unchanged (Fig. 7B). Finally, after 24 hrs of administration of SkQR1, an increase in the level of phosphorylated GSK-3β was detected (Fig. 7B), illustrating an apparent delayed causative effect of the tissue level of ROS on GSK-3β phosphorylation. SkQR1 also normalized renal EPO level dropped after kidney I/R and injection of a well-known nephrotoxic agent gentamicin. This indicates the central role of the kidney EPO synthesis in the realization of SkQR1-mediated nephroprotection.

There is evidence that general antioxidants can induce the generation of EPO in cells [52,53]. It seems plausible that during treatment with the mitochondria-targeted antioxidant SkQR1, direct antioxidative effects and induction of ischemic preconditioning signaling pathways are linked in synergy due to the targeted delivery of this compound to mitochondria.

We conclude that SkQR1 affords renal protection under different pathologic conditions with some limitations specific for each pathology. These limitations cover the non-toxic concentration range used and the time frame of SkQR1 application in order to afford effective protection.

5. Disclosure

The authors have declared that no conflict of interest exists.

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