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Ferritin oxidation and proteasomal degradation: Protection by antioxidants

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Abstract

The accumulation of oxidatively damaged proteins is a well-known hallmark of aging and several neurodegenerative diseases including Alzheimer's, Parkinson's and Huntigton's diseases. These highly oxidized protein aggregates are in general not degradable by the main intracellular proteolytic machinery, the proteasomal system. One possible strategy to reduce the accumulation of such oxidized protein aggregates is the prevention of the formation of oxidized protein derivatives or to reduce the protein oxidation to a degree that can be handled by the proteasome. To do so an antioxidative strategy might be successful. Therefore, we undertook the present study to test whether antioxidants are able to prevent the protein oxidation and to influence the proteasomal degradation of moderate oxidized proteins. As a model protein we choose ferritin. H_2O_2 induced a concentration dependent increase of protein oxidation accompanied by an increased proteolytic susceptibility. This increase of proteolytic susceptibility is limited to moderate hydrogen peroxide concentrations, whereas higher concentrations are accompanied by protein aggregate formation.

Protective effects of the vitamin E derivative Trolox, the pyridoindole derivative Stobadine and of the standardized extracts of flavonoids from bark of Pinus Pinaster Pycnogenol® and from leaves of Ginkgo biloba (EGb 761) were studied on moderate damaged ferritin.

Keywords: *Oxidative stress, ferritin, antioxidants, protein oxidation, proteasome*

Abbreviations: *AAS, aminoadipic semialdehyde; AOx, antioxidant; BHT, butylated hydroxytoluole; BSA, bovine serum albumin; DEAE, diethylaminoethyl; DNPH, dinitrophenylhydrazine; EDTA, ethylenediaminetetraacetic acid; EGb 761, Ginkgo biloba extract; ELISA, enzyme linked immunosorbent assey; FINH$_2$, fluoresceinamine; FPLC, fast protein liquid chromatography; Gb, Ginkgo biloba; GGS, γ-glutamic semialdehyde; MCO, metal catalyzed oxidation; PBS, phosphateous buffer saline; Pyc, Pycnogenol®; RNS, reactive nitrogen species; ROS, reactive oxygen species; SDS/PAGE, sodium-dodecyl-sulfate polyacrylamide gel electrophoresis; Sto, Stobadine; TCA, trichloroacetic acid; Tro, Trolox*

Introduction

Reactive oxygen species (ROS) and other oxidants like hypochloric acid and reactive nitrogen species (RNS) are able to damage several cellular structures like DNA, lipids and proteins. The accumulation of oxidatively damaged proteins is a well known hallmark of several neurodegenerative diseases like Alzheimer's [1–8], Parkinson's [9–11] and Huntigton's disease [12–14]. Also the aging process is connected to the accumulation of oxidized and miss-folded proteins [2,15–26].

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As cellular function is disturbed by accumulated protein aggregates [19,21–23,27–30] the elimination of oxidized proteins via degradation is a favored outcome. The main proteolytic system responsible for the degradation of oxidized proteins is the proteasomal system. In a series of publications we [22,23,31–35] and others [19,27,29,30,36–39] were able to demonstrate that the proteasome is able to selectively recognize and degrade such oxidatively modified proteins. It was shown by us and others that the formation of cross-linked protein aggregates is able to decrease the proteasomal activity in several model systems as well as during diseases [19,21–23,28,29,40–42]. Since the ongoing accumulation of protein aggregates might be a self-accelerating process, it is important to find antioxidants which are able to inhibit further protein oxidation and prevent protein cross-linking [43]. Therefore, we undertook the present study to test whether various antioxidants are able to prevent protein oxidation. As measure for protein oxidation we used a common method of detecting protein-bound carbonyl groups by an ELISA and immunoblot technique and the recognition of the oxidized proteins by the proteasome, a very sensitive tool to determine oxidative changes in the protein structure [34,44].

The fact that free iron has a large potential to convert relatively low-reactive ROS into highly reactive ones and the knowledge that ferritin itself is a highly oxidizable protein with known changes in the proteolytic susceptibility after oxidation [45–48] lead us chose ferritin as a model protein for the here presented study. Furthermore, the content of ferritin in specific regions of the brain is altered during aging or Alzheimer's and Parkinson's disease [10,49,50].

Antioxidants like vitamin C are able to reduce oxidative damage in proteins [51,52] but on the other hand they will interact with the Fenton system. Therefore, we chose simple substances of known antioxidant properties; Stobadine (*cis*-(−)2,3,4,4a,5,9b-hexahydro-2,8-dimethyl-1H-pyrido[4,3-b]indole) [53–55] and vitamin E derivative Trolox as well as the two standardized extracts of flavonoids Pycnogenol® [56,57] and EGb 761 [58–60] with mutual biological properties for our investigations.

