Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Hemoglobin induces cytotoxic damage of glycine-preserved renal tubules

M. R. Moussavian,[1] J. E. Slotta,[1] O. Kollmar,[1] M. D. Menger,[2] M. K. Schilling[1] and G. Gronow[3]

1 Department of General, Visceral, Vascular and Pediatric Surgery, University of Saarland, Homburg/Saar, Germany
2 Institute for Clinical and Experimental Surgery, University of Saarland, Homburg/Saar, Germany
3 Department of Physiology, University of Kiel, Kiel, Germany

Keywords
hemoglobin, hypoxia/reoxygenation, isolated tubuler segments, organ preservation.

Correspondence
Prof. Martin K. Schilling MD, FRCS, Department of General, Visceral, Vascular and Pediatric Surgery, University of Saarland, D-66421 Homburg/Saar, Germany. Tel.: ++49 6841 31000; fax: ++49 6841 31002; e-mail: chschil@uniklinikum-saarland.de

Received: 21 March 2007
Revision requested: 11 April 2007
Accepted: 19 July 2007

doi:10.1111/j.1432-2277.2007.00538.x

Summary

In isolated tubular segments (ITS) of rat kidney cortex, we studied the effect of hemoglobin (Hb) on reoxygenation damage. All tubules were suspended in Ringer's solution containing 5-mM glycine and oxygenated for 30 min with 95% O_2:5% CO_2, followed by a 30-min period with 95% N_2:5% CO_2, and final reoxygenation for 60 min. Untreated tubules served as controls. Different concentrations of free Hb and equivalent amounts of intact erythrocytes were added to the incubation medium. Secondly, we added deferoxamine (DFO) to Hb and erythrocytes. Membrane leakage and lipid peroxidation were measured by lactate dehydrogenase and glutamate dehydrogenase and the development of thiobarbituric acid reactive substances. Cell function was quantified by gluconeogenesis and intracellular potassium accumulation. Hb exerted concentration-dependent cytotoxic effects indicated by significantly increased enzyme leakage rates, lipid peroxidation and a significantly decreased cell function ($P < 0.05$), in ITS during hypoxia, and subsequent reoxygenation. Moreover, we found that toxicity of both Fe^{2+} and Fe^{3+} ions increased with rising concentration. However, Fe^{2+} showed a higher tissue toxicity than Fe^{3+}. DFO reduced significantly the reoxygenation damage of free Hb and iron ions. Our data clearly demonstrate a pronounced cytotoxic effect of free Hb in ITS, which critically depended on the reduction state of the iron ions.

Introduction

In clinical situations with impaired or interrupted perfusion such as organ preservation or in polytraumatized patients with severe myoglobinemia, hypoxia leads to cellular stress, followed by massive damage upon reoxygenation [1]. In ITS, short normothermic hypoxia has been demonstrated to cause irreversible damage [2,3]. Adenosine triphosphate (ATP) consumption is increased followed by a restriction of Na^+-K^+-ATPase and subsequent loss of volume regulation [4].

During reoxygenation, disintegration of cellular membranes causes a loss of electrolytes followed by cell swelling, cellular death and loss of organ function. A reduced function of Na^+-K^+-ATPase generates a drop of membrane voltage and an intracellular uptake of Ca^{2+}, which, in turn, activates phospholipases inducing higher levels of free fatty acids, lysosomal enzymes, and phopholipids [5]. Furthermore, in reoxygenated kidneys, xanthine dehydrogenase is converted to xanthine oxidase, which, in turn, stimulates the production of free oxygen radicals [6] and thus initiates further membrane damage. Glycine whose cytoprotective qualities have already been demonstrated in numerous studies [7,8] does not protect against oxygen radicals [2].

It is well known that heme proteins can cause kidney dysfunctions [9,10] even under physiological conditions. Under pathological conditions coinciding with tissue hypoxia, heme proteins are described to cause functional restrictions and morphologic damage in the kidney,

which is mostly attributable to reoxygenation [11]. It was shown that heme proteins and/or the iron in the protein complex catalyze oxygen into more aggressive metabolites [12] namely: the superoxide-, peroxide- and hydroxy radicals. Among these, conversion of the hydroxyl radical (OH·) to water (or hydroxylized organic compounds) has a remarkably high redox potential of 2.33 V. Thus, OH· is a strong biological oxidant with an extremely short half-life and very short diffusion ways. It initiates radicalization reactions and oxidizes nearly all biological molecules in its surrounding. Under physiological conditions, however, OH· formation is low in cell metabolism. In contrast, superoxide ion and H_2O_2 are 'normal' waste products of the mitochondrial oxygen reduction. They are detoxified by cellular protective enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione oxidase (GSH), and 'scavengers'. During reoxygenation, this cellular protective system can break down, resulting in an accumulation of O_2^-, H_2O_2, and especially OH· [13].

The purpose of this study was to examine the effects of exogenous Hb or intact erythrocytes on reoxygenated ITS. Additionally, the impact of Hb-engaged or exogenous iron ions on reactive oxygen species (ROS) formation was analyzed [14].

Materials and methods

Animals

Wistar rats of either sex (300–350 g) were housed in single-cages at 22–24°C with a 12/12-h dark–light cycle, and were kept on water and standard chow *ad libitum*. After approval by the local animal care committee, the experiments were conducted according to the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH publication 86–23, revised 1985).

Isolation of tubule segments

Animals were anesthetized by an i.p. injection of pentobarbital sodium (60 mg/kg; Narcoren, Merial, Hallbergmoos, Germany). After median laparotomy, ligation of the superior mesenteric artery and celiac trunc and cannulation of distal aorta, kidneys were flushed with 25 ml chilled, oxygenated medium I (Table 1). After incision of the inferior cava vena, 2 ml chilled collagenase-solution (Worthington CLS II, Freehold, NJ, USA; Table 1) was injected. After decapsulating both resected kidneys, the cortex was cut into 1.0 mm thick slices and the marrow was separated. Kidney cortex was chopped homogeneously and the resulting particles were continuously stirred in 30 ml oxygenated collagenase solution (37°C). After 30 min, the tubule segments were filtered through a sieve (pore-size 1 mm). Subsequently, the suspension was centrifuged at 50 *g*, the sediment resuspended and washed once more in medium I, centrifuged and finally oxygenated (95% O_2/5% CO_2 = carbogen) in ice-cold medium II. Under moderate shaking, the isolated tubules were then gassed for 30 min with carbogen at 37°C for recovery from the preceding collagenase treatment [15]. Thereafter, the tubule suspension was centrifuged twice at 4°C for 30 s at 50 *g* and the cell precipitation resuspended in chilled medium II (Table 1). The precipitate, replenished with medium II to 6.5 ml, finally served as a standard for ITS in the following experiments.

Table 1. Chemical composition of used incubated media. The collagenase solution und medium I contained hydroxyethyl piperazinyl ethansulfonacid (HEPES) as buffer and were used for isolation of ITS, in medium II physiological bicarbonate buffer was used for incubation of the ITS.

