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Ziya Akçetin · Alexander Busch
Guido Kessler · Hans Heynemann
Jürgen Holtz · Hans-Jürgen Brömme

Evidence for only a moderate lipid peroxidation during ischemia-reperfusion of rat kidney due to its high antioxidative capacity

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Abstract The extent of lipid peroxidation after ischemia-reperfusion (I-R) injury in rat kidney has been controversial. After I, xanthine oxidase (XO) is thought to be the main oxygen radical-generating system and malondialdehyde (MDA) is considered to be a marker of lipid peroxidation (LPO). In young rats (10 weeks old) a unilateral warm I of 40 and 60 min duration with subsequent R up to 1 h was conducted. Beside the "footprints" of oxidative stress, the cytosolic antioxidative capacity, expressed as superoxide anion (SOA) scavenging capacity, and the renal catalase were also investigated. There was only a moderate and transient increase of renal MDA 5 and 10 min after the onset of reoxygenation (133.57/70.67 and 97.84/91.57 vs. 49.47 nmol/g ww in preischemic controls). ATP breakdown (to 83/65 from 2947 nmol/g ww) with consecutive accumulation of hypoxanthine (up to 1105 nmol/g ww) at the end of ischemic period and the subsequent rapid decline of hypoxanthine by XO during reperfusion were used for an assessment of the SOA-generating capacity of these kidneys. Superoxide dismutase (SOD) activity, glutathione (GSH) and the high activity of catalase (18000 U/g ww) remained nearly unchanged during R. Only 1/25–1/50 of the kidney cytosol was able to scavenge the whole amount of SOA generated by the total XO activity of rat kidney. Thus, it could be analytically and stoichiometrically shown that after IR there is only a moderate oxidative stress in kidneys of young rats; this is due to their high SOA-scavenging capacity compared with their SOA-generating ability.

Key words Oxygen radicals · Ischemia-reperfusion · Lipid peroxidation · Oxidative stress · Antioxidants

Z. Akçetin (✉) · A. Busch · G. Kessler · H. Heynemann
Department of Urology, University of Halle-Wittenberg,
Magdeburger Strasse 16, D-06097 Halle, Germany

J. Holtz · H.-J. Brömme
Department of Pathophysiology,
University of Halle-Wittenberg, Halle, Germany

Introduction

The temporary discontinuation of renal blood supply is a consequence of diverse clinical conditions such as renal transplantation, aortic aneurysm surgery or hypotension due to resuscitation. The tissue changes caused by ischemia are well known. Upon depletion of energy rich phosphates (ATP), the tissue concentration of their degradation products (hypoxanthine) rises [10, 15, 17, 29, 41, 43]. Hypoxanthine is the substrate for the xanthine oxidase reaction, by which superoxide anions (SOA) are produced [11] in the presence of oxygen. SOA is further metabolized by the superoxide dismutase (SOD) reaction to hydrogen peroxide, which can be detoxified, e.g., by catalase and/or glutathione peroxidase. But it can also be the substrate for the Fenton reaction, by which the hydroxyl radical, a highly reactive substance, is produced, or it can react with the NO-radical to form peroxynitrite; both are capable of damaging tissue due to their ability to peroxidize lipid membranes [3, 13, 34, 40, 44, 45]. Therefore, the reperfusion, although it is essential for tissue survival, increases the tissue damage caused by the ischemia [26]. On the other hand, the kidney is equipped with a large antioxidant defence arsenal. Radical-mediated cellular damage would be expected to occur when oxygen radical production exceeds the cellular detoxification capacity. In the study presented here, the tissue footprints of oxidative stress after unilateral warm ischemia of the rat kidney were examined. The goals of this study were to determine whether an oxidative stress with significant lipid peroxidation (LPO) occurs and to analyze the stoichiometry of the discussed reactions.

