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Oxidation of bovine serum albumin initiated by the Fenton reaction—effect of EDTA, *tert*-butylhydroperoxide and tetrahydrofuran

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Abstract

Oxidation of bovine serum albumin (BSA) was investigated using different oxidants: The water-soluble azo-initiator 2,2′azo-bis-(2-amidinopropane) hydrochloride (AAPH), a combination of $FeCl_3$ and ascorbate or the Fenton oxidant consisting of $FeCl_2$, H_2O_2 and EDTA. In addition, the effects of exogenous compounds such as *tert*-butyl hydroperoxide (*t*BuOOH) or solvents such as tetrahydrofuran (THF), often used in model systems, was evaluated. The extent of protein damage was studied by measuring protein carbonyl groups and protein hydroperoxides. The interaction between Fenton oxidant and EDTA, THF or *t*BuOOH was further characterized using spin trapping electron spin resonance (ESR) spectroscopy. The results showed that the extent of protein oxidation depended on the oxidant used. The Fenton oxidant was the most reactive of the initiators tested. However, in the absence of EDTA, the Fenton system produced protein carbonyl groups on BSA equivalent to that obtained with the other oxidants, however, significantly more protein hydroperoxide was produced. Surprisingly, it was also found that addition of *t*BuOOH or THF to BSA reduced protein damage when the oxidation was initiated with the Fenton oxidant. ESR investigation showed that EDTA played a key role in the generation of free radicals. It was also revealed that in an EDTA containing system both *t*BuOOH and THF were able to react with radicals without inducing protein damage in effect protecting BSA from oxidative damage.

Keywords: *Protein oxidation, BSA, Fenton, THF, t BuOOH, ESR, spin trap*

Introduction

Oxidative damage to lipids has been extensively studied [1,2], and recently the interest in oxidation of biological macromolecules such as DNA and protein has grown significantly [3,4]. Awareness of the role of oxidative stress in the pathology of many diseases such as Alzheimer's [5] and aging [6,7] has also increased. Now accumulation of oxidized proteins is being recognized as one of the markers of oxidative stress. Oxidation has been shown alter protein structure, function and impair enzyme activity [8,9]. Investigations, intended to get an insight into the mechanism behind oxidative modification of biomolecules, have been designed to avoid disturbing biological materials and subsequently many studies have been performed on model systems [10,11]. Today, different types of free radicals are used to initiate and simulate oxidation of biological systems. Various free radical initiators have advantages and disadvantages. Initiators can be synthetic molecules such as 2,2′azo-bis-(2-amidinopropane) hydrochloride (AAPH), generating carbon centered alkyl

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or peroxyl radicals. Another common method of radical formation, based on iron chemistry and decomposition of peroxides, produces reactive peroxyl and alkoxyl radicals (i.e. Fe^{3+}/ascorbate, Fenton chemistry, heme proteins). Fenton oxidants (i.e. Fe^{2+} and H_2O_2, and sometimes EDTA) are some of the most popular oxidants, believed to simulate *in vivo* oxidation. Fenton oxidants generate hydroxyl radicals, which are among the most reactive species in biological systems [12,13]. However, the initiation mechanisms behind the chemistry of *in vivo* free radical mediated oxidation is very complex.

We have aimed at investigating the effects of Fenton oxidants on proteins in detail. *In vivo* degradation of lipids and protein peroxides is also known to be a key event in the initiation of biological damage [14,15]. Some studies have been performed using exogenous organic peroxides such as *tert*-butyl hydroperoxide (*t*BuOOH), cumene hydroperoxide or benzoyl peroxide as models for oxidative stress inducing agents [16–18]. These compounds have been reported to be good for modeling oxidative stress, however, the reactions may not directly reflect the events taking place *in vivo* and may also have some impact on the kinetics of radical production and free radical damage. It is often necessary to test the antioxidative activity of lipophilic antioxidants, such as carotenoids, by adding them to assay systems as solutions in organic solvents such as tetrahydrofuran (THF) or dimetyl sulfoxide (DMSO) [19,20]. Such small exogenous (solvent) molecules may react with radicals and are likely to affect the course of free radical generation as well as participate in the radical reactions generating interference thereby impairing conclusions drawn from experimental work.

In the present study, bovine serum albumin (BSA) was used as the macromolecule target. Protein carbonyls and peroxides were measured after initiation of oxidation with: AAPH, Fe^{3+}/ascorbate, and Fenton oxidant (Fe^{2+}, EDTA and H_2O_2). The effect of the exogenous peroxide, *t*BuOOH and the solvent THF, were evaluated on the free radical generating systems. In addition, and in order to gain a deeper understanding of the reaction mechanisms, electron spin resonance (ESR) spectroscopy was used to identify the free radicals formed during the course of oxidation when Fenton chemistry was applied.

Materials and methods

Materials

BSA, hydrogen peroxide, *t*BuOOH, 2-methyl-2-nitrosopropane (MNP), 4-pyridyl-1-oxide-*N*-*tert*-butylnitrone (POBN) and xylenol orange were from Sigma (St Louis, MO, USA). Analytical grade THF was from MERCK (Darmstadt, Germany) and 2,4-dinitrophenylhydrazine (DNPH), fluoresceinamine isomer II ($FINH_2$), AAPH and sodium cyanoborohydride were from Aldrich (Milwaukee, WI, USA). 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was from Fluka (Buchs, Switzerland). All chemicals were of analytical grade and double deionized water was used throughout.

