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ORIGINAL PAPER

The carboxyproxyl-derived spin trap (CP-H) is an appropriate detector-compound for oxidative stress

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Abstract Reperfusion of ischemic tissue disturbs the balance between reactive oxygen species (ROS) and the cellular antioxidative defense. This imbalance is known as oxidative stress. In this study the spin trap 3-carboxy-2,2,5,5-tetramethylpyrrolin-1-hydroxide (CP-H) with its ESR-detectable paramagnetic analogue 3-carboxy-2,2,5,5-tetramethylpyrrolin-1-oxyl (•CP) was analyzed in vitro and in vivo. In preliminary in vitro experiments we studied the interaction of CP-H with reactive compounds like hydroxyl radicals (•OH) and alkylperoxyl radicals (ROO•) which are formed during organ reperfusion or tissue reoxygenation. The increase in the peak intensity of the ESR signal of the •CP-radical was used as a measure for CP-H oxidation by the above-mentioned oxidizing radicals. It could be clearly shown that •OH as well as ROO• induce CP-H oxidation. The intensity of the ESR signal (•CP) depends on the concentration of the applied oxidant. In a further set of in vitro experiments we analyzed some factors influencing the stability of the generated •CP. Cellular reductants are able to interact with many radicals whereby their paramagnetic signal intensity decreases. We could show that glutathione (GSH) up to 5 mM does not influence •CP concentration. On the other hand, ascorbate at a concentration of 0.6 mM significantly reduces 55% of •CP within 60 min to the ESR-silent CP-H. At 1 mM ascorbate the •CP derived ESR signal is reduced within 60 min by 90%. Lower concentrations of ascorbate (0.1–0.3 mM) do not significantly decrease signal intensity within 1 h. Homogenization of ischemic rat kidney in the presence of an air-equilibrated buffer obviously induces the formation of oxidizing radicals which in turn are able to convert diamagnetic CP-H into paramagnetic •CP. The intensity of the formed •CP was analyzed in a 600 g supernatant with ESR spectroscopy at 25°C. It could be demonstrated that at least 3.0 ± 0.5 μM •CP is formed 15 min after starting tissue homogenization and reoxygenation. Subsequent measurements of the •CP concentration indicated that its signal intensity continuously decreases. After 75 min a residual •CP concentration of 0.7 ± 0.3 μM was monitored. Removal of mitochondria from the homogenate by centrifugation at 6,000*g* decelerates the disappearance of •CP but does not block it completely. In summary it could be shown that the marker (CP-H) is able to indicate the formation of oxidizing radicals during reoxygenation of ischemic tissue. This method underestimates the amount of produced oxidizing radicals. One reason for this is the reduction of •CP by some cellular reductants. Other reasons will be discussed. We assume that the used method allows a nearly real-time determination of radical production during organ reoxygenation.

Keywords Radicals · Spin trap · Carboxyproxyl · Oxidative stress · Rat kidney

Abbreviations

AAPH	2,2′-Azo-bis-2-amidinopropane hydrochloride
•CP	3-Carboxy-2,2,5,5-tetramethylpyrrolin-1-oxyl

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CP-H	3-Carboxy-2,2,5,5-tetramethylpyrrolin-1-hydroxide
DMPO	5,5-Dimethylpyroline-1-oxide
ESR	Electron spin resonance
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HPLC	High-pressure liquid chromatography
NADPH	Nicotinamide adenine dinucleotide phosphate
$\bullet O_2^-$	Superoxide anion radical
•OH	Hydroxyl radical
PMN	Polymorphonuclear leukocytes
ROO•	Alkylperoxyl radical
ROS	Reactive oxygen species

Introduction

Due to the inability to cover the high demand on organs in transplantation medicine, it has become more and more important to prevent organ failure in the host. Primary tissue alterations are a result of ischemia reperfusion injury, which can also appear in organ-maintaining tumor surgery. Reperfusion of an ischemic tissue increases ROS production. ROS include superoxide anion radicals ($\bullet O_2^-$), hydroxyl radicals (•OH), and hydrogen peroxide (H_2O_2) as well as a variety of organic radicals such as alkyl and peroxyl radicals [1, 2]. The increasing imbalance between radical production and antioxidant defense, called oxidative stress, characterizes this injury [3, 4]. Generation of ROS and non-radical oxidants after reperfusion can directly induce PMN activation and adhesion to vascular endothelium triggering a subsequent leukocyte-mediated injury which in turn amplifies and accelerates further organ damage [5].