Materials and methods

Unilateral warm renal ischemia was conducted on male Wistar rats (10 weeks old) for 40–60 minutes under pentobarbital anesthesia (100 mg/kg bw i.p.). After reperfusion times of 0, 5, 10, 30 and 60 min (n = 10 for each reperfusion group), the left nephrectomy

was performed. Kidneys from rats not exposed to ischemia-reperfusion, but to anesthesia were taken as controls. Part of the kidney was stopped frozen in situ in liquid nitrogen for quantitative determination of ATP (bioluminescence assay kit CLS II, Boehringer Mannheim, Germany), xanthine oxidase activity, hypoxanthine (by using the standard spectral photometric methods) and catalase activity [1]; the remaining part of the kidney was used to analyze malondialdehyde (MDA), SOD and glutathione (GSH) (lipid peroxidation, SOD, GSH assay kits, Calbiochem, La Jolla, Calif.).

In a second experimental setup, supernatant (representing mainly the cytosol) from kidney homogenates was added at different volumes and dilutions for inhibiting the chemiluminescence (CL)-generation by the xanthine oxidase (XO) reaction with a defined XO activity (3 mU) using lucigenin as a CL-enhancer to quantify the cytosolic antioxidative capacity of the rat kidney. The kidney was washed and minced in ice-cold 20 mM TRIS-HCl and homogenized using a Potter-homogenizer with a Teflon pestle and diluted with ice-cold TRIS-HCl to approximately 10% (W/v). After the centrifugation of the diluted homogenates at 3000 *g* for 10 min at 4°C, the supernatant was centrifuged again at 21 000 *g* for 20 min at 4°C. The resulting supernatant was used undiluted or diluted to scavenge SOA generated via XO + X reaction in a luminometer. The reaction mixture contained 915 µl Phosphate buffered saline (PBS)-Hepes, pH 8,0, 5 µl XO (3 mU in final mixture (fm), 50 µl Lucigenin (50 µM in fm), 20 µl xanthine (0,2 mM in fm) and 10 µl supernatant at different dilutions. The reaction was started by adding the xanthine. After 5 min of CL-registration, the SOA-dependent lucigenin-CL declined gradually by administration of different dilutions of supernatant. The CL was measured by using a luminometer (Berthold, Luminal LB 9501, Wildbad, Germany).

The results were expressed as mean ± SEM. The statistical significance was determined using the unpaired Student's *t*-test.

During the animal experiments, principles of laboratory animal care were followed, as was the current version of the "German Law on the Protection of Animals".

Results

The measured footprints of oxidative stress are listed in Table 1. The ATP content in ischemic kidney decreased from 2947 ± 313 nmol/g ww to less then 3% after 40 min and 2% of the initial value after 60 min, respectively. A major part of the utilized ATP was broken down to hypoxanthine (553 ± 111 and 1105 ± 255 nmol/g ww), which was quantitatively metabolized within 5–10 minutes after reoxygenation (Figs. 1, 2). The XO activity of ischemic kidneys was about 60 mU/g ww; the high catalase activity was not influenced by ischemia (Table 2). Renal SOD activity (about 25 U_{525}/g ww) as well as GSH content remained unchanged during the reperfusion period after 40 min of ischemia; after 60 min of ischemia there was a slight decrease (nonsignificant) of the GSH content and a temporary decrease of the SOD activity 5 min after reoxygenation; 10 to 60 min after reoxygenation SOD activity returned to ischemic values. Renal MDA was, after ischemia, significantly higher than in the nonischemic kidney; a slight increase – compared with pre-ischemic values – was observed 5 and 10 min after reoxygenation. Changes in the MDA concentrations were, in sum, inhomogenous