Material and methods

Proteasome isolation

Proteasome was isolated from erythrocytes of outdated human blood conserves according to Hough et al. [61]. Erythrocytes were lysed in 1 mM dithiothreitol. After removal of membranes and non-lysed cells by centrifugation the proteasome was isolated by DEAE-chromatography, sucrose-density gradient ultracentrifugation and separation on a Mono Q column of a FPLC system. The purity of the preparation was controlled by both one-dimension SDS/PAGE and non-denaturing polyacrylamide electrophoresis. Non-denaturing electrophoresis was followed by overlaying the gel with a 200 μM Suc-LLVY-MCA solution for detection of peptidase activity. The proteasome preparation was diluted finally to a concentration of 0.35 mg/ml.

Ferritin treatment with oxidants and antioxidants

We treated ferritin (1 mg/ml) with various concentrations of hydrogen peroxide in a 20 mM phosphate buffer (pH 7.2) for 2 h at 25°C. This incubation was either performed in the presence of the indicated amounts of antioxidants or without antioxidants. In the case of the incubation of antioxidants after the hydrogen peroxide treatment a 2 h-incubation with antioxidants followed the oxidation step. The incubation with antioxidants followed immediately the oxidation. Afterwards oxidants and/or antioxidants were removed by dialysis for 16 h at 4°C vs. 2 l dialysis buffer (10 mM potassium phosphate containing 10 mM KCl, pH 7.4).

Measurement of protein degradation

Isolated ferritin was radiolabeled by reductive methylation with [^{3}H]-formaldehyde and sodium cyanoborohydride as described by Jentoft and Dearborn [62], and then dialyzed extensively. Afterwards the protein was stored at −80°C until use in oxidation experiments. Degradation of [^{3}H]-labeled ferritin by the proteasome was measured in 50 mM Tris–HCl (pH 7.8), 20 mM KCl, 5 mM MgOAc and 0.5 mM dithiothreitol. Percentage of ferritin degradation was determined by liquid scintillation in supernatants of trichloroacetic acid precipitated proteins, and calculated according to the following formula: (TCA-soluble radioactivity − background radioactivity)/(total radioactivity − background radioactivity) × 100.

Total protein carbonyl measurements

For the determination of protein carbonyl groups the method of Buss et al. [44] with modifications of Sitte et al. [34] was used.

Sample preparation. After the 2 h of treatment of ferritin with H_2O_2 the hydrogen peroxide was removed by dialysis and BHT (final concentration 1 mM) was added.

Protein carbonyl determination. About 15 mg of the protein solution were derivatized with three volumes of DNPH solution (10 mM in 6 M guanidine hydrochloride, 0.5 M potassium phosphate buffer,

pH 2.5) by incubation at room temperature for 45 min. The sample was diluted (1:200) with coating buffer (10 mM sodium phosphate buffer containing 140 mM NaCl, pH 7.0), vortexed and four aliquots of each sample were transferred into Nunc Immunosorp plates. Plates were incubated overnight at 4°C. The next day the derivatization solution was removed and each well was blocked with 0.1% reduced BSA in PBS for 1.5 h at room temperature in dark; then biotinylated anti-DNP antibody (1:1000 dilution in 0.1% reduced BSA, 0.1% Tween 20 solution) was added and incubated for 1 h at 37°C. Wells were washed three times with PBS. Then the samples were incubated with the secondary antibody, anti-rabbit-IgG-peroxidase (γ-chain specific), (1:10,000 in 0.1% reduced BSA, 0.1% Tween 20 solution), incubated for 1 h in the dark at room temperature and washed three times with PBS. The developing solution (50 mM Na_2HPO_4 with 24 mM citric acid), containing 0.6 mg/ml *o*-phenylenediamine and hydrogen peroxide (stock, diluted 1:2500), was added to each well. The plate was incubated at 37°C for 30 min. The reaction was stopped with 2.5 M sulfuric acid. Absorbances were determined at 492 nm. A standard curve of oxidized BSA was included in each plate. Blanks of PBS and BHT without protein were subtracted from standard and sample absorbances.

Oxidized BSA was prepared by modifying solved BSA with hypochlorite. The carbonyl content of the oxidized BSA was determined according to Buss et al. [44]. Reduced BSA was obtained by sodium cyano borohydryte reduction [44].

Quantification of glutamic semialdehyde and aminoadipic semialdehyde by HPLC

The quantification of glutamic semialdehyde (GGS) and aminoadipic semialdehyde (AAS) was performed by a modified HPLC method [63]. The concentrations of GGS and AAS were calculated by comparison of the peak areas of the prepared standards with the areas of the samples.