Adjuvant solution	Medium I (mmol/l)	Medium II (mmol/l)	Collagenase (mmol/l)
NaCl	138.0	110.0	138.0
KCl	4.4	4.4	4.4
$MgCl_2 \times 6H_2O$	1.2	1.2	1.2
$NaHPO_4$	2.0	2.0	2.2
$CaCl_2 \times 2H_2O$	2.5	2.5	52.2
$NaHCO_3$	0.0	25.0	0.0
Na-lactate	0.0	10.0	0.0
HEPES	10.0	0.0	10.0
Bovine albumin	0.0	0.5 g/100 ml	0.0

Incubation media

For incubation of the ITS, a modified Krebs ringer bicarbonate solution was used (Table 1). With the exception of medium I, all solutions contained the physiological buffer system $CO_2/H_2CO_3/HCO_3^-/H^+$, excluding an influence of organic buffer molecules on reactive oxygen metabolites. Medium I was used merely for first-time washing of the cells and during processing in the collagenase treatment solution (2 mg/ml collagenase = 350 U/ml; Worthing CLS). For pH stabilization, hydroxyethyl-piperazinyl-ethansulfonacid (HEPES-buffer) was added. Medium II contained 10 mM lactate as a substrate for gluconeogenesis and additionally 0.5% of albumin.

The raised calcium concentration in the collagenase solution catalyzed collagenase activity [16].

The pH in medium I was set up at 4°C, in medium II and the collagenase solution at 37°C by a Schott-glass-electrode (Polzin, Kiel, Germany) on pH 7.35. All suspensions were supplemented with 5 mmol/l glycine [17]. With addition of other substances, the equivalent molar mass of NaCl was lowered in the media. Thus, a steady isosmotic extra-cellular concentration existed in all solutions.

Testing procedure

After preincubation and repeated washing, ITS were resuspended at 37°C in 50 ml Erlenmeyer-flasks in 5 ml desoxygenated medium II in a concentration of 1 mg cell protein/ml. Consecutively, 30 min hypoxia were performed ($pO_2 < 1$ mmHg) by gassing with a nitrogen-containing gas mixture (95% N_2/5% CO_2).

After hypoxia, reoxygenation was performed for 60 min with carbogen.

Measuring methods

Samples were taken before hypoxia, after 30 min hypoxia and after 60 min reoxygenation and centrifuged immediately for 6 s at 20 000 *g*. The precipitate was used for K^+ and protein determination, the supernatant served for measurements of lactate dehydrogenase (LDH) and glutamate dehydrogenase (GLDH) enzyme activity, glucose concentration, and the content of thiobarbituric reactive substances (TBA-RS). Suspension was mixed immediately with 0.5 ml ice cold 10% trichloracetic acid (TCA). All used reagents were from p.A.-quality and were purchased from Merck (Darmstadt, Germany) and Serva (Heidelberg, Germany).

For ascertainment of the intracellular K^+ concentration, the precipitate was mixed with 1 ml aqua destillatum, shaken and centrifuged for 30 min with a glass bead on a rotation mixer (5432 Eppendorf, Germany). The K^+ concentration in the supernatant was determined using flame photometer (K 701 A, Eppendorf, Germany) according to a linear standard curve from 0.1 to 0.5 mmol K^+/l.

Enzyme activities and glucose concentration in the supernatant were measured spectrophotometrically with standard test kits (Boehringer, Mannheim, Germany). Precipitates were analyzed for protein content by a modified Lowry-method according to Gronow *et al.* [2,7]. Oxidative degradation of unsaturated fatty acids caused by oxygen radicals was determined by the formation of TBA-RS: 0.5 ml cell suspension was homogenized with 0.5-ml TCA, then added to 0.5 ml TBA (0.6%) and heated for 20 min at 95°C.

During this procedure, adducts from the fatty acid-oxidation formed with TBA a red color complex. After cooling the mixture to 20°C, the red color complex was extracted in 1.5 ml *n*-butanol. Extinction (E) was measured spectrophotometrically at 500 and 535 nm. The difference in absorbance yielded in a range between 1 and 4 nmol/ml the TBA-RS content according to a linear calibration curve made up with 1,1,3,3-tetraethoxypropan (TEP, Fluka, Neu-Ulm, Germany) [2,18].

For the extraction of dissolved Hb, 5 ml of rat blood was taken without addition of anticoagulatory substances from the inferior vena cava, centrifuged and washed four times in Krebs–Ringer-Bicarbonate medium. Sedimented erythrocytes were lyzed with aqua destillatum and the hemoglobin (Hb) content in the solution was measured spectrophotometrically at 546 nm. This concentrated Hb solution was diluted with medium II to a defined final test concentrations of 0.03 vol.%. A control group of ITS from the same cell suspension served as control.

All data are expressed as mean ± SD. Statistical comparison between two groups was performed by a paired *t*-test, multiple comparisons were performed by one-way ANOVA with Bonferroni *post hoc* correction using a statistical software (SIGMA STAT, Jandel Scientific, San Raffael, CA, USA). A *P*-value < 0.05 was assumed to indicate a significant difference.

Results

Cellular integrity

To determine the impact of hypoxia and reoxygenation on cellular integrity, loss of LDH and GLDH was analyzed by measuring the enzyme activities in the incubation medium. Quantitative analysis showed a significant loss of LDH from ITS during hypoxia ($P < 0.05$; Fig. 1a), indicating a severe cellular injury. LDH loss from ITS gassed for 90 min with carbogen (control) was constant throughout the experiment (1.5 ± 0.2 mU/mg/min).

Spectrophotometric analysis of GLDH activity in the incubation medium showed a significant increase in GLDH loss during hypoxia from ITS ($P < 0.05$; Fig. 1b), which was even more distinct during reoxygenation. Persistently carbogen-gassed control ITS showed a constant GLDH loss of 0.31 ± 0.04 mU/mg/min.

Cellular functions

The intracellular K^+ concentration of control ITS, gassed for 90 min with carbogen, was ~390 nmol/mg (Fig. 2a) and remained constant throughout the duration of the experiment. Hypoxia induced a significant decrease in intracellular K^+ concentration to 220 nmol/mg ($P < 0.05$; Fig. 2a). During reoxygenation, intracellular potassium concentration re-increased, but did not reach baseline levels.

Gluconeogenesis (GNG) rate in negative control ITS was 4.34 ± 0.35 nmol/mg/min throughout the experiment. Incubation of ITS under hypoxia induced a significant diminution of GNG by 97% to 0.1 ± 0.1 nmol/mg ($P < 0.05$; Fig. 2b). Sixty minute reoxygenation of ITS was followed by a significant increase of GNG.