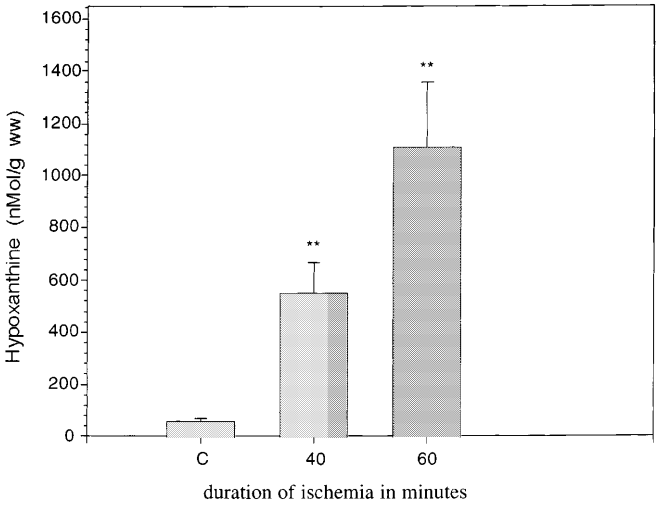

Fig. 1 Hypoxanthine accumulation in the rat kidney during warm ischemia. $^{**}P < 0.01$, $n = 5$; *C* nonischemic control rats

Table 1 Footprints of oxidative stress. Expressed as $x \pm$ SEM; controls vs. reperfusion; I_{40} and I_{60}, 40 and 60 min ischemia; R_x, *x* min after reperfusion; C, controls. *ATP* adenosine triphosphate, *SOD* Superoxide dismutase, *GSH* glutathione, *MDA* malondialdehyde

		C	R_0	R_5	R_{10}	R_{30}	R_{60}
I_{40}	ATP (nmol/g ww)	2947 ± 313	83 ± 14*	169 ± 16*	1060 ± 152*	745 ± 178*	701 ± 21*
I_{60}	ATP (nmol/g ww)		65 ± 17*	655 ± 113*	371 ± 75*	880 ± 211*	682 ± 139*
I_{40}	SOD (U_{525}/g ww)	25.32 ± 1.34	22.80 ± 1.65 (n. s.)	29.64 ± 2.86 (n. s.)	34.56 ± 0.75**	29.48 ± 1.37 (n. s.)	13.44 ± 1.68**
I_{60}	SOD (U_{525}/g ww)		24.94 ± 1.14 (n. s.)	15.10 ± 1.11**	23.06 ± 2.38 (n. s.)	31.97 ± 2.92 (n. s.)	26.49 ± 3.76 (n. s.)
I_{40}	GSH (µmol/g ww)	1.60 ± 0.08	0.92 ± 0.02*	1.07 ± 0.05*	1.02 ± 0.07*	0.97 ± 0.06*	1.18 ± 0.02*
I_{60}	GSH (µmol/g ww)		1.44 ± 0.04 (n. s.)	1.01 ± 0.04*	1.14 ± 0.05*	1.00 ± 0.04*	1.04 ± 0.03*
I_{40}	MDA (nmol/g ww)	49.47 ± 3.19	82.78 ± 6.22*	133.57 ± 12.62*	97.84 ± 6.37*	90.08 ± 10.95*	154.65 ± 28.54*
I_{60}	MDA (nmol/g ww)		126.26 ± 5.41*	70.67 ± 2.46*	91.57 ± 5.48*	53.64 ± 4.62 (n. s.)	100.78 ± 5.46*

$^{*}P < 0.001$; $^{**}P < 0.01$; n.s. not significant

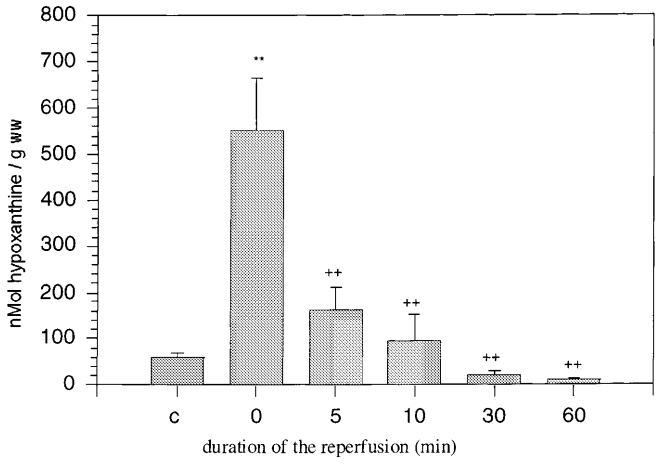

Fig. 2 Utilization rate of hypoxanthine during reoxygenation after 40 min of ischemia. *c* nonischemic control rats, controls vs. ischemic kidneys $**P < 0.01$, ischemic vs. reperfused kidneys $++P < 0.01$, $n = 5$