•OH appears to be one of the most aggressive and destructive radicals formed in biology [6, 7]. Under aerobic conditions •OH are generated mainly by the Haber–Weiss reaction (Eq. 3), whereby hydrogen peroxide is reductively split into •OH and OH^- by ferrous ions (Fenton reaction, Eq. 1). In the presence of superoxide anion radicals ($\bullet O_2^-$) or other reductants (e.g., ascorbate) the ferric ions can be re-reduced to ferrous ions which in turn are able to generate more •OH (Eq. 2) [4, 6].

$$H_2O_2 + Fe^{2+} \rightarrow \bullet OH + OH^- + Fe^{3+} \quad (1)$$

$$Fe^{3+} + \bullet O_2^- \rightarrow Fe^{2+} + O_2 \quad (2)$$

$$H_2O_2 + \bullet O_2^- \rightarrow \bullet OH + OH^- + O_2 \quad (3)$$

Superoxide anion radicals and hydrogen peroxide can be produced by a variety of cellular reactions. Xanthine oxidase, NADPH oxidase, monoamino oxidase, as well as the respiratory chain of mitochondria are important sites of cellular $\bullet O_2^-$ and H_2O_2 formation [6–10]. •OH is strongly electrophilic and so it is able to oxidize nearly every substance in its immediate vicinity, e.g., enzymes, nucleic acids, lipids, antioxidants, as well as some marker molecules used for detection of oxidative stress. For the measurement of radical formation during tissue reoxygenation a variety of different methods has been developed. Some studies used spin traps like 5,5-dimethylpyroline-1-oxide (DMPO) [8] or alpha-phenyl *N-tert*-butyl nitrone [10], while others analyzed the so-called footprints of oxidative stress. Such footprints are often more or less stable end products formed after interaction of ROS with nucleic acids or lipids like 8-hydroxydeoxyguanosine [11], deoxyribose degradation products [12], or malondialdehyde [13, 14]. Further methods described the formation of hydroxylated aromatic compounds [15]. None of the cited methods appears to be optimal to reflect adequately the ongoing oxidative stress during reperfusion. Especially some of the footprint methods need time-consuming sample preparations, sometimes are not specific, or do not always form stable products [16]. The use of DMPO for measurement of $\bullet O_2^-$ is limited by the short half-life of the adduct [17] and hydroxyl radical adducts of DMPO are highly vulnerable against cellular reductants [2].

The aim of this study was to analyze the suitability of CP-H as a marker for oxidative stress. We studied the influence of in vitro generated hydroxyl- and alkylperoxyl radicals on CP-H, investigated the stability of the formed •CP against cellular reductants like GSH and ascorbate, and characterized the application of this marker in experiments of reoxygenation of ischemic rat kidney tissue.

Materials and methods

3-Carboxy-2,2,5,5-tetramethylpyrrolin-1-oxyl (•CP) was supplied by Sigma–Aldrich (Taufkirchen, Germany). 3-Carboxy-2,2,5,5-tetramethylpyrrolin-1-hydroxide (CP-H) was obtained from Prof. Kirilyuk, Institute of Organic Chemistry (Novosibirsk, Russia). Ascorbic acid (sodium salt) was obtained from Fluka Chemie AG (Buchs, Switzerland). Except 2,2′-Azo-bis-2-amidinopropane hydrochloride (AAPH) supplied by Polysciences (Warrington, USA) all other chemicals were purchased from Sigma–Aldrich (Taufkirchen, Germany).

A 10 mM stock solution of •CP or CP-H dissolved in HPLC grade water was freshly prepared every day. 100 mM stock solutions of Fe^{2+} and H_2O_2 and all other chemicals were freshly dissolved every day in HPLC grade water. Sodium ascorbate was dissolved in HPLC-grade water to give a final concentration of 100 mM. In the absence of catalytic metals (Fe^{2+} or Cu^+) ascorbate does not autoxidize [18]. Dilutions from the stock were done immediately before use. The measurements were performed at 25°C.

Male Wistar rats (body weight 200–250 g) were purchased from Harlan Bioservice for Science GmbH (Walsrode, Germany), housed in a temperature-controlled room with 12 h light and darkness per day, free access to water, and standard rat chow. Rats were anesthetized i.p. with 1.5 ml/kg Rompun (20 mg/ml) and 2 ml/kg Ketavet (100 mg/ml). Additionally 1,000 U Heparin were given i.p. After full anesthesia the abdomen was opened and the left kidney was excised by cutting the distal Arteria and Vena renalis. Thereafter the kidney was incubated for 20 min at 37°C in 0.9% NaCl (warm ischemia). Subsequently the weight of the kidney was recorded, the organ was cut into small slices, and transferred into a glass tube of a 20 ml Potter–Elvehjem homogenisator containing the eightfold volume of the organ weight of an air-equilibrated homogenization buffer (0.25 M saccharose, 25 mM potassium chloride (KCl), 50 mM tris(hydroxymethyl)-aminomethane (TRIS) pH 7.4, and 0.5 mM ethylene-diamine-tetraacetate (EDTA)). An appropriately sized Teflon pestile was placed in the tube of the homogenistor and the dissected kidney tissue was carefully homogenized by 10 strokes over 5 min. During homogenization the ischemic tissue was vigorously mixed with the air-saturated buffer. Residual cell debris as well as nuclei but not the organelles were removed by centrifugation (10 min at 25°C and 600*g*) before the resulting supernatant was used for ESR-measurements. In another set of experiments mitochondria were additionally removed by centrifugation at 6,000*g*.