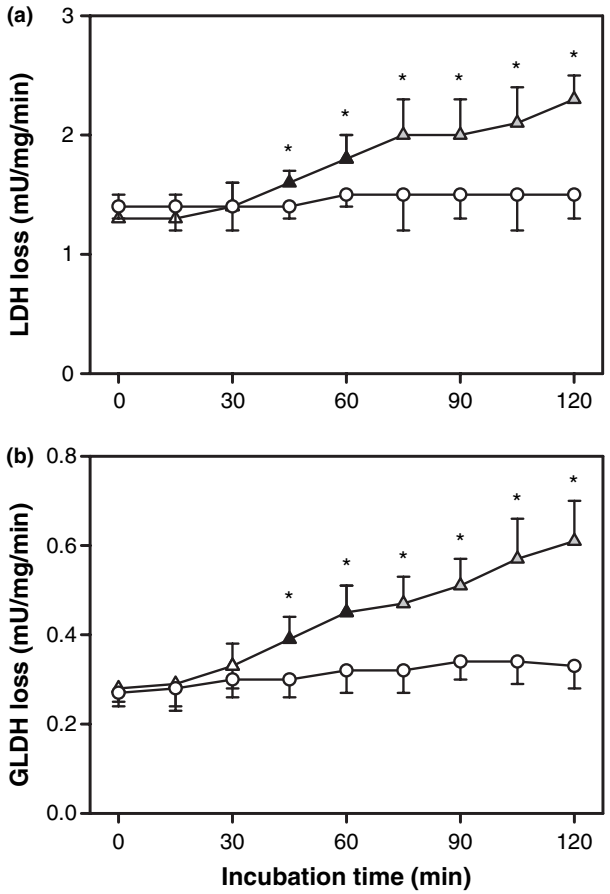

Figure 1 Membrane integrity of isolated tubular segments (ITS), measured by lactate dehydrogenase (LDH) (a) and glutamate dehydrogenase (GLDH) (b) concentrations in culture medium during 30 min oxygen preincubation (white triangles), 30 min extreme hypoxia (black triangles) and 60 min reoxygenation (gray triangles). ITS under carbogen gassing served as controls (white circles). Data are given as mean ± SD; * $P < 0.05$ vs. control, $n = 15$.

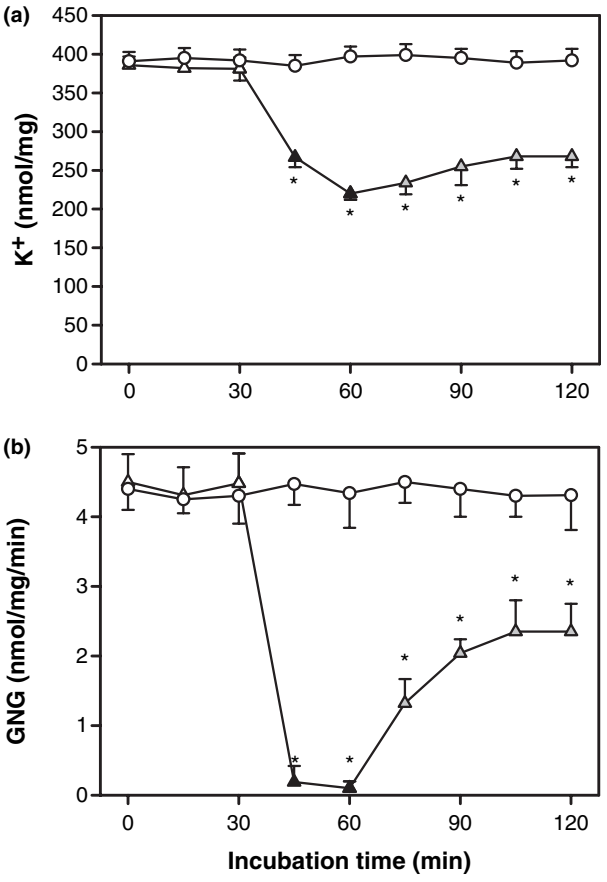

Figure 2 Cellular function of ITS, measured by intracellular K^+ content (a) and gluconeogenesis rate (b) during 30 min oxygen preincubation (white triangles), 30 min extreme hypoxia (black triangles) and 60 min reoxygenation (gray triangles). ITS under carbogen gassing served as controls (white circles). Data are given as mean ± SD; * $P < 0.05$ vs. control, $n = 15$.

Lipid peroxidation

Lipid peroxidation was quantified by measuring the formation of TBA-RS. In negative control ITS, TBA-RS increased linearly from 0.59 ± 0.20 nmol TEP/mg at the beginning of the experiment to 1.26 ± 0.21 nmol TEP/mg (Fig. 3a). Hypoxia had no significant impact on lipid peroxidation. However, during reoxygenation, TBA-RS formation significantly increased in ITS and reached 2.99 ± 0.26 nmol TEP/mg ($P < 0.05$; Fig. 3a). Addition of a mixture of SOD (100 U/ml) and CAT (250 U/ml) was capable of significantly preventing this increase in lipid peroxidation in ITS during reoxygenation ($P < 0.05$; Fig. 3a).

To determine oxidative stress, in separate experiments 0.03 vol.% of hydrogen peroxide was added to the incubation medium at the beginning of hypoxia. Addition of H_2O_2 caused a significant increase in TBA-RS during hypoxia ($P < 0.05$), which was even more prominent upon reoxygenation ($P < 0.05$; Fig. 3b). Addition of SOD and CAT significantly attenuated hypoxia and reoxygenation-induced lipid peroxidation ($P < 0.05$; Fig. 3b).

Impact of free heme proteins

In the next series of experiments, the influence of extracellular heme proteins was examined and cellular integrity and active cell functions in ITS were analyzed quantitatively.

Firstly, the influence of free Hb on enzyme loss and lipid peroxidation was examined at increasing Hb concentrations. ITS without Hb served as negative controls. After 60 min reoxygenation, LDH and GLDH loss from

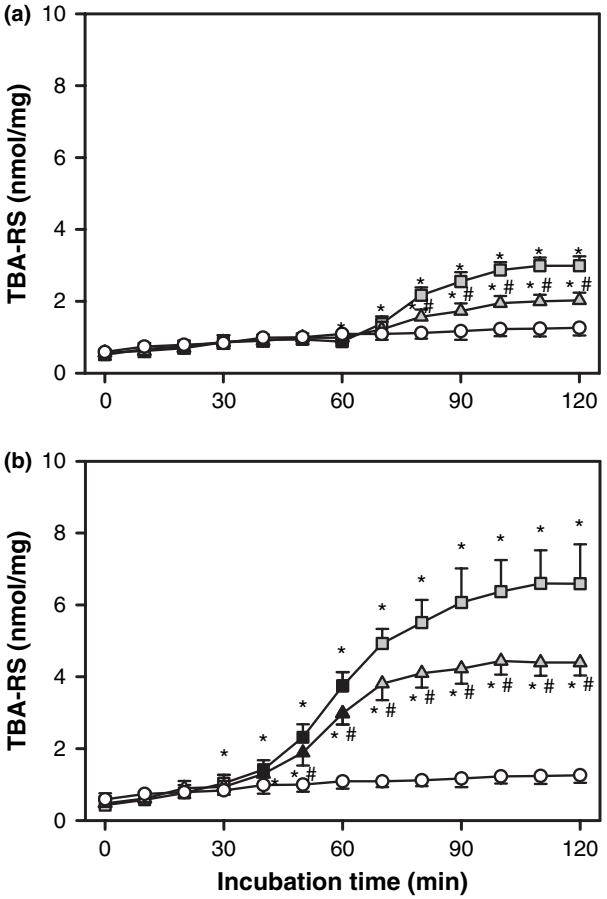

Figure 3 Lipid peroxidation in ITS, measured by thiobarbituric reactive substances formation during 30-min oxygen preincubation (white symbols), 30 min extreme hypoxia (black symbols) and 60 min reoxygenation (gray symbols) without (squares) or with (triangles) catalase (CAT) and superoxide dismutase (SOD) under control conditions (a) and after co-incubation with H_2O_2 (b). ITS under carbogen gassing served as controls (white circles). Data are given as mean ± SD; $^*P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. without CAT and SOD; $n = 12$.