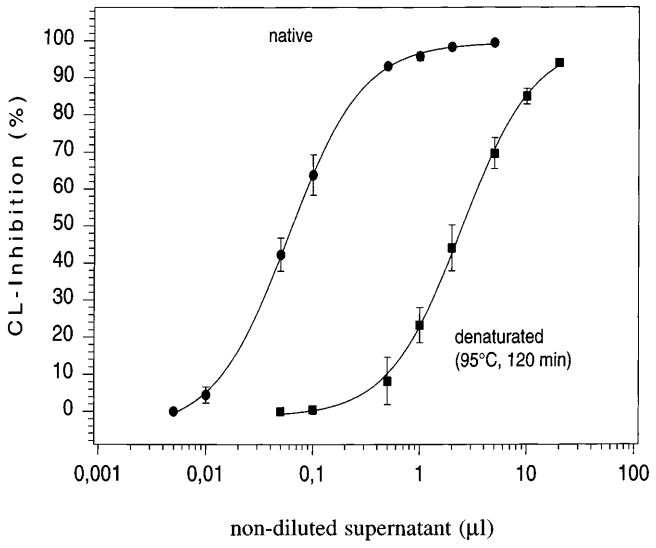

Fig. 3 Dose–response curves of native and heat-denaturated supernatant (mainly the cytosolic fraction of the kidney; for more detail see Materials and methods). *CL* chemiluminescence

and without further tendency to change. Supernatant (0.5–1.0 µl) was able to neutralize quantitatively the SOA, which was generated by 3 mU XO. This high scavenging capacity was lost after heating the supernatant to 95°C (Fig. 3).

Discussion

The extent of LPO in the kidney after ischemia-reperfusion (IR) is udner debate in literature. Although several investigators reported on increased renal MDA concentrations after IR as an index of LPO [7, 12, 19, 20, 25, 31], other groups have disputed extensive LPO [4, 6, 9].

The classical determination of MDA by the thiobarbituric acid (TBA) method is compromised by many interfering agents and has a poor reproducibility [16]. This can lead to an overestimation of the MDA content. Trapped erythrocytes due to the no-reflow phenomenon [2, 24, 32] could be identified as a source for false-positive high MDA values after IR [8, 9, 18]. Joannidis et al. [18] could show a temporary increased arteriovenous MDA difference in ischemic kidneys only 5 min after reperfusion. Gamelin and Zager [9] were unable to detect increased MDA tissue levels after 15 min of reperfusion following a 45-min interval of renal ischemia. In an in vitro model of proximal tubular ischemia, Borkan and Schwartz [4] could not demonstrate an enhanced LPO, although alterations of respiratory function could be detected. Cristol et al. [6] could demonstrate only a moderate LPO after 60 min of ischemia in rat kidney.

In accordance with these findings, our analysis of footprints of oxidative stress show only evidence for a slight to moderate LPO. The finding of slightly increased renal MDA concentrations in the early reperfusion period in our experiments is in accordance with our hypoxanthine measurements. At the end of the ischemic period we could demonstrate the highest hypoxanthine concentrations.

After 5–10 min hypoxanthine was completely metabolized. Hypoxanthine is the substrate for the XO reaction, therefore, during this time significant amounts of SOA radical were expected. The further utilization of the toxic SOA could reduce renal SOD activity. Since the radicals show specific toxic side-effects, the SOD is the enzyme which is directly involved in the metabolism of SOA and could therefore be affected. However, enhanced gene expression for SOD due to the oxidative stress is also reasonable.

