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Comparison of Protein Oxidation and Aldehyde Formation During Oxidative Stress in Isolated Mitochondria

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Oxidative stress is known to cause oxidative protein modification and the generation of reactive aldehydes derived from lipid peroxidation. Extent and kinetics of both processes were investigated during oxidative damage of isolated rat liver mitochondria treated with iron/ascorbate. The monofunctional aldehydes 4-hydroxynonenal (4-HNE), n-hexanal, n-pentanal, n-nonanal, n-heptanal, 2-octenal, 4-hydroxydecenal as well as thiobarbituric acid reactive substances (TBARS) were detected. The kinetics of aldehyde generation showed a lag-phase preceding an exponential increase. In contrast, oxidative protein modification, assessed as 2,4-dinitrophenylhydrazine (DNPH) reactive protein-bound carbonyls, continuously increased without detectable lag-phase. Western blot analysis confirmed these findings but did not allow the identification of individual proteins preferentially oxidized. Protein modification by 4-HNE, determined by immunoblotting, was in parallel to the formation of this aldehyde determined by HPLC. These results suggest that protein oxidation occurs during the time of functional decline of mitochondria, i.e. in the lag-phase of lipid peroxidation. This protein modification seems not to be caused by 4-HNE.

Keywords: Lipid peroxidation, aldehydes, protein oxidation, carbonyl, mitochondria, rat liver

Abbreviations: DNPH, 2,4-dinitrophenylhydrazine; GSH, reduced glutathione; 4-HNE, 4-hydroxy-2,3-*trans*-nonenal (4-hydroxynonenal); 4-HDE, 4-hydroxydecenal; MDA, malondialdehyde; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid; TLC, thin layer chromatography

INTRODUCTION

Mitochondria are discussed as sources and targets of reactive oxygen species (ROS) under

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many physiological and pathological conditions. ROS are able to oxidize all macromolecular constituents of mitochondria like DNA, (phospho-)lipids and proteins. DNA damage may lead to functional decline of mitochondria over longer time periods. In contrast, impairment of mitochondrial membranes by lipid peroxidation as well as oxidative modification of proteins may also cause immediate loss of function. For example, it is thought that ROS are involved in the induction of mitochondrial permeability transition and release of cytochrome c during the apoptotic process.[1]

Oxidative stress in isolated rat liver mitochondria by treatment with Fe^{2+}/ascorbate results in an exhaustion of the antioxidative defense systems, followed by a loss of active respiration, inactivation of the respiratory chain enzymes and breakdown of the inner membrane potential.[2–4] It is widely acknowledged that substantial lipid peroxidation is ultimately linked to the stress induced by Fe^{2+}/ascorbate. Lipid peroxidation is generally accompanied by a formation of secondary products derived from chain cleavage within the fatty acid residues of phospholipids. A considerable part of these products resembles aldehydes.[5] Whereas the bifunctional malondialdehyde (MDA) became mainly important as a marker of lipid peroxidation, the interest in the monofunctional aldehydes arose from the finding that among these aldehydes are highly reactive compounds, most notably 4-hydroxynonenal (4-HNE), possibly mediating some of the deleterious effects of lipid peroxidation in biological systems.[5,6] The formation and the pattern of the monofunctional aldehydes in the liver have been studied extensively during peroxidation of microsomes and in hepatocytes.[7,8] Since hepatocytes exhibit high activities of cytosolic aldehyde dehydrogenase an effect of aldehydes on mitochondrial functions is likely to depend on the generation of these compounds within the mitochondria.[9] For isolated liver mitochondria, however, the pattern and the kinetics of monofunctional aldehyde formation in the course of Fe^{2+} induced oxidative stress has not been reported until now.

Another possible target of free oxygen radicals in this model are the mitochondrial proteins. The functional impairment of proteins by ROS occurs either directly by oxidation of amino acid side chains[10] or by secondary reactions with aldehydic products of lipid peroxidation, most notably 4-HNE,[11] or glycoxidation.[12] All of these primary and secondary reactions can result in the occurrence of carbonyl-groups in the protein molecule. Therefore, the assessment of protein-bound carbonyls by derivatization with 2,4-dinitrophenylhydrazine 2,4-(DNPH) is a widely used marker for oxidative protein modification.[13]

The aim of the present study was to compare the oxidative modification of proteins to the formation of mono- and bifunctional aldehydes with special interest in the pattern of monofunctional aldehydes generated in isolated mitochondria during oxidative stress. Western blotting was performed to identify proteins containing DNPH reactive carbonyl groups as well as 4-HNE modified histidine residues in order to distinguish between primary and secondary processes of oxidative protein damage.