ITS were found significantly increased at Hb concentrations from 0.000 g/dl to 1.000 g/dl ($P < 0.05$) compared with hypoxic ITS (Fig. 4a,b). Moreover, Hb was found to exert dose-dependently cytotoxic effects upon hypoxia/reoxygenation in concentrations up to 0.1 g/dl, whereas even an Hb concentration of 0.001 g/dl caused a severe mitochondrial damage, as indicated by a significantly elevated GLDH loss. In contrast, a significant LDH release was not found until 10-fold higher Hb concentrations (0.010 g/dl). Interestingly, a high Hb concentration (2 g/dl) was even effective to attenuate enzyme loss significantly from both hypoxic and reoxygenated ITS ($P < 0.05$) and to protect from rexoxygenation injury (Fig. 4a,b).

Analysis of ITS function showed that Hb concentrations >0.100 g/dl significantly increased intracellular potassium during hypoxia ($P < 0.05$; Fig. 4c). Reoxygenation caused a striking increase in intracellular K^+ ($P < 0.05$), which was significantly reduced in the presence of Hb in concentrations from 0.001 to 0.100 g/dl ($P < 0.05$). A quantity of 0.100 g/dl Hb completely abolished reoxygenation-induced increase in intracellular K^+. Still higher Hb concentrations cause again a significant increase of intracellular potassium during reoxygenation, which was even significantly higher at 2.000 g/dl compared with that at 0.000 g/dl (Fig. 4c).

Gluconeogenesis was not impaired during 30-min hypoxia. Upon reoxygenation, GNG was found significantly increased compared with hypoxia. Presence of Hb in concentrations up to 0.100 g/dl significantly reduced GNG. At a concentration of 2.000 g/dl, we observed a significant protection of GNG by Hb ($P < 0.05$; Fig. 4d).

Quantification of lipid peroxidation showed that incubation of ITS with increasing concentrations of Hb did not alter TBA-RS formation during 30-min hypoxia (Fig. 4c). However, reoxygenation of hypoxic ITS led to a significant increase in TBA-RS formation in the presence of Hb at concentrations above 0.010 g/dl ($P < 0.05$), which was more distinct with increasing Hb concentrations.

Impact of intact erythrocytes

In a second experimental series, the effect of intact erythrocytes on hypoxic/reoxygenated ITS was compared with that of equimolar concentration of free Hb.

Addition of intact erythrocytes to incubation medium had no effects on cellular integrity, cell functions in carbogen-gassed ITS, but lipid peroxidation was found significantly increased. In contrast, addition of free Hb caused a significant impairment of cellular integrity and cell functions, and caused lipid peroxidation (Fig. 5a–e).

After 30 min extreme hypoxia and 60 min reoxygenation, ITS showed a significant reoxygenation damage in control ITS as well as in ITS incubated with RBC or Hb. Presence of 0.03 vol.% intact erythrocytes did not affect cellular integrity. However, addition of 0.100 g/dl free Hb significantly increased enzyme loss compared with control ITS as well as to ITS incubated with intact red blood cells (RBC) (Fig. 5a,b). Addition of RBC to ITS resulted in a slight, but significant reduction in intracellular K^+ and GNG upon hypoxia/reoxygenation. Again, cell functions were significantly reduced by free Hb compared with ITS incubated with RBC or control ITS (Fig. 5c,d). After hypoxia/reoxygenation of ITS, intact erythrocytes did not significantly influence lipid peroxidation. Likewise, the presence of 0.100 g/dl free Hb significantly increased lipid peroxidation ($P < 0.05$; Fig. 5e).

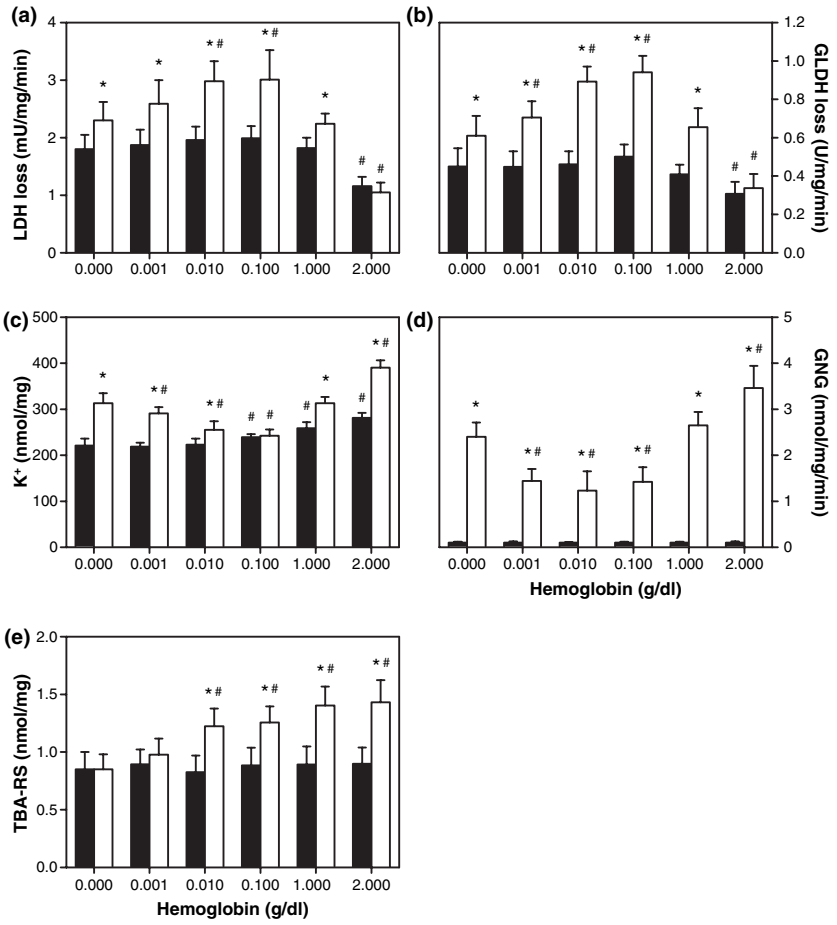

Figure 4 Membrane integrity, cell function, and lipid peroxidation in ITS, determined by LDH (a), GLDH (b) loss, intracellular K^+ content (c) and gluconeogenesis rate (d) and thiobarbituric reactive substances formation (e) after 30 min extreme hypoxia (black bars) and 60 min reoxygenation (white bars) in addition of increasing hemoglobin concentrations. Data are given as mean ± SD; $^*P < 0.05$ vs. hypoxia, $^\#P < 0.05$ vs. 0.000 g/dl Hb; $n = 15$.