Oxidation of amino acid homopolymers and bovine serum albumin. The amino acid homopolymers (poly-Arg, poly-Lys) were oxidized with a MCO system (ascorbate/iron/EDTA) described by Amici et al. [64]. About 20 mg of the protein or homopolymer were dissolved in 2 ml of 50 mM phosphate buffer (pH 7.0) and oxidized for 1 h at 37°C by adding 200 µl of 25 mM EDTA, 100 µl 625 mM ascorbic acid and 200 µl 25 mM $FeCl_3$. The ascorbate and $FeCl_3$ solutions were freshly prepared. Adding trichloracetic acid to a final concentration of 10% stopped the incubation reaction. The protein or homopolymer was separated by centrifugation and resolved in 50 mM phosphate buffer (pH 7.0).

Derivatization of the protein carbonyl group with fluoresceinamine ($FINH_2$). About 100 µl of the oxidized sample were heated in a water bath for 1 min at 100°C. Afterwards, 12.8 µl of 0.25 M $FINH_2$ in 0.52 M NaOH, 10 µl of 0.4 M $NaCNBH_3$ in 50 mM phosphate buffer and 37.2 µl of phosphate buffer were added to a final volume of 160 µl. $FINH_2$ and $NaCNBH_3$ solutions were freshly prepared. The mixture was incubated at 37°C for 1 h. Finally, by addition of phosphate buffer, the volume was adjusted to 1 ml and the protein or homopolymers were precipitated by adding trichloracetic acid to a final concentration of 10%. The centrifuged sample was dissolved in phosphate buffer. This procedure was repeated up to 5 times until all free $FINH_2$ was removed. After the last precipitation the sample was dissolved in 1 ml of 0.1 M NaOH for 15 min at 37°C. By means of centrifugation for 4 min any insoluble material was pelleted.

Acid hydrolysis and HPLC. About 200 µl of the oxidized and derivatized samples were hydrolyzed with 300 µl of 30% HCl for 24 h at 110°C. The hydrolysates were filtered through 0.45 µm filters and analyzed by HPLC (System Gold, Beckman). About 50 µl of the sample were injected onto a Supelcosil LC-18 (250 × 4.6 mm; 5 µm) column (Supelco), which was equilibrated using 2 mM formic acid (pH 3.2), at 1 ml/min. A linear gradient from 0 to 50% acetonitrile in 2 mM formic acid was developed up to 15 min. The eluent was monitored at 454 nm.

Results

The incubation of ferritin with H_2O_2 increased the amount of protein carbonyls in a concentration dependent manner. The highest oxidant concentrations doubled the protein bound carbonyl content of the ferritin compared to the untreated sample. This could be demonstrated by both, by the used ELISA-technique (Figure 1A) as well as by immunobloting (Figure 1B). The highest H_2O_2 concentration (50 µmol per mg ferritin) not only increased amount of carbonyls, but also carbonyl containing dimers and multimers were observed. The additional bands which were not observed at lower H_2O_2 concentrations belong to multimerisation products of ferritin and to protein fragmentation products due to the strong oxidative damage.

Incubation of ferritin with H_2O_2 of 20 µmol/mg protein over several hours lead to an increasing carbonyl content within the protein as shown in Figure 2. The presence of H_2O_2 raised the protein modification by a factor of 3. Even catalase, a H_2O_2 decomposing enzyme, did not prevent ferritin completely from further carbonyl formation. This further increase of protein carbonyls after removal of

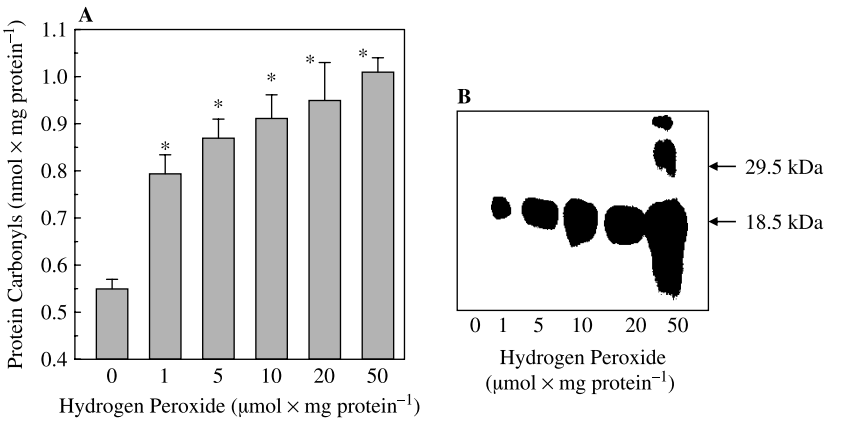

Figure 1. Oxidative carbonyl formation in ferritin. A, The quantification of protein bound carbonyls in ferritin after treatment with the indicated amounts of H_2O_2. The incubation time was 2 h. B, The same increase of DNPH derivatized carbonyls by immunoblot. At the highest oxidant concentration also dimerisation is observed. The blot shows one representative of two blots. The values in panel A represent the mean ± SE of 5 independent measurements ($\star p < 0.05$).