METHODS

Preparation of Mitochondria

Liver mitochondria were prepared from adult fasted Wistar rats by differential centrifugation, suspended in 0.25 M sucrose at pH 7.4 and kept on ice.[14] Mitochondrial protein concentration was determined by a modified Biuret method.[15] Functional integrity of the mitochondrial preparations was assessed by oxygraphic measurement of respiration with succinate as substrate in presence and absence of ADP and the purity was checked by electron microscopy.[16]

Iron/Ascorbate Mediated Peroxidation

Freshly prepared functionally intact mitochondria were incubated in a medium containing

100 mM KCl and 10 mM Tris-HCl at pH 7.7. Mitochondrial protein in the medium was adjusted to 5 mg/ml. Oxidative stress was induced as previously described by 40 μM $FeSO_4$ and 0.5 mM ascorbate.[2] Iron/ascorbate was omitted in the controls. During shaking in open air at 25°C samples were taken for the analyses. Thiobarbituric acid reactive substances (TBARS) as a gross indicator of lipid peroxidation were determined according to Buege and Aust.[17] GSH was determined according to Tietze.[18]

Spectrophotometric Quantification of Protein-bound Carbonyls

For assessment of protein carbonyls, the reaction with 2,4-DNPH, with subsequent spectrophotometric quantification at 370 nm according to Reznick and Packer[13] and as described by our group for rat liver mitochondria[20] was employed.

Western Blotting for Detection of Protein-bound 2,4-DNPH and Protein-bound 4-HNE

This method for the detection of DNPH reactive proteins after separation by SDS-PAGE and subsequent transfer onto a nitrocellulose membrane was adopted from Levine *et al.*[19] and performed as described in detail for rat liver mitochondria.[20] Immunostaining was performed using an anti-DNP-IgG (Sigma, Deisenhofen, Germany) in a 1:2000 dilution in TBS-Tween 20 and a peroxidase-conjugated anti-rabbit-IgG (Boehringer, Mannheim) in 1:3000 dilution. Visualization of carbonyl-containing protein bands occurred by development with H_2O_2/diaminobenzidine.

For the detection of protein-bound 4-HNE 100 μl of the samples were solubilized in a solution containing 12% SDS, 6% 2-mercaptoethanol, 50 mM Tris, pH 7.8 and 30% glycerol. After heating at 95°C for 5 min samples were allowed to cool down and aliquots of 10 μg protein were applied to the gels. Electrophoresis and blotting were performed as described.[21] The primary antibody, raised against HNE-histidine,[22] was used in a 1:500 dilution. Peroxidase-conjugated anti-mouse Fab-fragment (Boehringer, Mannheim) was diluted 1:3000. Chemiluminescence was detected on polaroid-films using the ECL-mini-camera.

Separation and Quantification of Aldehydes

Aldehydes were determined by the method of Esterbauer and Cheeseman,[23] which consists in a derivatization of the compounds by 2,4-DNPH, subsequent separation of the hydrazones by thin layer chromatography (TLC) into classes of different polarities and a final separation of the eluted compounds from the TLC-spots of interest by HPLC.

Determination of Fatty Acids[24]

Mitochondrial lipids were extracted by the Folch-extraction procedure.[25] After saponification and crown ether catalyzed derivatization of the fatty acids to their p-bromo-phenacylesters individual fatty acids were quantified by HPLC as outlined in detail.[24]

RESULTS

Qualitative evidence for the aldehydes formed in the course of lipid peroxidation was obtained by TLC and HPLC. The TLC-chromatograms (see Figure 1 for schematic explanation) revealed additional spots of DNPH reactive substances in chloroform-extracts of mitochondria peroxidized for 90 min. In agreement with Esterbauer,[5] fractions 3 and 5, present after iron/ascorbate treatment as well as in the controls, resembled contaminations by formaldehyde and acetone (fraction 5) as well as osazones together with free DNPH. The additional fractions found in

the peroxidized samples corresponded to the TLC-fractions of unpolar aldehydes i.e. alkanals, alkenals and alkadienals (fraction 6) and 4-hydroxyenals (fraction 4). Fraction 2, obviously containing highly polar compounds, was not further analyzed because no authentic standards had been available. The separation of the eluted TLC-spots by HPLC allowed the identification of the majority of the peaks from fraction 6 and of a number of peaks from fraction 4 by retention times and UV/VIS-spectra as compared to authentic standards. As shown in Figure 2A, n-hexanal and 4-HNE represent, with concentrations of about 4 nmol/mg protein and 3 nmol/mg protein respectively, the major part of monofunctional aldehydes generated in mitochondria at the end of the experiments. The other aldehydes detected, i.e. n-pentanal, n-nonanal, n-heptanal, 2-octenal and 4-hydroxydecenal, were generated to considerably lower extent reaching maximally 0.5 nmol/mg mitochondrial protein. TBARS (MDA) were generally produced at 5- to 6-fold higher concentrations than the predominant monofunctional aldehydes.