Impact of exogenous iron ions

To investigate the cytotoxic effects of exogenous iron ions on hypoxic/reoxygenated ITS, cells were incubated with increasing concentrations of Fe^{2+} and Fe^{3+} ions.

Quantification in LDH loss after 30 min hypoxia and 60 min reoxygenation showed a significantly increasing enzyme loss with increasing Fe^{2+} concentrations with an LDH loss of 5.95 ± 0.43 mU/mg/min at 1 mM compared with 1.72 ± 0.38 mU/mg/min at 0 mM ($P < 0.05$; Fig. 6a). Fe^{3+} also showed a dose-dependent increase of LDH loss from ITS, which was significantly higher from concentrations above 0.010 mM compared with 0.000 mM ($P < 0.05$ Fig. 6a). However, LDH loss was significantly lower at concentrations of 0.100 and 1.000 mM compared with Fe^{2+} ($P < 0.05$; Fig. 6a). In parallel with LDH loss, incubation of ITS with iron ions caused a significantly increasing lipid peroxidation after hypoxia/reoxygenation with increasing concentrations ($P < 0.05$; Fig. 6b). Again, incubation of ITS with Fe^{3+} showed a significantly reduced TBA-RS formation compared with ITS incubated with Fe^{2+} at any concentration ($P < 0.05$; Fig. 6b). Cytotoxic effects of free iron ions were also analyzed by intracellular potassium and GNG. Quantitative analysis revealed that increasing concentrations of iron ions significantly impaired cellular functions ($P < 0.05$; Fig. 6c,d). ITS incubated with Fe^{2+} showed a dramatic drop of intracellular potassium at 1 mM ($P < 0.05$; Fig. 6c) and an even more distinct reduction in GNG ($P < 0.05$; Fig. 6d). However, incubation of ITS with Fe^{3+} caused a significantly less pronounced impairment of cellular functions ($P < 0.05$; Fig. 6c,d).

Masking of Hb effects with deferoxamine (DFO)

To further evidence that cytotoxic effects of free Hb are mediated by Fe^{2+}, we added a highly potent iron chelator DFO in an equimolar concentration of 0.1 mM. Cells were incubated with 0.1 g/dl free Hb with or without additional DFO (Fig. 7a–e).

Addition of Hb to the reoxygenated ITS caused massive increase of enzyme leakage ($P < 0.05$). These alterations were significantly inhibited by DFO and were found even lower than control levels ($P < 0.05$; Fig. 7a,b). Moreover,

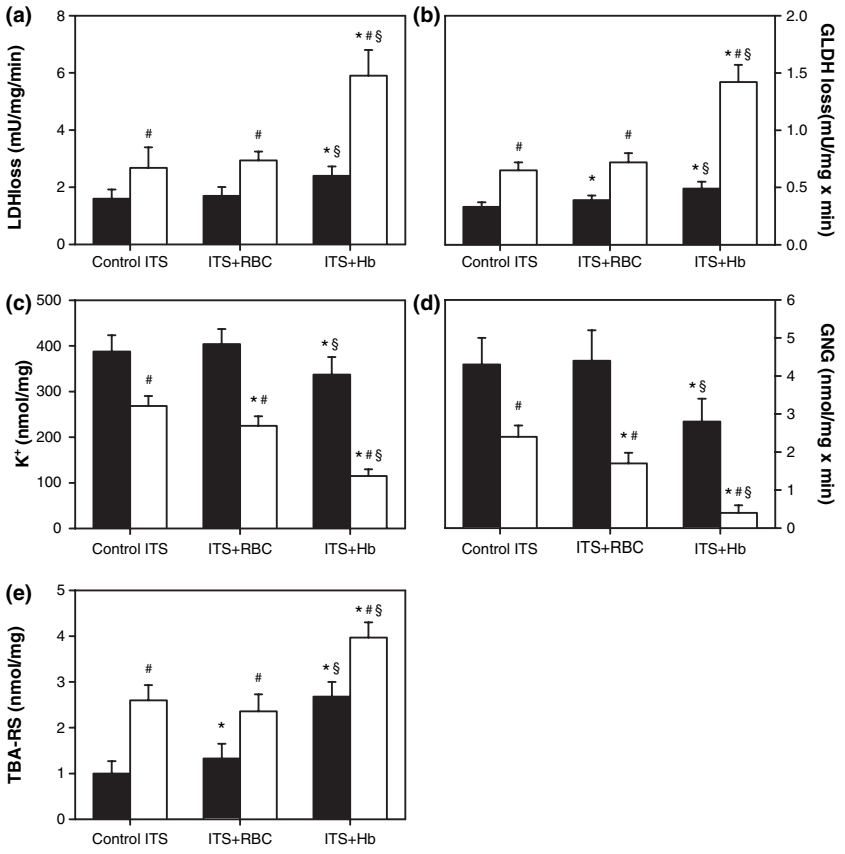

Figure 5 Membrane integrity, cell function, and lipid peroxidation in ITS, determined by LDH (a), GLDH (b) loss, intracellular K^+ content (c) and gluconeogenesis rate (d) and thiobarbituric reactive substances formation (e) in carbogen-gassed ITS (black bars) and ITS after 30 min extreme hypoxia and 60 min reoxygenation (white bars) in the absence (control ITS) and in the presence of equimolar concentrations of intact red blood cells (RBC; 0.03 vol.%) or free hemoglobin (0.100 g/dl). Data are given as mean ± SD; $^*P < 0.05$ vs. control ITS, $^{\#}P < 0.05$ vs. carbogen gassed ITS, $^{\S}P < 0.05$ vs. ITS+RBC; $n = 15$.

the presence of free Hb massively impaired active cell functions of reoxygenated ITS ($P < 0.05$). Again, additional DFO completely compensated cellular dysfunctions ($P < 0.05$; Fig. 7c,d). Lipid peroxidation was also found significantly increased in the presence of hb. This increase was completely prevented by adding DFO ($P < 0.05$; Fig. 7e).

Discussion

The pathophysiology of reactive oxygen metabolites has gained increasing importance in modern medicine. Cellular damage appears even under normal oxygen tension, especially in the absence of protective antioxidative enzymes [19]. If a temporal oxygen lack occurs, after reoxygenation [20,21] the balance between generation and elimination of ROS can break down. Herein, heme proteins can play a supporting role [22]. In patients with nephritic failure triggered by heme proteins, it became evident that kidney dysfunction occurred not directly upon hemolysis, but days or even weeks later [23] indicating secondary injury after hemolysis. Also, in organ transplantation, flushing of kidneys before transplantation significantly improves graft survival. Obviously, there is a link between the quantity of remaining erythrocytes in the preserved organ and organ function after restoration of the circulation [24,25].