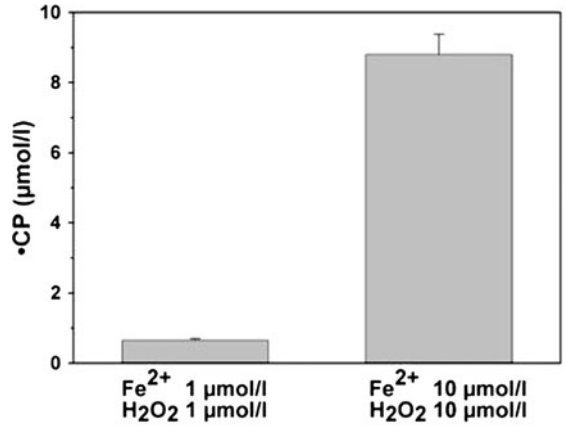
An ESR spectroscope MS100 from Magnettech GmbH (Berlin-Adlershof, Germany) was used for monitoring the formation of •CP. Glass capillaries purchased by Brand (Wertheim, Germany) containing 50 µl of the sample were sealed with plasticine and the ESR spectra were analyzed at the following instrument settings: center field 3,368 G, sweep width 100 G, sweep time 60 s, modulation amplitude 1,000 mG, receiver gain 50, and power attenuation 6 db. Since the cavity of the ESR spectrometer cannot be heated to 37°C, kidney homogenization as well as the ESR measurements was performed at ambient temperature (25 ± 2°C). The ESR signal of •CP was analyzed using the peak-to-peak amplitude of the low-field line (I_0). According to Schößler et al. [19] calculation, the •CP concentration was done using •CP as spin standard.

Data are presented as mean ± standard error of mean (SEM) of at least 3–8 independent measurements. Data were considered to be significant at $p < 0.05$ (*t*-test).

Results

Fenton-generated •OH are able to oxidize CP-H to •CP in vitro. The formation of •CP can be easily monitored by ESR spectroscopy. As a result of the oxidation of CP-H a three-lined ESR spectrum appears. Its signal intensity is a measure of the formed •CP (insert in Fig. 1b). The extent of CP-H oxidation depends on the amount of the formed •OH which in turn depends on the concentration of transition metals and hydrogen peroxide. Increasing the concentration of Fe^{2+} increases the quantity of the formed •CP ($p < 0.001$). In the absence of either H_2O_2 or Fe^{2+} CP-H cannot be oxidized (not shown).

Since •OH can initiate lipid peroxidation some other oxidizing radicals are formed during organ reperfusion also. Thus AAPH at a final concentration of 50 mM produces



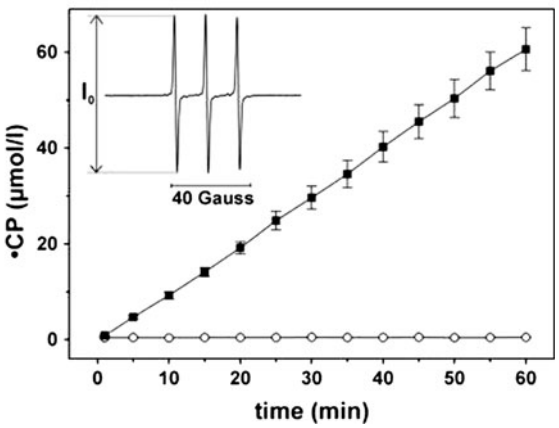


Fig. 1 CP-H and radicals. **a** CP-H and •OH. All batches contain 10 µl of CP-H (10 mmol/l) dissolved in water. The *first bar* shows measurements with 10 µl of Fe^{2+} (10 µmol/l), 10 µl H_2O_2 (10 µmol/l) and 70 µl water, the *second bar* shows the same batch with 10 µl Fe^{2+} (100 mmol/l) and 10 µl H_2O_2 (100 mmol/l). All samples were measured at 25°C. Data are given as mean ± SEM, $n = 5$, $p < 0.001$. **b** CP-H and R•/ROO• (originated from AAPH). The batch contains 10 µl of CP-H (10 mmol/l) dissolved in water without AAPH (*open circles*) or with 10 µl AAPH (500 mmol/l) (*filled squares*) and 90 or 80 µl water at 25°C. At this temperature the thermal decay of AAPH to R• and in the presence of oxygen to ROO• is of constant rate. Data are given as mean ± SEM, $n = 3$, $p < 0.001$. The insert documents the ESR spectrum of •CP

alkyl- and peroxyl radicals by its thermal decay at a constant rate of about 2 µM/min at 25°C [20]. Both AAPH derived radicals are able to oxidize CP-H to •CP in vitro. Figure 1b demonstrates that the rate of CP-H oxidation is proportional to the rate of the decay of AAPH. As a result •CP accumulates in the batch over time. Without AAPH (open circles) no CP-H oxidation takes place.

In a further set of experiments we analyzed different cellular reductants in relation to their ability to reduce •CP. GSH up to a concentration of 5 mM was not able to rereduce •CP to CP-H (Fig. 2). Contrary to GSH, ascorbate reduced •CP to an ESR-silent derivative (CP-H) at concentrations above 300 µM (Fig. 2). At 600 µM (filled triangles) •CP is reduced to CP-H by 45% ($p < 0.001$) within 60 min. 1 mM (open triangles) ascorbate reduces 90% of •CP within 50 min ($p < 0.001$).