As discussed below, we believe that the XO is the main SOA-generating system after IR in the rat kidney. The SOA generation rate of XO is measured as 60 nmol/min per gram. Compared with the determined SOA-utilisation rate of about 25 µmol/min per gram ww, the SOA-generation rate is about 400-fold lower. Therefore, the unchanged SOD-values after IR are not surprising.

Table 2 Renal enzyme activity (expressed as $x \pm$ SEM; n.d. not determined)

	Controls	Ischemic kidneys	
		40 min	60 min
Xanthine oxidase (mU/g ww)	n.d.	60 ± 18	62 ± 23
Catalase (U/g ww)	18 150 ± 3435	17 900 ± 4100	17 700 ± 5235

The SOA-generation is also limited by the amount of hypoxanthine at the onset of reperfusion. Based on about 1100 nmol/g ww after 60 min of reperfusion and a mean weight of 1.3 g/rat kidney, the calculated maximal SOA amount is 2.8 μmol. In the case of a disturbed antioxidative capacity of the kidney, 2.8 μmol SOA would in the presence of Fe^{2+} or Cu^{1+} lead to the generation of significant amounts of hydroxyl radical [26] and therefore cause extensive LPO [14].

The oxidative stress is a disturbance in the prooxidant–antioxidant balance in favor of the former. In our second experimental setup, we demonstrated that 0.5–1.0 μl supernatant from kidney homogenate could quantitatively neutralize the SOA generated by a XO activity of 3 mU. This accounts nearly for 5% of the total XO activity of a rat kidney. Stoichiometrically, 10–20 μl supernatant could therefore neutralize the whole amount of SOA generated by the total XO activity of a rat kidney. Since we estimated the cytosol of a rat kidney as 400 μl/g ww, the total SOA-utilizing capacity of a rat kidney was calculated to be 25 to 50-fold higher than the SOA-generating capacity. Nath et al. [28] also pointed out the high antioxidative capacity of 3-weeks-old rats. They were not able to show LPO in these rats, but after dietary inhibition of the antioxidative enzymes LPO was demonstrated. By lowering this SOA-scavenging capacity of the supernatant after heating, we could demonstrate that the mean antioxidative capacity is represented by proteins, most likely SOD. Hydrogen peroxide, the product of the SOD reaction, can be detoxified mainly by catalase and/or glutathione peroxidase. The high catalase activity in renal tissue and the nearly unchanged GSH content during the reperfusion period in our experiment argue for a more important role of the catalase in the detoxification of hydrogen peroxide rather than the glutathione peroxidase. The finding of a decline of GSH after ischemia and unchanged levels in the first 1–2 h after reoxygenation is in accordance with other investigators' findings [35, 38].

In our stoichiometric considerations XO was emphasized as the main SOA-generating system. Mitochondria are under normal circumstances capable of producing significant amounts of reactive oxygen species [5, 37]. But in the early reperfusion period after ischemia the oxygen concentration will not be high enough for mitochondrial SOA generation [37]. Oxygen radicals are also formed by polymorphonuclear granulocytes; however, in the very early reperfusion period there is no significant leukocyte migration [42] and in many studies no cytoprotection was observed, either in which neutrophils were depleted [30, 33, 39] or monoclonal antibodies against adhesion molecules were administered [36]. We are thus left with XO as the main SOA generator in the very early reperfusion period.

Conclusions

In the very early reperfusion period after ischemia, XO is the main oxygen radical-generating system. It is able to produce significant amounts of SOA, which is a prerequisite to a subsequent extensive LPO. That only slight to moderate LPO was observed after IR of the rat kidney is due to the demonstrated high cytosolic antioxidative capacity of young rats used in our experiment. The conflicting results from transplantation studies with or without impact of antioxidants on the outcome and on the LPO should again be discussed in the light of this high antioxidative capacity of young kidneys [21, 22, 23].

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