Moreover, *in vitro* the addition of cross-linked Hb to ITS has been shown to cause decreasing Na^+ resorption, reduced glomerulus filtration ratio, and considerable enzymes losses. Primarily, this was ascribed to small quantities of Hb, which were filtered in glomeruli and moved into the tubule lumen increasing intratubular pressure and subsequently lowering glomerular filtration rate [17]. Therefore, not only mechanical effects can cause cellular damages in kidneys cortex [26], but also an addition of heme proteins. Heme proteins, like myoglobin or Hb, are known to promote the formation of ROS [27] under certain conditions. Oxygen metabolites like O^{2-} and $O_2^{2-}$are produced even under physiological conditions in small amounts by the mitochondrial respiratory chain [28]. After hypoxia/reoxygenation, however, cellular protective mechanisms, like SOD and CAT break down [29] and short-living, extremely oxidizing active OH-radicals can be formed. These oxidize biomolecules, resulting in membrane fragmentation, lipid peroxidation, and inactivation of enzymes and ion pumps by cross linking the proteins [30].

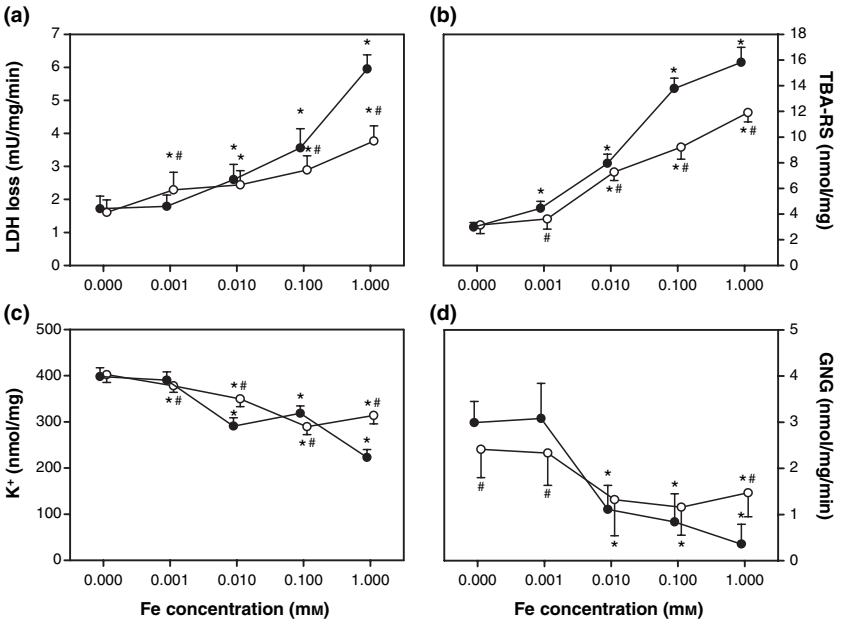

Figure 6 LDH loss (a), lipid peroxidation (b) and cell function of ITS, assessed by intracellular K^+ (c) and gluconeogenesis rate (d), in the presence of increasing Fe^{2+} (black circles) and Fe^{3+} (white circles) ions after 30 min extreme hypoxia and 60 min reoxygenation. Data are given as mean ± SD; $^*P < 0.05$ vs. 0.000 mM; $^{\#}P < 0.05$ vs. Fe^{2+}; $n = 15$.

In the presence of glycine, whose cytoprotective and nephroprotective qualities have already been demonstrated in numerous studies [7,8], we found distinct cell damages after reoxygenation indicated by raised enzyme levels and decreased cell functions. That indicates that the examined reoxygenation damage must be glycine-insensitive. Lipid peroxidation increased arithmetically in untreated ITS during 90 min carbogen gassing, demonstrating that there is a low, but detectable lipid peroxidation already under physiological conditions of cell incubation. This hypoxia/reoxygenation-induced increase of lipid peroxidation, enhanced by H_2O_2, was reduced by adding a mixture of oxygen metabolite-converting enzymes, which indicated that the increase in lipid peroxidation during reoxygenation was caused at least partially by O_2^- and O_2^{2-}. Thus, OH-radicals contribute critically to the reoxygenation damage.

Moreover, our results show that also in the absence of exogenous heme proteins, released iron ions can induce ROS under rexoxygenation conditions. The fact that myoglobin-bound iron exerts cytotoxic effects has already been demonstrated several times [31]. In accordance with these observations, we show a concentration-dependent dichotomic effect of exogenous Hb on reoxygenated ITS. Low concentrations of Hb (<0.1 g/dl) damage the cells with lasting effect. In contrast, higher Hb concentrations (>0.1 g/dl) significantly reduced enzyme leakage and clearly improved cellular functions. This paradox of protecting effects of high Hb concentrations might be caused by the reduction in released oxygen: the Hb-bond oxygen might attenuate hypoxia. Compared with normal oxygen consumption of ITS of nearly 40 nmol O_2/mg protein per min [32], an Hb concentration of 2 g/dl with a maximum oxygen load of about 0.12 mmol O_2/2 g hb/dl (Hüfner number) or 1.2 μmol O_2/ml is theoretically adequate to supply the ITS for the whole hypoxic period. This fact might also explain the paradoxical increase in K^+ during hypoxia and the measurable GNG in extreme oxygen starvation. At high concentrations, Hb acts as a redox partner via Fenton reaction especially in a concentration of 0.1 g hb/dl.

The cytotoxic properties of Hb are detectable not only during reoxygenation, but also during carbogen gassing. Enzyme losses and lipid peroxidation were increased in ITS incubated with 0.1 g hb/dl after hypoxia/reoxygenation. In the presence of intact erythrocytes, we found no significant differences compared with the corresponding controls regarding membrane integrity; however, cellular functions were decreased. One of the reasons for lipid peroxidation and membrane damage in the presence of Hb might be intracellular iron ions that are released from phagocytosed and lysosomally degraded Hb [33]. This degradation might lead to ROS formation causing a destruction of the cell membrane, indicated by an increased lipid peroxidation and the high loss of cytoplasmatic LDH and intramitochondrial GLDH. Fe^{2+} can inflict a more severe damage to cells than Fe^{3+} because of its electron-transmitting effect [34]. Accordingly, the cell damage in the presence of added Fe^{2+} was found more pronounced than in presence of Fe^{3+}. Fe^{2+} caused a higher lipid peroxidation than an equivalent quantity of Fe^{3+} in concentrations up to 0.01 mM. The damaging