H_2O_2 gives a hint on ongoing intramolecular oxidation reactions.

In further experiments we tested whether the oxidation of ferritin is accompanied by increased proteolytic susceptibility. Interestingly an almost linear increase of the proteolytic susceptibility of ferritin until a treatment level of 20 μmol hydrogen peroxide per mg ferritin was measured (Figure 3). The higher extent of ferritin oxidation at H_2O_2 concentration of 30 μmol/mg protein results in a decrease of proteolytic susceptibility of the oxidized ferritin as demonstrated in Figure 3. The fact that the protein modification is still increasing with higher oxidant concentrations while the recognition and degradation of the model protein is declining can be explained by the increasing aggregation of ferritin in the presence of high oxidant concentrations which is also seen by the dimerisation in Figure 1B.

In order to test the effect of antioxidants on the proteolytic susceptibility of ferritin we used a concentration of H_2O_2 that induced maximal protein degradation (20 μmol/mg). The concentration dependent effects of antioxidants on the carbonyl content of ferritin are demonstrated in Figure 4. All tested antioxidants were able to decrease the oxidative damage of the model protein by H_2O_2 in a concentration dependent manner. Stobadine and Trolox in the concentrations used induced more intensive decrease of protein carbonyls compared with flavonoid extracts. But protein bound carbonyls were

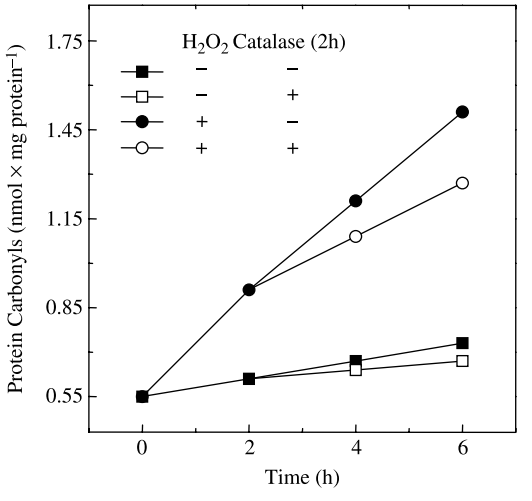

Figure 2. Time dependence of protein carbonyl formation. Ferritin was treated with 20 μmol hydrogen peroxide per mg protein. The concentration of protein carbonyls was measured at the indicated time points. As indicated catalase (10 μg per mg ferritin) was added after 2 h in order to remove the remaining hydrogen peroxide. The values are the mean of three independent experiments with SE less then 0.3 nmol protein carbonyls per mg protein.

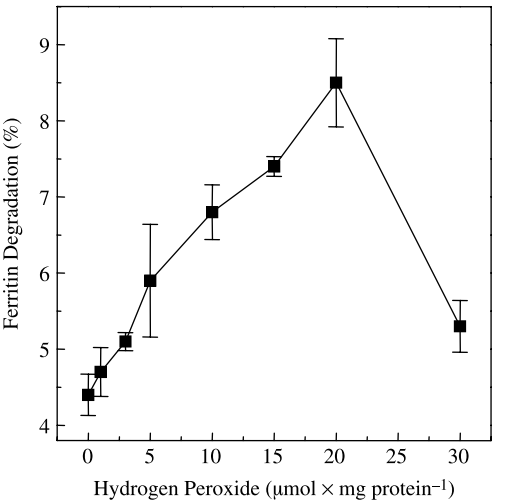

Figure 3. Proteasomal susceptibility of ferritin in dependence to the oxidation state. The proteolytic susceptibility towards the isolated proteasome was tested after treatment of ferritin with the indicated amounts of hydrogen peroxide and 2 h incubation. For further description see "Material and Methods". The values are the mean ± SE of five independent experiments.