Since kidney homogenate contains beside both reductants some other reducing systems, e.g., electron transporting chains of mitochondria and of endoplasmic reticulum, it can reduce added •CP ($p < 0.001$) in vivo. 15 min after the addition of •CP to the homogenate, 27 ± 0.8% of the radical were reduced, and about 46 ± 1.4% after 60 min (Fig. 3, open squares) ($p < 0.001$). In the absence of homogenate the concentration of •CP did not change within 60 min at 25°C. (Fig. 3, open circles). Depletion of mitochondria from homogenate by centrifugation at 6,000*g* significantly diminishes its reducing capacity. After 60 min no more than 34 ± 2.6% of the initially present 90 µM •CP were reduced (Fig. 3, filled squares) ($p < 0.001$).

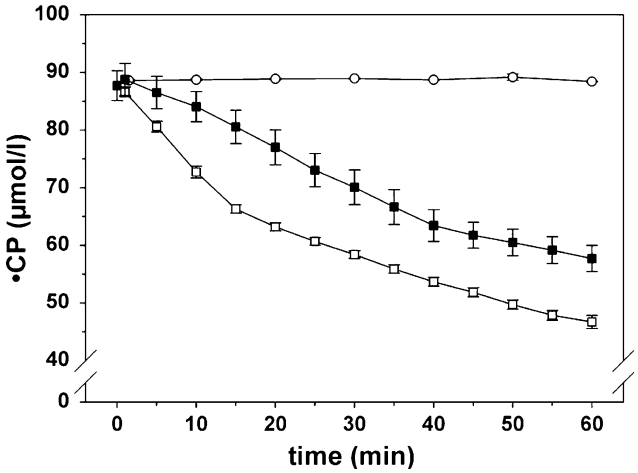


Fig. 3 Influence of the reoxygenated rat kidney homogenate on •CP. The batch contains 9 µl •CP (1 mmol/l) dissolved in water (*open circles*) with 91 µl of 600 g supernatant of rat kidney homogenate (*open squares*) or 6000 g supernatant of rat kidney homogenate (*open squares*) at 25°C within 60 min. Data are given as mean ± SEM, $n = 6$, $p < 0.001$

In an alternative experiment CP-H (1 mM) was added to the oxygen equilibrated homogenization buffer before the kidney was homogenized and therefore reoxygenated. As a consequence radicals were produced and the transformation of CP-H to •CP started (Fig. 4, short dash, rising part). As homogenization, equilibration with air and centrifugation took altogether 15 min, no measurements were possible during this period of time. So the short dash line in Fig. 4 symbolizes the theoretical development of the concentration

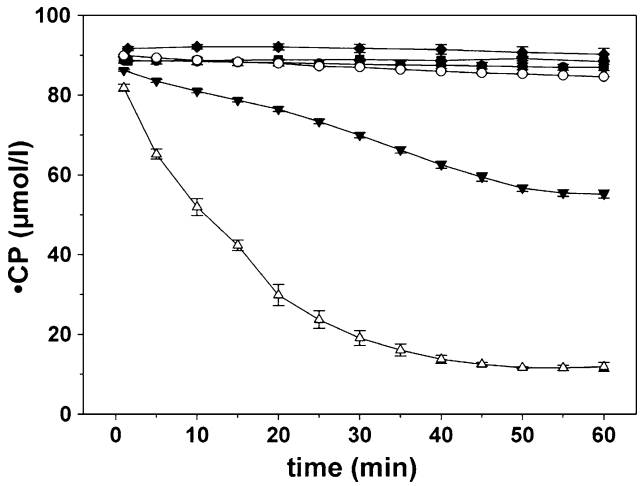


Fig. 2 •CP and cellular reductants. The batch contains 9 µl •CP (1 mmol/l) dissolved in water, and different concentrations of ascorbate or GSH measured at 25°C: *filled circles* control, •CP without ascorbate or GSH; *filled diamonds* •CP reduction by 0.1 mmol/l ascorbate; *open circles* •CP reduction by 0.3 mmol/l ascorbate; *filled triangles* •CP reduction by 0.6 mmol/l ascorbate; *Open triangles* •CP reduction by 1 mmol/l ascorbate; *filled squares* •CP reduction by 5 mmol/l GSH. Data are given as mean ± SEM, $n = 6$, $p < 0.001$