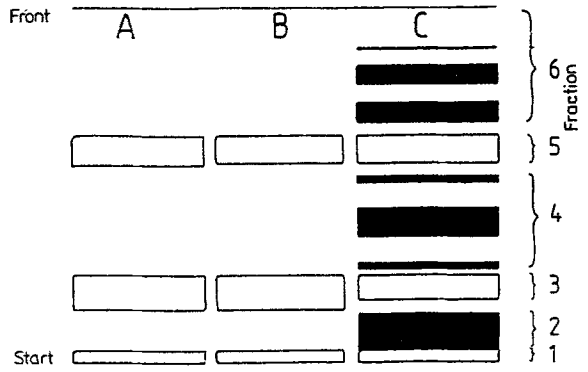

FIGURE 1 Schematic presentation of mitochondrial DNPH reactive substances separated by thin layer chromatography according to Esterbauer.[5] (A) Buffer + Fe^{2+}/ascorbate (blank), (B) Mitochondria, Fe^{2+}/ascorbate omitted (control), (C) Mitochondria, peroxidized with Fe^{2+}/ascorbate for 90 min with new spots shown in black. (1–6) Fractions as described in the text.

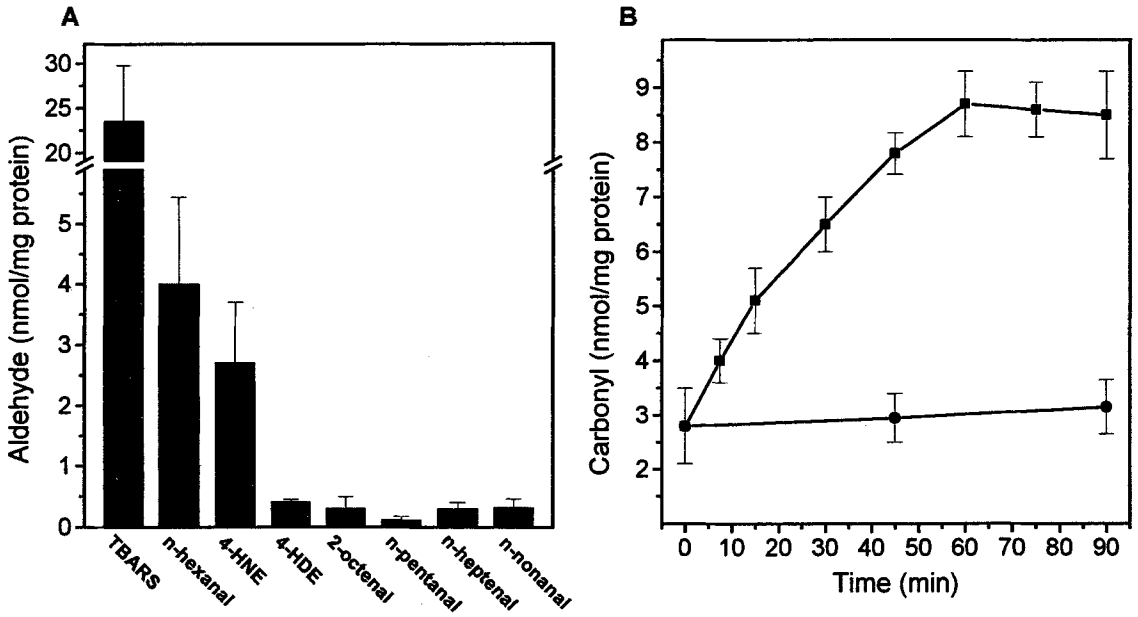

FIGURE 2 Aldehyde pattern and oxidative protein modification in Fe^{2+}/ascorbate-induced lipid peroxidation in rat liver mitochondria. (A) Thiobarbituric acid reactive substances (TBARS) and DNPH reactive monofunctional aldehydes as determined by HPLC after 90 min peroxidation. 4-hydroxynonenal (4-HNE), 4-hydroxydecenal (4-HDE). (B) DNPH reactive protein carbonyls determined by the spectrophotometric method.[13] (●) control, (■) Fe^{2+}/ascorbate. Data present mean values ± S.E. from at least 4 independent experiments.