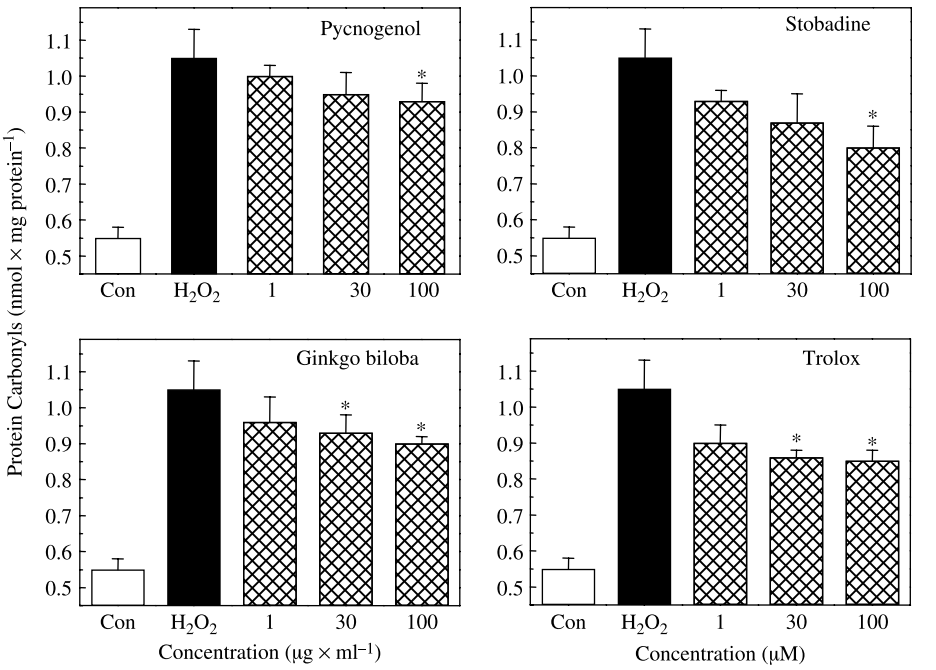

Figure 4. Influence of antioxidants on ferritin oxidation. Ferritin was treated with 20 μmol H_2O_2 per mg ferritin. After 2 h of ferritin oxidation were added the various concentrations of the indicated antioxidants. The protein carbonyl formation was measured by the ELISA method described above. The values represent the mean ± SE of three independent experiments ($\star p < 0.05$ antioxidant vs. hydrogen peroxide alone).

even at the highest antioxidant concentration only reduced to 50% of the hydrogen peroxide induced protein oxidation. So the tested antioxidants were only at high concentrations effective in preventing ferritin from oxidative damage.

As glutamic semialdehyde and aminoadipic semialdehyde in sum is expected to represent about one third of the total protein bound carbonyls we also measured the antioxidant capacity on these two compounds in detail (Figure 5). Figure 5A shows a typical HPLC chromatogram of an untreated sample and a blank reference. Beside the two measured semialdehydes three unidentified peaks were detected. H_2O_2 oxidation introduced preferably glutamic semialdehyde into the protein (Figure 5B). Therefore, the relation between GGS and AAS is changed due to

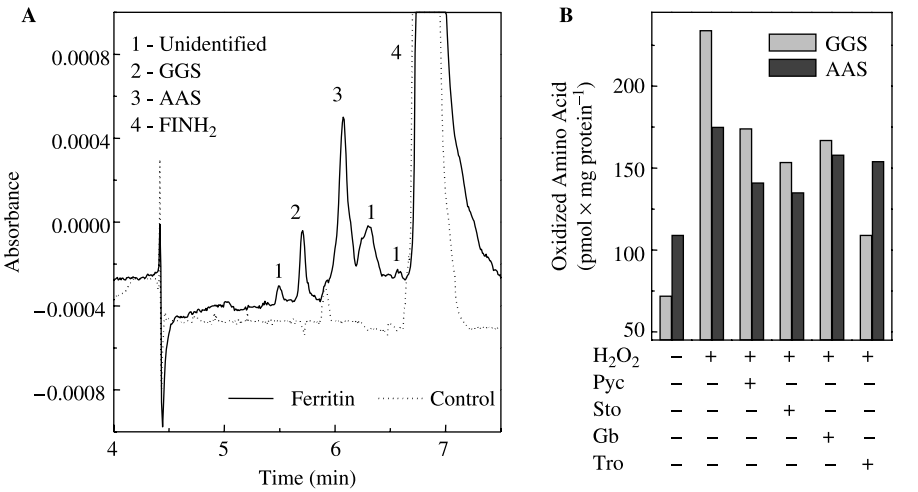

Figure 5. Measurement of glutamic semialdehyde and aminoadipic semialdehyde. Panel A shows a typical chromatogram of a non-oxidized ferritin (straight line). The peaks of the two quantified substances glutamic semialdehyde (GGS) and aminoadipic semialdehyde (AAS) as well as three unidentified Peaks and the $FINH_2$ peak are indicated. A blank control (dotted line) is also shown. Panel B is showing the GGS- and AAS-formation by treatment of ferritin with 20 μmol hydrogen peroxide per mg ferritin with or without antioxidant addition. Stobadine and Trolox were used at 30 μM concentrations, whereas Pycnogenol and Gingko biloba were used at 30 μg per ml. The values are the mean of two independent experiments. Abbrevations: Pyc, Pycnogenol®; Gb, Gingko biloba; Sto, Stobadine; Tro, Trolox.

the oxidation. All antioxidants reduced the GGS as well as the AAS content. While Pycnogenol® and Stobadine reduced both oxidation products, EGb 761 and Trolox were relatively ineffective in preventing ferritin from AAS generation. In contrast to all measured antioxidants Trolox was very effective in reducing the GGS-production and, therefore, it has the greatest effect in protecting the ferritin from AAS and GGS in sum.