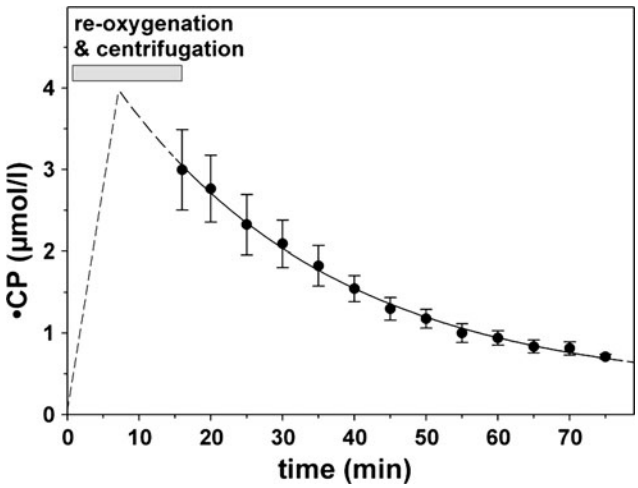


Fig. 4 Influence of the reoxygenated rat kidney homogenate on CP-H. Oxidation of CP-H to •CP by radicals produced during reoxygenation of the ischemic rat kidney at 25°C. With homogenization of an ischemic tissue, the amount of oxygen increases, therefore this has to be seen as a reoxygenation. 10 µl of CP-H (10 mmol/l) was added to 90 µl homogenate. Homogenization and measurements were done at 25°C. Data are given as mean ± SEM, $n = 8$, $p < 0.001$. Theoretical amount of •CP before and after measurements as *short dashed line*

of •CP, whereby the supposed slope as well as the position of the turning point can not be exactly determined. The first determined concentration of •CP by ESR was 3.0 ± 0.5 μM 15 min after reoxygenation. In the following 75 min most of the initially formed •CP was reduced by the homogenate (Fig. 4) ($p < 0.001$). With the kinetic of this homogenate induced decline of •CP the maximal concentration can be extrapolated. It appears to be about 5 μM.

Discussion

ROS are unavoidably produced after starting reperfusion/reoxygenation of an ischemic organ. Furthermore, they are at least partially responsible for tissue damage and might be involved in organ failure [6, 21, 22]. A direct measurement of generated radicals is complicated because of their high reactivity and short half-life. Therefore, the search for effective markers indicating the onset and the duration of ROS production are of special interest not only for transplantation medicine. A large variety of spin traps and so-called footprints of oxidative stress has been already analyzed [16, 23]. But none of them fulfills all criteria requested for an optimal marker [2].

ROS are often formed within cells but not all ROS can leave the place of their production. A suitable detector should reach the place of ROS generation. According to Fink et al. [24] CP-H can cross membranes and hence penetrates into cells. Furthermore it has been shown that CP-H appears to be able to interact with oxidizing radicals, at least, in vitro [24–26].

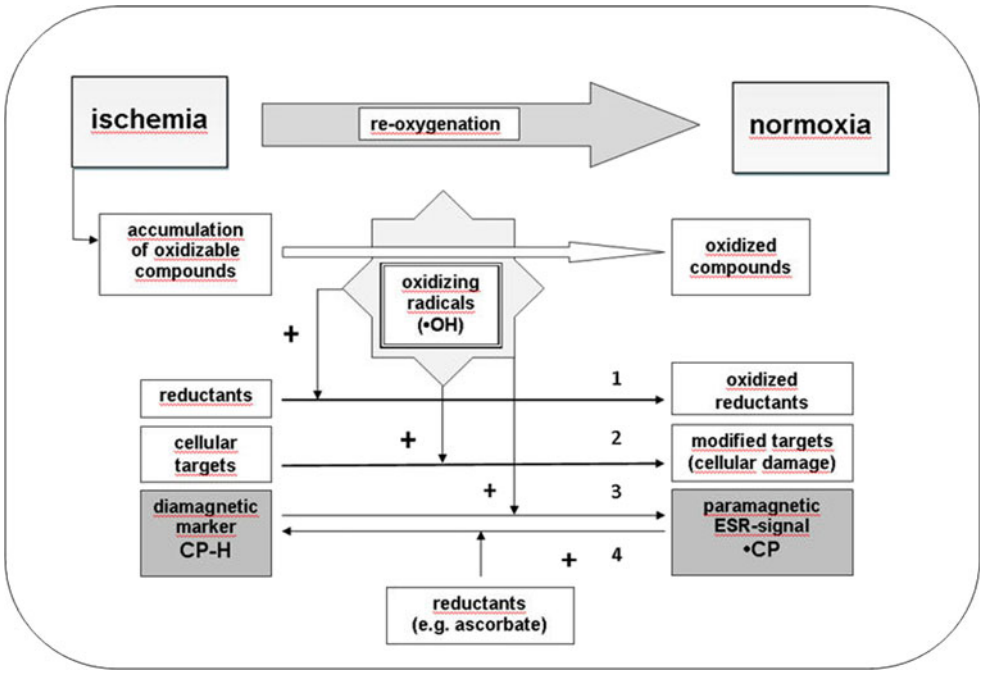
Among others, hydroxyl radicals as well as alkyl- or alkylperoxyl radicals are formed after the onset of reperfusion [27–30]. Therefore, we analyzed primarily the interactions of those formed radicals with CP-H in vitro (Fig. 1a, b). The product of a hydroxyl-, alkyl-, or alkylperoxyl radical-initiated oxidation of the diamagnetic CP-H is again a radical (•CP). Therefore, the time course of its appearance can be recorded by ESR spectroscopy. As seen from Fig. 1b the concentration of generated •CP is obviously a function of the produced oxidizing radicals. Furthermore, the half-life of •CP is considerably longer than that of •OH or ROO• (Fig. 3, open circles).