The generation of free radicals is a physiological process resulting in detectable levels of oxidatively modified proteins in tissues and organelles. The basal level of DNPH reactive protein-carbonyls in isolated rat liver mitochondria was about 2.7 nmol/mg protein and remained unchanged during the control incubations (Figure 2B). The treatment of mitochondria with iron/ascorbate resulted in a continuous 3-fold increase of oxidatively modified proteins (Figure 2B).

In Figure 3 the time course of protein oxidation was compared to the decrease of mitochondrial GSH content and the occurrence of quantitatively important lipid peroxidation products i.e. n-hexanal, 4-HNE and TBARS. In this model the formation of TBARS, including MDA as the quantitatively most important compound, is characterized by a lag-phase of about 20–30 min followed by an exponential increase and a plateau phase. For the monofunctional aldehydes the length of the lag-phase was apparently not different to that of TBARS. After the exponential increase, however, a distinct plateau phase as seen for the TBARS was not observed. The aldehyde concentrations rose continuously until the end of the experiments after 90 min. Mitochondrial GSH decreased from 6.7 nmol/mg protein to 0.4 nmol after 75 min. The loss of GSH was, however, only about 5% during the first 20 min of peroxidation. Most importantly and in contrast to the aldehyde generation and GSH depletion, oxidative protein modification obviously occurred without any lag-phase reaching plateau levels after 1 h.

To substantiate the finding of only weak aldehyde formation during the initiation phase of oxidative damage the pattern of fatty acids, representing the substrates for aldehyde formation, was determined. The saturated fatty acids, in particular stearic acid, declined only slowly during the whole experiment (Figure 4). In correspondence to the kinetics of lipid peroxidation a massive loss of unsaturated fatty acids,

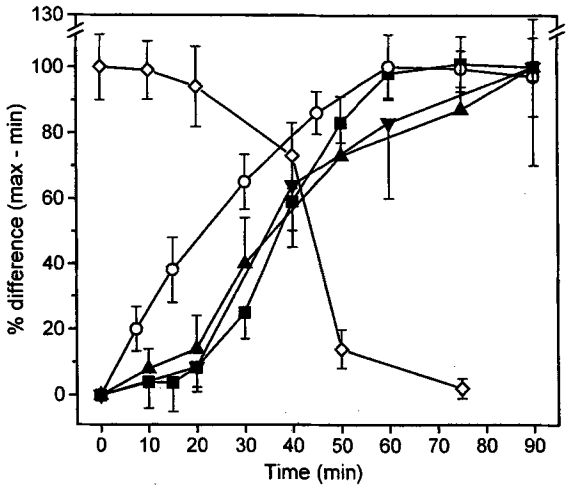

FIGURE 3 Time course of increases of protein-bound carbonyls and aldehydes as well as GSH-decline during oxidative stress in mitochondria. DNPH reactive protein carbonyls were determined by a spectrophotometric method[13] and aldehydes determined by TLC-HPLC. The GSH-level, determined according to Tietze,[18] was 6.7 ± 0.65 nmol/mg protein at the begin of the experiments. To compare the formation rates of the parameters, the differences of the measured values between the control group and the group showing the highest absolute levels was defined as 100%. Values are calculated from means ± S.E. of at least 4 experiments. (◇) GSH, (○) Protein-bound carbonyls, (■) TBARS, (▲) 4-hydroxynonenal, (▼) n-hexanal.

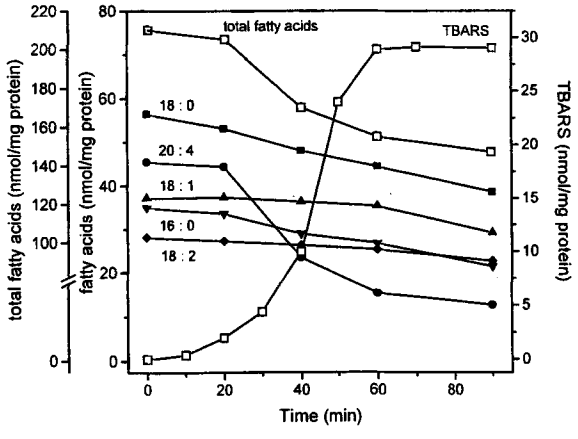

FIGURE 4 Fatty acid pattern of rat liver mitochondria during Fe^{2+}/ascorbate induced peroxidation compared to the generation of TBARS. (16:0) palmitic acid (18:0) stearic acid, (18:1) oleic acid, (18:2) linoleic acid, (20:4) arachidonic acid. Demonstrated is one representative experiment of total $n = 6$.

mainly of arachidonic acid, starts in parallel with the onset of massive TBARS formation (Figure 4).