The tested antioxidants Trolox and Stobadine as well as the flavonoid extracts were able to decrease the proteolytic susceptibility of oxidized ferritin in a concentration dependent fashion (Figure 6). Pycnogenol® and Stobadine were more effective, preventing the ferritin degradation by more then 60%. Trolox and the EGb 761 were less effective in reducing the proteolytic degradation by the proteasome in concentrations used. Higher doses of Pycnogenol® were almost completely able to normalize the degradation rate of the protein (Figure 6).

In order to test whether the preventive effect of the antioxidants is due to an interaction of these compounds with hydrogen peroxide rather then with the initially damaged protein intermediates we used two different experimental setups. In the first we added the antioxidants at the same time as the hydrogen peroxide and in the second setup we added the antioxidants 2 h after the addition of hydrogen peroxide together with catalase, which removes the remaining hydrogen peroxide. In Figure 7A the effects of antioxidants on protein carbonyl formation are depicted in these two setups. The proteolytic susceptibility towards the proteasomal degradation is shown in Figure 7B. As expected the antioxidant effect was more pronounced if antioxidants were added to ferritin together with hydrogen peroxide. A direct interaction of the antioxidant with the hydrogen peroxide is possible in the case of flavonoid extracts and, therefore, a reduction of the oxidative burden towards the protein cannot be excluded. The addition of antioxidants after the removal of hydrogen peroxide resulted in some decline in the proteolytic susceptibility of the oxidized ferritin. In both cases the effect of the antioxidants on the proteasomal degradation of the model protein ferritin is greater than the effect of reducing the protein bound carbonyl formation. If the antioxidants are used at the same time as the oxidant the proteolytic susceptibility is in the same range than those of the untreated sample. In contrast the carbonyl formation is only reduced by around 50%.

Discussion

Some oxidants damage proteins only slowly but if iron is present the relative low reactive ROS can be converted into highly reactive hydroxyl radicals via Fenton-reaction [65–67]. As hydroxyl radicals have a much higher potential to damage proteins it is important to keep the concentration of free iron as low as possible. Therefore, iron which is on the other hand important for the biosynthesis of the heme group and other proteins is stored in ferritin the main iron-storage protein [68–70].

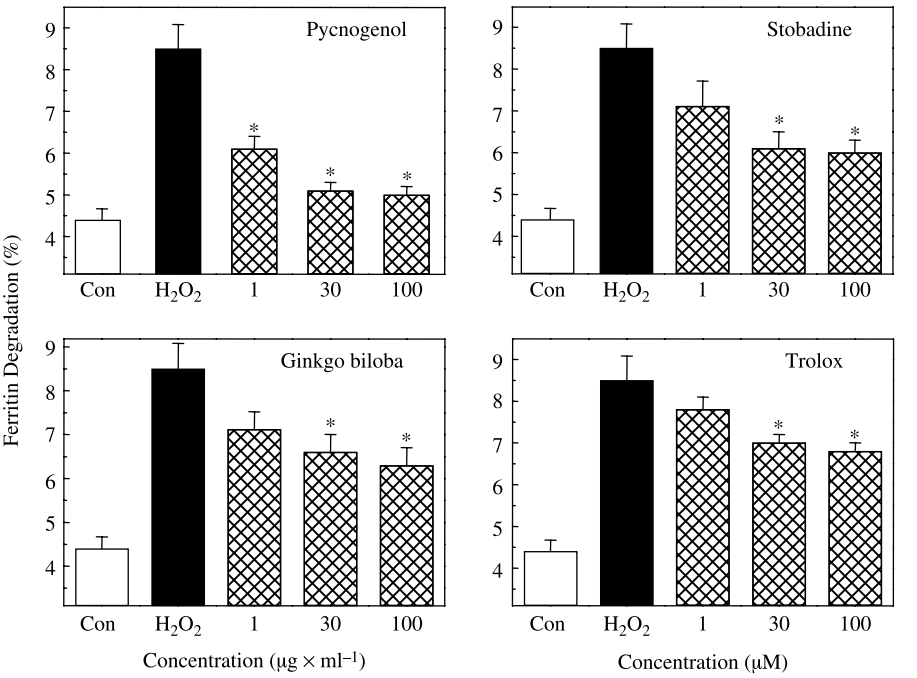

Figure 6. Influence of the antioxidants on the proteasomal susceptibility of ferritin. Ferritin oxidation and antioxidant treatment was performed as described in the legend to Figure 4. After the oxidation procedure the measurement of proteolytic susceptibility was performed as described in "Material and Methods". The values represent the mean ± SE ($n = 3$, $\star p < 0.05$ antioxidant vs. hydrogen peroxide alone).