In previous experiments we were able to show that ROS generation reaches a maximum within the first 10 min after initiating kidney reperfusion [13, 31, 32]. For that reason we limited the time of reoxygenation during tissue homogenization and centrifugation to about 15 min (see Fig. 4). Homogenization of kidney tissue with an air-saturated buffer brings oxygen into intense contact with the ischemic tissue. At 25° C the oxygen concentration of an aqueous buffer amounts to 260 μmol/l as has been verified in earlier experiments [33]. In principle, reoxygenation during homogenization should be comparable to the onset of reperfusion. A similar conclusion was made in a study with ischemic rat hearts: they were homogenized and the content of malondialdehyde was analyzed [34]. Every ROS generation during organ or tissue reoxygenation requires the presence of oxidizable substrates produced during ischemia (see Scheme 1). This statement was supported in a former study: we analyzed the accumulation of hypoxanthine in rat kidneys as a function of the duration of ischemia. Within 60 min the content of hypoxanthine continuously increased. After initiation of reperfusion this compound was almost completely transformed to uric acid within 10 min by xanthine oxidase (XO), whereby molecular oxygen is reduced to ROS. Based on these data we used an ischemic period of 20 min before homogenizing and reoxygenating the kidney for 15 min. In reperfused rat kidneys XO appears to be involved in ROS generation [35, 36]. In humans its role in radical formation has been challenged [37].

The formation of oxidizing radicals during kidney homogenization and the subsequent transformation of CP-H to •CP is demonstrated in Fig. 4. Fifteen minutes after starting homogenization, the •CP concentration in the supernatant amounts to 3.0 ± 0.5 μmol/l. Some possible reasons for this low signal concentration are described below.

GSH up to a concentration of 5 mmol/l and ascorbate up to 0.3 mmol/l did not reduce •CP. However, at concentrations between 0.3 and 0.6 mmol/l ascorbate started to reduce •CP in a time depending manner and at 1 mmol/l the ESR signal of •CP diminished within 45 min of exposure by about 86 ± 0.6% (compare Fig. 2). Since the concentration of ascorbate in blood plasma is merely higher than 0.14 mmol/l [38, 39] extra-cellular •CP would not be influenced by this reductant. However, its intracellular concentration can range between 0.4 and 1.2 mmol/l [40, 41]. At this level of ascorbate •CP can partially be transformed to its ESR-silent derivative, as shown in Fig. 2. But this is, in general, the fate of spin adducts used for detection of oxidative stress which either have to compete with other cellular material for oxidizing radicals or are vulnerable to cellular reductants [2].

Ascorbate is able to neutralize many radicals. During that reaction the ESR-detectable ascorbyl radical is produced. Its formation has been used as a measure of oxidative stress [32, 42]. An advantage of this method is that ascorbate is not toxic and may be added in vivo in considerably high concentrations. Unfortunately the ascorbyl radical does not accumulate during reoxygenation, since it is only a transiently formed intermediate. By dismutation it is transformed back to ascorbate as well as to dehydroascorbate. The latter is a labile compound which itself rapidly decays [42]. So the detectable low and transient ESR signal of the ascorbyl radical can be used as a reliable marker of



Scheme 1 Principle modification of cellular compounds during reoxygenation

oxidative stress only under definite conditions. On the other hand, ascorbate is also a pro-oxidative agent as it reduces, e.g., Fe^{3+} to Fe^{2+}. This enhances the Fenton reaction and, therefore, can increase the generation of hydroxyl radicals [43–45]. For all these reasons the ascorbyl radical appears to be a possible but not an ideal marker for detecting oxidative stress. In our experiments (Figs. 2, 4) we could not detect the ascorbyl radical since its signal is completely masked by the second line of the •CP signal.

To evaluate ROS production during reoxygenation of an organ or tissue, the interaction of partially opposite effects has to be taken into consideration (see reactions 1–4 in Scheme 1). Strongly oxidizing radicals are non-selective. That means they can be neutralized by nearly every cellular compound including nucleic acids, lipids, proteins (reaction 2), antioxidants, like GSH or ascorbate (reaction 1), or exogenously added marker compounds, like, e.g., CP-H (reaction 3). But in order to interact with radicals the target compound has to be located in the immediate vicinity of radical generation. Detoxification of dangerous oxidizing radicals means that simultaneously a chemical modification of the target takes place (oxidation, addition to double bonds, etc.). A radical triggered target modification often induces a decrease in their functionality which contributes to cell damage. Basically the reaction rate constant as well as the local concentration of individual detoxifying compounds determines the probability of their interaction with oxidizing radicals. Therefore, the concentration of the transformed marker (e.g., •CP, reaction 3) reflects obviously only a small fraction of all cellular targets hit by oxidizing radicals. On the other hand, antioxidants like ascorbate are reductants. They can donate either an electron or a hydrogen atom to a radical (e.g., to •CP). As a consequence, the intensity of the ESR signal of •CP will decrease in the same extent as •CP is reduced to CP-H by a suitable reductant (reaction 4). Therefore, the monitored concentration of •CP represents only the resulting concentration of the four reactions summarized in Scheme 1 and, hence, underestimates the amount of originally generated oxidizing radicals during reoxygenation.