Besides the possibility to evaluate the quantitative data of the spectrophotometric assay for protein-bound carbonyls, Western blotting using an anti-DNP-antibody is potentially useful for identification of individual proteins modified selectively during oxidative stress. As shown in Figure 5A, the content of DNPH-reactive proteins increased gradually in accordance to the quantitative results from Figure 2B. However, single proteins preferentially modified during iron/ascorbate stress were not observed in this approach using SDS-PAGE in one dimension. It should be noted that the Coomassie-stain (Figure 5C), also considered as "loading control", did not reveal major differences in the electrophoretic protein pattern between the samples.

Dot-Blot analysis using antibodies directed against HNE-modified histidine residues of proteins with colorimetric detection showed that the content of HNE-modified proteins in the mitochondrial preparations was very low (not shown). Therefore, enhanced chemiluminescence detection was employed for these Western-Blots. Interestingly, untreated mitochondria contained some HNE-modified proteins, most distinctly a band at about 30 kDa (Figure 5B), which is not equally predominant in anti-DNP-stain and Coomassie-stain. It appears that the kinetics of selective modification of a number of proteins by 4-HNE during peroxidation went along with the increase of 4-HNE as determined by HPLC.

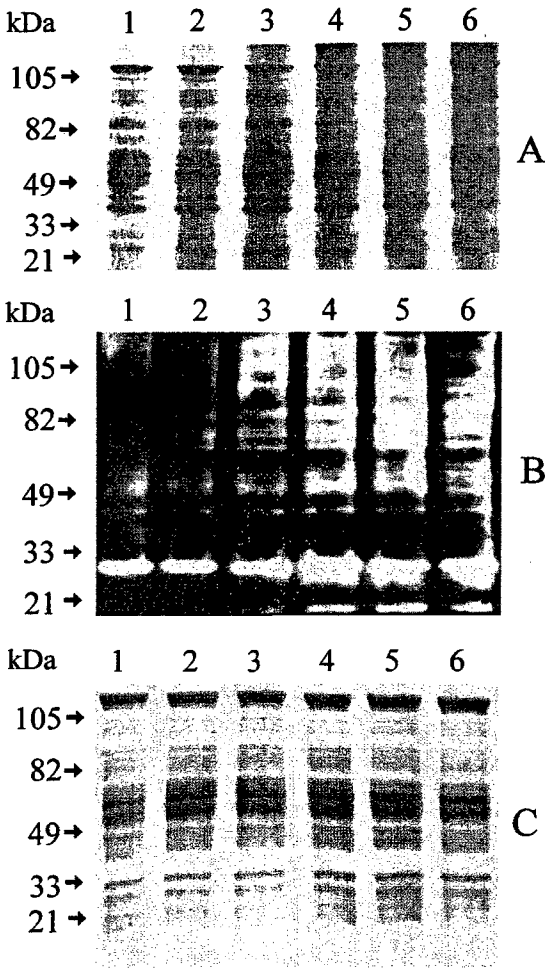

FIGURE 5 Western blots for the detection of oxidatively modified proteins. (A) Detection of DNPH reactive protein carbonyls with anti-DNPH-IgG. (B) Chemiluminescence detection of proteins with 4-HNE-modified histidine residues in the samples from (A). (C) Coomassie-stain of proteins to compare protein patterns and for "loading control" of the samples from (A) and (B). Iron/ascorbate incubation times: 1 = 0 min, 2 = 10 min, 3 = 20 min, 4 = 30 min, 5 = 45 min, 6 = 60 min. Shown is one representative of 3 independent experiments.