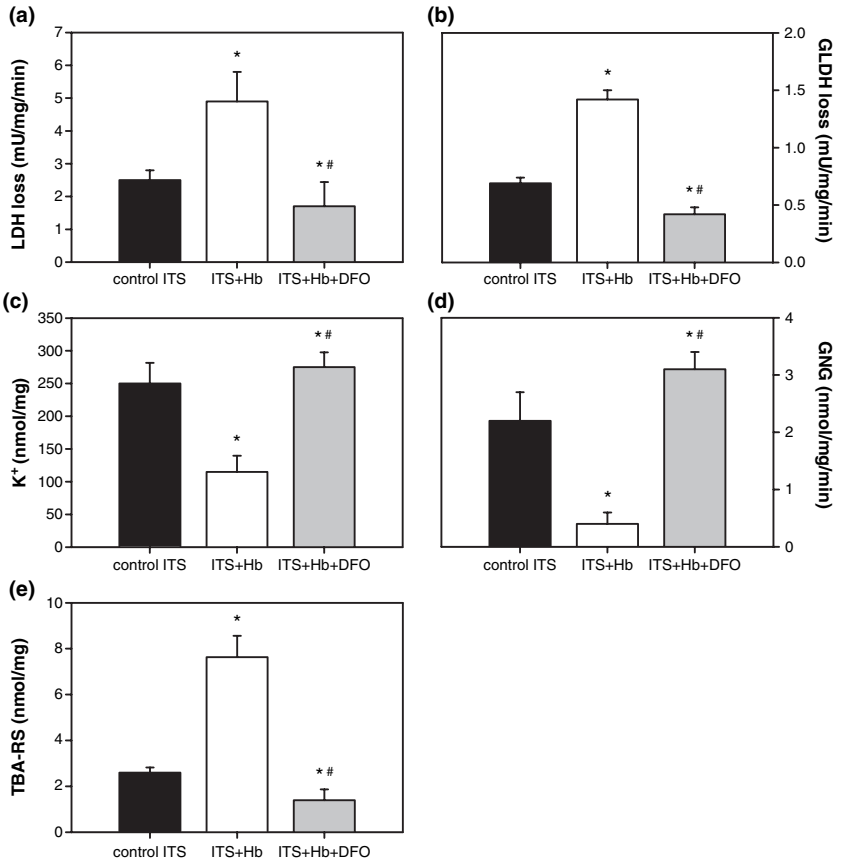

Figure 7 Membrane integrity, cell function and lipid peroxidation in ITS, determined by LDH (a), GLDH (b) loss, intracellular K^+ content (c) and gluconeogenesis rate (d) and thiobarbituric reactive substances formation (e) in carbogen-gassed ITS (controls; black bars) and ITS after 30 min extreme hypoxia and 60 min reoxygenation co-incubated with hemoglobin (Hb) in the absence (white bars) and in the presence (gray bars) of deferoxamine (DFO). Data are given as mean ± SD; $^*P < 0.05$ vs. control ITS, $^{\#}P < 0.05$ vs. Hb without DFO; $n = 15$.

effect of Fe^{3+} was based probably on redox reactions in the tissue, in which Fe^{3+} was transformed to Fe^{2+} by redox partners. The Fe^{2+}/Fe^{3+} complex can play here even the roll of a redox partner. The potential in the reaction with oxygen metabolites is decisive. In this cycle, iron acts as a catalyst, in that small quantities of released iron ions continuously promote the simultaneous development of reactive oxygen metabolites. Fe^{3+} and Fe^{2+} possess a redox potential of 0.75 mV, otherwise the oxidation O_2^-/O_2 releases only 0.33 mV. Thereby, Fe^{3+} oxidizes its redox partner while being reduced to Fe^{2+}. Increased consumption of ATP under hypoxic conditions enhances the activity of intracellular xanthine oxidases [35], producing hydrogen peroxide, which has a stronger redox potential than the Fe^{2+}/Fe^{3+} system. Fe^{2+} serves as reduction agent, H_2O_2 degenerates to the hydroxyl radical and hydroxide ion under formation of Fe^{3+}. This cycle is supported by the mitochondrial formation of O_2^- and the acidification of the tissue [36] during hypoxia. The formed H_2O_2 reacts with Fe^{2+} to the extremely reactive and oxidizing OH· (redox potential = +2.33 V).

The potent iron chelator DFO protected the ITS from cellular damage in the presence of Hb. Hydroxyl radicals affect not only intracellular lipid membranes, but also oxidize molecules in the cellular nucleus, as for example guanine to 8-hydroxyguanine. Latter prefers adenine in place of cytosine as its complementary nucleotide, causing serious transcription errors with a high corresponding mutation rate and degeneration [37]. Liu and Okada have impressively shown the total impact of iron ions in this reaction [38]. Accordingly, clinical studies involving patients with hemochromatosis demonstrated that a high percentage of the patients developed a primary liver cell carcinoma after a multiyear latency period [39].

Not only exogenous iron, but also iron ions from intracellular storage can contribute under reoxygenation conditions to the generation of ROS [40]. Even without addition of exogenous iron, lipid peroxidation and membrane leakage were significantly diminished and the cell function was improved. Even low concentrations of DFO are sufficient to bind all of the Hb released iron ions.

The equimolar addition of 0.1 mM DFO significantly reduced the cytotoxic effects of 0.1 g/dl Hb on membrane integrity, cell function, and lipid peroxidation. This is a clear indication that the reoxygenation damage of Hb is caused by bound Fe^{2+} whose deleterious effects were significantly reduced by DFO. The detrimental effect of free iron in liver preservation is well known. Kerkweg *et al.*

[41] demonstrated that DFO takes a central role to reduce cold-induced apoptosis of rat liver cells in the University of Wisconsin solution. The cell injury was decreased by DFO to 6–25% in hepatocytes and between 2% and 4% in liver endothelial cells.

In conclusion, our study shows cytoxic effects of released Hb on renal tubules based on the redox potential of iron ions catalyzing the hydroxy radical formation. In ITS, the cytotoxic effects of free Hb are critically concentration-dependent. This might explain acute and subacute kidney failure after transplantation and underlines the need for blood-free perfusion of organs, which critically affects the outcome of transplantations. Thus, scavenging of iron ions, e.g. by DFO, might be a beneficial approach for the composition of perfusion solutions.

Authorship

MRM and GG, study design; MRM, performing of experiments; MRM and JES, data analysis and writing; OK, MDM, MKS and GG, critical review and comments.