This underestimation of radical formation is not a specific property of CP-H, but appears to be a general feature of most spin traps such as DMPO. Nevertheless, CP-H and the ESR method used were able to detect the onset of oxidative stress as well as subsequent signal depletion.

We assume that the most effective interval to measure the formation of ROS with CP-H appears to be between the fifth and tenth minutes after reoxygenation because during this time segment the generation of •CP is high enough to be measured by ESR (Fig. 4).

In conclusion, it could be demonstrated that formation of •CP from CP-H needs the presence of oxidizing radicals. Strongly oxidizing radicals like hydroxyl- and alkylperoxyl radicals are usually formed during reperfusion of ischemic tissue or due to lipid peroxidation. So far the appearance of •CP from added CP-H is a marker of oxidative stress. We assume that the determined concentration of •CP inherently underestimates the real amount of primarily formed ROS because only a certain amount of the formed oxidizing radicals will transform CP-H to •CP, and because the reduction of •CP to its ESR-silent analogue will hold •CP at a low level. Nevertheless, the method described can easily be

performed, needs less time for preparation of samples and enables the measurement of oxidative stress even in turbid or colored solutions. We think that this method allows a nearly real-time determination of radical production during organ reoxygenation.

References

1. Baud L, Ardaillou R (1993) Involvement of reactive oxygen species in kidney damage. Br Med Bull 49:621–629
2. Halliwell B, Gutteridge J (1999) Free radicals in biology and medicine. Oxford University Press, Oxford
3. Sies H (1986) Biochemie des oxidativen stress. Angew Chem 98:1061–1075
4. Halliwell B, Gutteridge J (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J 219:1–14
5. Fraticelli A, Serrano C Jr, Bochner B, Capogrossi M, Zweier J (1996) Hydrogen peroxide and superoxide modulate leukocyte adhesion molecule expression and leukocyte endothelial adhesion. Biochim Biophys Acta 1310:251–259
6. Halliwell B, Gutteridge J (1990) Role of free radicals and catalytic metal ions in human disease: an overview. Methods Enzymol 186:1–85
7. Halliwell B (2006) Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiol 141:312–322
8. Kuppusamy, P., Chzhan, M. and Zweier, J. (2003) Principles of imaging. Theory and instrumentation. In: Berliner L (ed) In vivo EPR (ESR). Theory and applications. Biological magnetic resonance, vol. 18, Kluwer Academic/Plenum Publishers, New York, pp. 99–152
9. Symons M, Gutteridge J (1998) Free radicals and iron: chemistry, biology, and medicine. Oxford University Press, New York
10. Pincemail J, Defraigne J, Franssen C, Defechereux T, Canivet J, Philippart C, Meurisse M (1990) Evidence of in vivo free radical generation by spin trapping with alpha-phenyl *N-tert*-butyl nitrone during ischemia/reperfusion in rabbit kidneys. Free Radic Res Commun 9:181–186
11. Chen L, Gao Y, Li X, Shen D, Sun F (2005) Melatonin protects against MPTP/MPP+-induced mitochondrial DNA oxidative damage in vivo and in vitro. J Pineal Res 39:34–42
12. Halliwell B, Grootveld M, Gutteridge J (1988) Methods for the measurement of hydroxyl radicals in biomedical systems: deoxyribose degradation and aromatic hydroxylation. Methods Biochem Anal 33:59–90
13. Akcetin Z, Busch A, Kessler G, Heynemann H, Holtz J, Brömme H (1999) Evidence for only a moderate lipid peroxidation during ischemia- reperfusion of rat kidney due to its high antioxidative capacity. Urol Res 27:280–284
14. Draper H, Polensek L, Hadley M, McGirr L (1984) Urinary malondialdehyde as an indicator of lipid peroxidation in the diet and in the tissues. Lipids 19:836–843
15. Halliwell B, Kaur H, Ingelman-Sundberg M (1991) Hydroxylation of salicylate as an assay for hydroxyl radicals: a cautionary note. Free Radic Biol Med 10:439–441
16. Halliwell B, Whiteman M (2004) Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? Br J Pharmacol 142:231–255
17. Roubaud V, Sankarapandi S, Kuppusamy P, Tordo P, Zweier J (1997) Quantitative measurement of superoxide generation using the spin trap 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide. Anal Biochem 247:404–411
18. Buettner G (1988) In the absence of catalytic metals ascorbate does not autoxidize at pH 7: ascorbate as a test for catalytic metals. J Biochem Biophys Methods 16:27–40
19. Schößler W, Kirsch D, Lassmann G (1973) Die Bestimmung der Spinkonzentration mit der paramagnetischen Elektronenresonanz. Z Chem 13:364–372
20. Niki E (1990) Free radical initiators as source of water- or lipid-soluble peroxyl radicals. Methods Enzymol 186:100–108
21. Simm A, Brömme H (2005) Reactive oxygen species (ROS) and aging: Do we need them—can we measure them—should we block them? Signal Transductions 3:115–125
22. Halliwell B, Cross C (1994) Oxygen-derived species: their relation to human disease and environmental stress. Environ Health Perspect 102(Suppl 10):5–12
23. Brömme H, Loertzer H, Akcetin Z (2002) Der postischämische Reperfusionschaden Biochemische und methodische Grundlagen. Urologe A 41:159–163
24. Fink B, Dikalov S, Bassenge E (2000) A new approach for extracellular spin trapping of nitroglycerin induced superoxide radicals both in vitro and in vivo. Free Radic Biol Med 28:121–128
25. Dikalov S, Skatchkov M, Bassenge E (1997) Spin trapping of superoxide radicals and peroxynitrite by 1-hydroxy-3-carboxy-pyrrolidine and 1-hydroxy-2, 2, 6, 6-tetramethyl-4-oxo-piperidine and the stability of corresponding nitroxyl radicals towards biological Reductants. Biochem Biophys Res Commun 231:701–704
26. Dikalov S, Skatchkov M, Fink B, Bassenge E (1997) Quantification of superoxide radicals and peroxynitrite in vascular cells using oxidation of sterically hindered hydroxylamines and electron spin resonance. Nitric Oxide: Biol Chem 1:423–431
27. Baron P, Gomez-Marin O, Casas C, Heil J, Will N, Condie R, Burke B, Najarian J, Sutherland D (1991) Renal preservation after warm ischemia using oxygen free radical scavengers to prevent reperfusion injury. J Surg Res 51:60–65
28. Weight S, Bell P, Nicholson M (1996) Renal ischaemia–reperfusion injury. Br J Surg 83:162–170
29. Sorensen V, Nilsson U, Pettersson S, Schersten T, Sjoqvist P, Svensson L, Jonsson O (1996) Effect of a new inhibitor of lipid peroxidation on kidney function after ischaemia–reperfusion. A study on rat and rabbit kidneys. Acta Physiol Scand 157:289–297
30. Sussman M, Bulkley G (1990) Oxygen-derived free radicals in reperfusion injury. Methods Enzymol 186:711–723
31. Bauer S, Loertzer H, Fornara P, Mörke W, Brömme H (2005) Bewertung des AAPH-Zerfalls in Peroxylradikale bzw. der Generierung von Ascorbat-radikalen als Parameter zur Abschätzung von oxidativen Stress bzw. der Erfassung der antioxidativen Kapazität im Perfusionspuffer der Rattenniere. Transplantationsmedizin Suppl. II, 124
32. Loertzer H, Bauer S, Morke W, Fornara P, Bromme H (2006) Formation of ascorbate radicals as a measure of oxidative stress: an in vitro electron spin resonance-study using 2,2-azobis (2-amidinopropane) dihydrochloride as a radical generator. Transplant Proc 38:674–678
33. Wippich N, Peschke D, Peschke E, Holtz J, Bromme H (2001) Comparison between xanthine oxidases from buttermilk and microorganisms regarding their ability to generate reactive oxygen species. Int J Mol Med 7:211–216
34. Verbunt R, Egas J, Van der Laarse A (1996) Risk of overestimation of free malondialdehyde in perfused rat hearts due to homogenization artifacts. Cardiovasc Res 31:603–606
35. Sarnesto A, Linder N, Raivio K (1996) Organ distribution and molecular forms of human xanthine dehydrogenase/xanthine oxidase protein. Lab Invest 74:48–56
36. Linas S, Whittenburg D, Repine J (1990) Role of xanthine oxidase in ischemia/reperfusion injury. Am J Physiol 258:71171–71176