DISCUSSION

Considerable amounts of monofunctional aldehydes and oxidatively modified proteins are produced by isolated, iron/ascorbate stressed mitochondria. The pattern of aldehydes formed is very similar to those described for peroxidized microsomes and agrees to the fact that, although the phospholipid pattern of microsomes and mitochondria is different, the fatty acid pattern is rather similar.[26] In both organelles arachidonic acid, which makes up the main source for n-hexanal and 4-HNE, constitutes the predominant fatty acid with approximately 25% of total fatty acid content.[5,27] The different chemical pathways for the formation of MDA and monofunctional aldehydes, MDA by breakdown of

cycloendoperoxides and monofunctional aldehydes by reductive cleavage of lipohydroperoxides, may account for the differences in the extent and kinetics of the formation of both aldehyde classes.[5] The previously reported substantial amounts of lipohydroperoxides during the lag-phase of lipid peroxidation might, for example, explain the increase in monofunctional aldehydes at constant MDA-levels.[2]

The decline of most indicators of mitochondrial function occurs, however, before a significant amount of aldehydes has been generated by iron/ascorbate treatment.[2–4,28] Which ROS mediated processes could be involved? 4-HNE is able to react with sulfhydryl- and amino groups of proteins and therefore affecting the function of proteins by covalent modification.[29] For example, incubation of mitochondria with 4-HNE was shown to induce permeability transition[30] and to inhibit the adenine nucleotide translocator.[31] Since the formation of aldehydes, as shown by the present results, is a relatively late event in this model, it seems unlikely that aldehydes might exhibit their toxic effects in early phases of the experiment. This is supported by immunoblotting revealing that, although there are some 4-HNE-modified proteins already present at the begin of the experiments, binding of 4-HNE to proteins was in parallel to the increase of total 4-HNE determined by HPLC. GSH, which may bind covalently to 4-HNE and could give rise to a part of 4-HNE which is not detected by the method applied[29] declined during the lag-phase of lipid peroxidation by 5%. This corresponds to a GSH loss of about 0.33 nmol/mg protein. Even if all the lost GSH constitutes 4-HNE conjugates, this points to very low levels of free 4-HNE during the lag-phase of lipid peroxidation, too. Thus it appears unlikely, together with the slow decline of arachidonic acid, that 4-HNE is mediating the early functional impairment of mitochondria. In addition, exogenously added 4-HNE is needed in concentrations above 100 μM to affect mitochondrial respiration.[33] These inhibitory concentrations are not reached during the experiments reported here. n-Hexanal does not constitute a suitable candidate as a damaging agent in mitochondria, because the formation of thiazolin carboxylic acids depends on the presence of both free SH- and amino groups and demands extremely high concentrations of n-hexanal as well.[5,34]

In contrast to aldehyde generation the formation of protein-bound carbonyls did proceed continuously without any detectable lag-phase. Interestingly, submitochondrial particles treated with ADP/iron fail to exhibit different kinetics of TBARS-formation and protein oxidation mostly due to the missing of a lag-phase of lipid peroxidation.[38] This could be caused by the diminished contents of antioxidative systems derived from the mitochondrial matrix, i.e. GSH, glutathione peroxidase and superoxide dismutase, and demonstrates the importance of synergistic and consecutive actions of the complex defense of mitochondria against free radical attack.

The various staining patterns in the panels of Figure 5 demonstrate that proteins from functionally intact mitochondria are differentially oxidatively modified, i.e. exhibit different susceptibility towards ROS. Under the condition of severe oxidative stress induced by iron/ascorbate protein oxidation seemed to occur randomized and unspecific not indicating single, preferentially oxidatively modified proteins in early or later stages of mitochondrial damage. This could, however, also be due to the relatively low resolution of one-dimensional SDS-PAGE. It should be noted again, that mitochondrial proteins were not significantly modified by 4-HNE during the early phase of the experiments. This makes it likely, that the observed early increase of protein carbonyls may rather be due to direct oxidation of amino acids than to secondary modifications by aldehydes. Since free, redox-active iron is initiating the oxidative stress, metal catalyzed direct oxidation of amino acid side chains, possibly involving protein peroxide

caused chain reactions, oxidation may be the most important pathway for protein oxidation in this model.[10,35,36] Mitochondrial proteins are, however, not only highly susceptible to oxidative stress under conditions of high Fe^{2+} concentrations as applied here. Hypoxia as short as 5 min followed by reoxygenation can cause oxidative protein modification and functional alterations in isolated mitochondria with only minimally increased MDA-levels to about 2 nmol/mg mitochondrial protein.[20]

In conclusion, the severe oxidative stress in this model leads to early and random damage of mitochondrial proteins before secondary products of lipid peroxidation are produced. These aldehydes are therefore unlikely to contribute to the early functional impairment of mitochondria. Nevertheless lipid peroxidation is certainly important in the propagation of damage leading to desintegration of the membrane and structural alterations of membrane proteins,[37] especially of the complexes of the respiratory chain.[39]

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