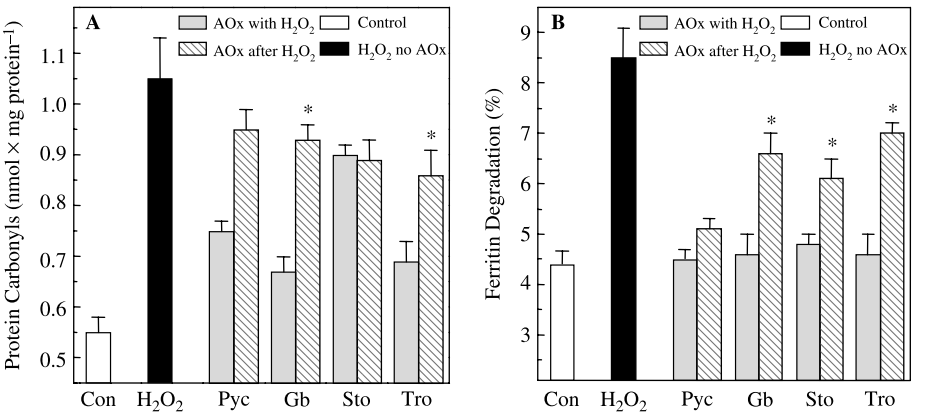

Figure 7. Influence of late antioxidant treatment on ferritin oxidation and degradation. Ferritin was oxidized as described above using again 20 μmol H_2O_2 per mg protein. Antioxidants (Stobadine, Trolox: 30 μM; Pycnogenol®, Gingko biloba: 30 μg per ml) were either added together with hydrogen peroxide (AOx with H_2O_2) or after 2 h of incubation (AOx after H_2O_2). In this case 10 μg catalase per mg ferritin was added. Protein oxidation and proteolytic susceptibility was measured after two hours presence of the antioxidant. Values are mean ± SE ($n = 3$, $\star p < 0.05$ AOx with H_2O_2 vs. AOx after H_2O_2). Abbrevations: Pyc, Pycnogenol®; Gb, Gingko biloba, Sto, Stobadine; Tro, Trolox.

Oxidatively damaged ferritin looses catalytical active iron ions. This is leading undoubtly to an accelerated oxidative damage of biological molecules. Therefore, it is important to prevent ferritin from oxidation. In this study we tried to reach this by preventing ferritin from the damaging effect of H_2O_2. In the presence of Stobadine, Trolox, Ginkgo biloba or Pycnogenol® the main iron storage protein ferritin was protected partly from carbonyl formation and almost complete from an elevated proteolytic degradation after oxidation with hydrogen peroxide.

Single compound antioxidants like the vitamin E derivative Trolox and the pyridoindole derivative Stobadine, as a known chain breaking antioxidant against lipid peroxidation, were used to study the preventive effects against ferritin oxidation. The antioxidant properties of Stobadine are summarized in Horáková et al. [71] and Horáková and Štolc [55]. Stobadine was described as a protective drug against reperfusion damage for isolated heart and also as cardioprotective drug with antihypoxic and antiarrhythmic effects [72]. Besides these compounds also the preventive effects of natural flavonoid extracts Pycnogenol® and EGb 761 against ferritin oxidation were studied. Pycnogenol® was reported to exert cardiovascular benefits [56] and may be useful for the prevention and/or treatment of vascular or neurodegenerative diseases associated with beta-amyloid toxicity [73]. EGb 761 is clinically important in cerebral insufficiency, atherosclerotic disease of peripheral artheries [74], has a protective effect on neurodegenerative diseases, cardiovascular dysfunction and ischemia reperfusion injuries [75,76].

As discussed in a number of publications the oxidative modification of proteins is accompanied by an unfolding of the protein structure and consequently by an exposure of hydrophobic peptide sequences to the protein surface which results consequently in a better recognition by the proteasome and, therefore, an enhanced degradation [77–79]. In order to minimize the surface area the exposed hydrophobic amino acids tend to aggregate and can than be cross-linked due to several reactions [80,81]. Higher oxidation states of proteins result in aggregated and covalently cross-linked proteins that are poor proteasomal substrates [21–23].

A very common oxidative modification of an amino acid within a polypeptide is the introduction of a carbonyl group into a side chain [81–83]. Protein bound carbonyls react easily with dinitrophenylhydrazine (DNPH) [84] which can be detected either spectrophotometrically, by an ELISA-technique or by immunobloting.

The protein bound aminoadipic semialdehyde (AAS) is formed during oxidative β-scission of the aliphatic side chains of alanine, valine and leucine [83,85]. γ-Glutamic semialdehyde (GGS) results from the oxidation of the two basic amino acids arginine and lysine as well as from proline. Both play an important role as oxidation products of polypeptides [86,87].