References

1. Aydogdu N, Atmaca G, Yalcin O, Taskiran R, Tastekin E, Kaymak K. Protective effects of L-carnitine on myoglobinuric acute renal failure in rats. *Clin Exp Pharmacol Physiol* 2006; **33**: 119.
2. Gronow G, Moussavian M, Malyusz M. Effect of hydroxyl radical scavengers in renal cortical cells. *Adv Exp Med Biol* 1999; **471**: 345.
3. Klause N, Arendt T, Lins M, Gronow G. Hypoxic renal tissue damage by endothelin-mediated arterial vasoconstriction during radioangiography in man. *Adv Exp Med Biol* 1998; **454**: 225.
4. Salvemini D, Cuzzocrea S. Oxidative stress in septic shock and disseminated intravascular coagulation. *Free Radic Biol Med* 2002; **33**: 1173.
5. Chen M, Xiao CY, Hashizume H, Abiko Y. Phospholipase A2 is not responsible for lysophosphatidylcholine-induced damage in cardiomyocytes. *Am J Physiol* 1998; **275**: 1782.
6. Salvemini D, Doyle TM, Cuzzocrea S. Superoxide, peroxynitrite and oxidative/nitrative stress in inflammation. *Biochem Soc Trans* 2006; **34**: 965.
7. Paller MS, Patten M. Protective effects of glutathione, glycine, or alanine in an in vitro model of renal anoxia. *J Am Soc Nephrol* 1992; **2**: 1338.
8. Sikujara O, Monden M, Toyoshima K, Okamura J, Kosaki G. Cytoprotective effect of prostaglandin I2 on ischemia-induced hepatic cell injury. *Transplantation* 1983; **36**: 238.
9. Gronow G, Kelting T, Skrezek C, vd Plas J, Bakker JC. Oxygen transport to renal tissue: effect of oxygen carriers. *Adv Exp Med Biol* 1987; **215**: 117.
10. Weinberg JM. The cell biology of ischemic renal injury. *Kidney Int* 1991; **39**: 476.
11. Zager RA, Sacks BM, Burkhart KM, Williams AC. Plasma membrane phospholipid integrity and orientation during hypoxic and toxic proximal tubular attack. *Kidney Int* 1999; **56**: 104.
12. Kim NH, Kang JH. Oxidative damage of DNA induced by the cytochrome C and hydrogen peroxide system. *J Biochem Mol Biol* 2006; **39**: 452.
13. Duann P, Datta PK, Pan C, Blumberg JB, Sharma M, Lianos EA. Superoxide dismutase mimetic preserves the glomerular capillary permeability barrier to protein. *J Pharmacol Exp Ther* 2006; **316**: 1249.
14. Huffman LJ, Miles PR, Shi X, Bowman L. Hemoglobin potentiates the production of reactive oxygen species by alveolar macrophages. *Exp Lung Res* 2000; **26**: 203.
15. Gronow GH, Cohen JJ. Substrate support for renal functions during hypoxia in the perfused rat kidney. *Am J Physiol* 1984; **247**: 618.
16. Hashimoto T, Hirata M, Itoh T, Kanmura Y, Kuriyama H. Inositol 1,4,5-trisphosphate activates pharmacomechanical coupling in smooth muscle of the rabbit mesenteric artery. *J Physiol* 1986; **370**: 605.
17. Gronow G, Klause N, Malyusz M. Restriction of hypoxic membrane defect by glycine improves mitochondrial and cellular function in reoxygenated renal tubules. *Adv Exp Med Biol* 1994; **361**: 585.
18. Jain SK, Morshed KM, Kannan K, McMartin KE, Bocchini Jr JA. Effect of elevated glucose concentrations on cellular lipid peroxidation and growth of cultured human kidney proximal tubule cells. *Mol Cell Biochem* 1996; **162**: 11.
19. Furukawa Y, O'Halloran TV. Posttranslational modifications in Cu, Zn-superoxide dismutase and mutations associated with amyotrophic lateral sclerosis. *Antioxid Redox Signal* 2006; **8**: 847.
20. Chen CF, Tsai SY, Ma MC, Wu MS. Hypoxic preconditioning enhances renal superoxide dismutase levels in rats. *J Physiol* 2003; **552**: 561.
21. Foresti R, Green CJ, Motterlini R. Generation of bile pigments by haem oxygenase: a refined cellular strategy in response to stressful insults. *Biochem Soc Symp* 2004; **71**: 177.
22. Martin FM, Bydlon G, Friedman JS. SOD2-deficiency sideroblastic anemia and red blood cell oxidative stress. *Antioxid Redox Signal* 2006; **8**: 1217.
23. Baliga R, Ueda N, Walker PD, Shah SV. Oxidant mechanisms in toxic acute renal failure. *Am J Kidney Dis* 1997; **29**: 465.
24. Gwozdzinski K, Janicka M. Oxygen free radicals and red blood cell damage in acute renal failure. *Biochem Soc Trans* 1995; **23**: 635.
25. Dittrich S, Groneberg DA, von Loeper J, *et al.* Influence of cold storage on renal ischemia reperfusion injury after non-heart-beating donor explantation. *Nephron Exp Nephrol* 2004; **96**: 97.

26. Paller MS. The cell biology of reperfusion injury in the kidney. *J Investig Med* 1994; **42**: 632.
27. Dhar-Mascareno M, Carcamo JM, Golde DW. Hypoxia-reoxygenation-induced mitochondrial damage and apoptosis in human endothelial cells are inhibited by vitamin C. *Free Radic Biol Med* 2005; **38**: 1311.
28. Gille L, Nohl H. The ubiquinol/bc1 redox couple regulates mitochondrial oxygen radical formation. *Arch Biochem Biophys* 2001; **388**: 34.
29. El-Sayed IH, Lotfy M, El-Khawaga OA, Nasif WA, El-Shahat M. Prominent free radicals scavenging activity of tannic acid in lead-induced oxidative stress in experimental mice. *Toxicol Ind Health* 2006; **22**: 157.
30. Hughes G, Murphy MP, Ledgerwood EC. Mitochondrial reactive oxygen species regulate the temporal activation of nuclear factor kappaB to modulate tumour necrosis factor-induced apoptosis: evidence from mitochondria-targeted antioxidants. *Biochem J* 2005; **389**: 83.
31. Zager RA, Burkhart K. Myoglobin toxicity in proximal human kidney cells: roles of Fe, Ca2+, H_2O_2, and terminal mitochondrial electron transport. *Kidney Int* 1997; **51**: 728.
32. Bertermann H, Gronow G, Weiss C. An improved technique for metabolic studies on isolated cortical cells and tubules of the rat kidney. *Curr Probl Clin Biochem* 1975; **4**: 76.
33. Gueye PM, Glasser N, Ferard G, Lessinger JM. Influence of human haptoglobin polymorphism on oxidative stress induced by free hemoglobin on red blood cells. *Clin Chem Lab Med* 2006; **44**: 542.
34. Valko M, Morris H, Cronin MT. Metals, toxicity and oxidative stress. *Curr Med Chem* 2005; **12**: 1161.
35. Griguer CE, Oliva CR, Kelley EE, Giles GI, Lancaster Jr JR, Gillespie GY. Xanthine oxidase-dependent regulation of hypoxia-inducible factor in cancer cells. *Cancer Res* 2006; **66**: 2257.
36. Littauer A, de Groot H. Release of reactive oxygen by hepatocytes on reoxygenation: three phases and role of mitochondria. *Am J Physiol* 1992; **262**: 1015.
37. Misiaszek R, Uvaydov Y, Crean C, Geacintov NE, Shafirovich V. Combination reactions of superoxide with 8-Oxo-7,8-dihydroguanine radicals in DNA: kinetics and end products. *J Biol Chem* 2005; **280**: 6293.
38. Liu M, Okada S. Induction of free radicals and tumors in the kidneys of Wistar rats by ferric ethylenediamine-*N,N'*-diacetate. *Carcinogenesis* 1994; **15**: 2817.
39. Fargion S, Mandelli C, Piperno A, *et al.* Survival and prognostic factors in 212 Italian patients with genetic hemochromatosis. *Hepatology* 1992; **15**: 655.
40. Hastie LE, Patton WF, Hechtman HB, Shepro D. Filamin redistribution in an endothelial cell reoxygenation injury model. *Free Radic Biol Med* 1997; **22**: 955.
41. Kerkweg U, Li T, de Groot H, Rauen U. Cold-induced apoptosis of rat liver cells in University of Wisconsin solution: the central role of chelatable iron. *Hepatology* 2002; **35**: 560.