37. Joannidis M, Gstraunthaler G, Pfaller W (1990) Xanthine oxidase: evidence against a causative role in renal reperfusion injury. Am J Physiol 258:232–236
38. Naidu KA (2003) Vitamin C in human health and disease is still a mystery? An overview. Nitrition J 2:1–10
39. Davies K, Sevanian A, Muakkassah-Kelly S, Hochstein P (1986) Uric acid–iron ion complexes. A new aspect of the antioxidant functions of uric acid. Biochem J 235:747–754
40. Rose R, Bode A (1993) Biology of free radical scavengers: an evaluation of ascorbate. Faseb J 7:1135–1142
41. Steffan J (1999) Bestimmung von L-Ascorbinsäure und Dehydro-L-ascorbinsäure in Humanplasma und Leukozyten mit HPLC/UV und HPLC/EC. In: Fachbereich Chemie und Chemietechnik vol. p. 178. Universität-Gesamthochschule Paderborn, Paderborn
42. Buettner GR, Jurkiewicz BA (1995) Ascorbate radical: a valuable marker of oxidative stress. In: Favier AE et al (eds) Analysis of free radicals in biological systems, Birkhäuser Verlag, Basel, pp. 145–164.
43. Udenfriend S, Clark C, Axelrod J, Brodie B (1954) Ascorbic acid in aromatic hydroxylation. I. A model system for aromatic hydroxylation. J Biol Chem 208:731–739
44. Halliwell B, Gutteridge J (2007) Free radicals in biology and medicine. Oxford University Press, Oxford
45. Burkitt M, Gilbert B (1990) Model studies of iron-catalysed Haber-Weiss cycle and the ascorbate-driven Fenton reaction. Free Radic Res Commun 10:265–280