As demonstrated earlier protein oxidation results in an increased proteolytic susceptibility of various substrate proteins [88,89]. This is also the case for ferritin oxidation by hydrogen peroxide [47,48] and could be demonstrated for the here presented conditions too (Figure 3).

Accumulation of oxidized protein material due to a failure of the degradation of damaged proteins by the proteasomal system has been proposed as one (if not the most important) factor for some severe diseases and ageing. Therefore, it is very important to keep the damage of oxidation sensitive proteins like ferritin as low as possible. This can be reached partially by the used antioxidants Stobadine, Trolox and the flavonoide extracts Pycnogenol® and EGb 761. On the

other hand our experiments show also that it is almost impossible to prevent the oxidative damage of ferritin completely.

As seen in Figure 2 the protein bound carbonyl content is raising constantly with time and even the removal of hydrogen peroxide by catalase is not able to prevent this damaging effect. Due to protein oxidation numerous reactive groups are introduced into the protein structure, including hydroperoxides, alkoxy- and peroxy-radicals, aldehydes and other. These chemically active groups undergo further reactions with proteinogenous components despite the presence of catalase, only able to remove the initializing hydrogen peroxide.

In the presence of transitional metals, (probably iron in ferritin), metal-ion catalyzed oxidation of amino acids by hydrogen peroxide is induced by multiple pathways where the main oxidant is a ferryl complex and major products are alpha-ketoacids and aldehydes [36,90]. This type of reaction is relatively insensitive to inhibition by free radical scavengers. Pycnogenol® and flavonoids included in EGb 761 and Pycnogenol® extracts were able to chelate transitional metals [56,91]. Flavonoids, included in standardized extracts of Pycnogenol® and EGb 761, were able in addition to their free radical scavenging properties, almost completely to block H_2O_2 toxicity against HT-22 cells [92] and protected also other bacterial and mammalian cells against cytotoxity induced by H_2O_2 [93]. EGb 761 directly scavenged H_2O_2 [94]. But in spite of that, they were able to decrease protein carbonyl generation of ferritin induced by H_2O_2 only slightly. Stobadine is not able to scavenge H_2O_2 nor chelate iron. Both Stobadine and Trolox are able to scavenge hydroxyl radicals [95–97], inhibit lipid peroxidation, to quench singlet molecular oxygen, and scavenge peroxyl radicals [54,55,98]. Trolox is also able to detoxify the ferryl complex. Antioxidants, except Stobadine, were able to decrease protein carbonyl generation more efficiently when added simultaneously with hydrogen peroxide in comparison when added after oxidation (Figure 7). This might be explained by H_2O_2 scavenging or iron chelating effects of Pycnogenol® and EGb 761 and by the ability of Trolox to detoxify the ferryl complex. Stobadine has no ability to scavenge hydrogen peroxide, chelate iron or detoxify the ferryl complex and thus no difference between Stobadine added with H_2O_2 and after H_2O_2 was observed.

The impact of the antioxidants on the degradation of highly oxidized ferritin was not investigated in this study. As it is of great importance for the treatment of neurodegenerative diseases if the accumulation of oxidative protein aggregates could be reversed further studies should be addressed on the influence of antioxidants on the proteasomal degradation of highly oxidized proteins. If it is possible to increase the proteasomal susceptibility of highly cross-linked protein aggregates this would be a milestone in the treatment of Alzheimer's, Parkinson's and other neurodegenerative disease.

Proteins may be oxidatively modified by primary and secondary mechanisms. Primary mechanisms of protein oxidation may involve site-specific metal ion-catalyzed reactions [36,99]. This type of protein modification is relatively insensitive to inhibition by free radical scavengers [36]. Other primary mechanisms involve the interaction of free radicals released from various sources, what can naturally be prevented by antioxidants. Secondary mechanisms of oxidative protein injury can be mediated by the products of lipid oxidation, namely by MDA or HNE [100]. Secondary oxidation products like those of the lipid peroxidation, 4-hydroxynonenal and malondialdehyde are also able to oxidize proteins and to crosslink them [100–102]. Our experiments give no answer to the question if also secondary oxidation effects are prevented by the investigated antioxidants, but nevertheless they show that Stobadine, Trolox, Pycnogenol® and EGb 761 are able to protect a model protein *in vitro* against oxidative damage.

Oxidation of ferritin leads to an increased amount of protein bound carbonyls and to a reduced susceptibility for proteasomal degradation. The antioxidants Stobadine and Trolox as well as the flavonoide extracts Ginkgo biloba and Pycnogenol® are able to reduce the oxidative damage of H_2O_2 on ferritin in a concentration dependent manner. Further they are able to reduce the proteolytic susceptibility almost to the level of the non oxidized protein at least if the antioxidants were applied at the same time as the oxidant.

Acknowledgements

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