Incubation

BSA (10 mg/ml) was incubated in a shaking water bath at 37°C in 50 mM phosphate buffer pH 7.4 and THF or *t*BuOOH were added to the reaction mixture prior to initiation. THF represented 1% of the total volume whereas *t*BuOOH was added to the protein solution to give a final concentration of 5 mM. The reactions were initiated by adding AAPH (50 mM) or $FeCl_3$ and ascorbic acid (0.10 and 25 mM, respectively) or $FeCl_2$, EDTA and hydrogen peroxide (1, 1 and 2 mM, respectively). After 3 or 6 h incubation, a 2.5 ml sample was taken out and eluted on a PD 10 column (Amersham Biosciences, Uppsala, Sweden) pre-equilibrated with phosphate buffer (50 mM, pH 7.4) to remove the initiating agents. The protein concentration was determined in the collected protein fractions (3.5 ml) which were further analyzed for protein bound hydroperoxides and carbonyls groups.

Protein hydroperoxides (FOX1)

The protein fraction (50 μl) was incubated with 950 μl of ferrous ammonium sulfate/xylenol orange reagent (2.5 and 1 mM, respectively) and incubated at room temperature for 30 min. The absorbance was read at 560 nm on a UV-160 Shimadzu spectrophotometer using xylenol orange reagent as a blank. Standard curves were prepared using hydrogen peroxide.

Protein carbonyl groups

The protein carbonyl content was determined using DNPH, as previously described by Levine et al. [21]. The protein fraction (200 μl) was precipitated with trichloroacetic acid (TCA) and incubated for 10 min with DNPH (10 mM) in 2 M HCl. A blank incubated without DNPH was run in parallel for each sample. After precipitation with TCA the pellet was washed three times with ethanol/ethylacetate (1:1 v/v) containing 10 mM HCl. The carbonyl content was calculated as mol carbonyl per mol BSA using the absorbance at 370 nm for the dinitrophenylhydrazone derivate as described.

Protein determination

Protein content of the protein fraction was determined using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA) according to recommendations by the manufacturer.

ESR measurements

Spin trapping. A spin trap (DMPO, POBN or MNP; 20 mM) was added to the reaction mixture prior initiation of oxidation. Immediately after initiation the reaction mixture was transferred into ESR capillary tubes (Wilmad, Buena, NJ, USA) and placed into the cavity of a JES FR 30 ESR spectrophotometer (JEOL, Tokyo, Japan). The settings were: microwave power 4 mW, modulation amplitude 0.1 mT, sweep time 30 s, sweep width 5 mT, and time constant 0.3 s. Spectra were recorded at room temperature after 2, 5 and 10 min of incubation.

Simulations. Simulation and fitting of ESR spectra was performed by the PEST WinSIM program [22]. The intensities of the ESR spectra were calculated relative to the intensity of the built-in Mn^{2+}-standard by using doubly integrated spectra.

Statistics

The experiments were carried out at least in triplicate (unless otherwise stated) on separate days. The results are given as mean values of triplicates with indication of the standard deviation. Evaluation of statistical significance of differences was performed using the Student's *t*-test.

Results

Protein oxidation

Incubation of BSA with the different initiators AAPH, Fe^{3+}/ascorbic acid, or Fe^{2+}/H_2O_2/EDTA (Fenton oxidant) resulted in the formation of a significantly high level of oxidative modification of BSA measured as carbonyl groups and peroxides (Figure 1). However, after 3 h of incubation the Fenton initiating agent gave approximately five times as many peroxides and twice as many carbonyl groups on BSA, when compared to the other two initiators. Addition of 1% THF to the reaction mixture gave ≈50% reduction in carbonyl group production on BSA and significantly reduced the level of hydroperoxides when initiation was performed with the Fenton initiator. This effect was not observed when oxidation was initiated with either AAPH or Fe^{3+}/ascorbic acid (Figure 1). Similarly, addition of *t*BuOOH to the reaction mixture halved the amount of carbonyl groups and bound peroxides formed on BSA when oxidation was initiated with the Fenton oxidant (Figure 1A,B). Increasing the THF content to 4% or *t*BuOOH to 10 mM gave similar results as with lower concentrations (data not shown).

The interaction between the Fenton oxidants (i.e. Fe^{2+}, H_2O_2 and EDTA) and THF or *t*BuOOH was further investigated. Results showed that in the absence of EDTA, the protein carbonyl groups remained at the same level irrespective of the presence of THF or *t*BuOOH in the reaction mixture (Figure 2A). However, the level of carbonyl groups formed remained lower than when initiation was performed in the presence of EDTA (Figure 2A). Correspondingly, in the absence of EDTA protein bound peroxides increased to a similar level in all treatments (Figure 2B). Incubation in the absence of H_2O_2 did not result in significantly altered carbonyl group levels unless THF was present (Figure 2A). In contrast, the level of protein peroxides remained the same unless incubated with *t*BuOOH, or in the absence of H_2O_2 (Figure 2B). Interestingly, comparison of the results obtained when H_2O_2 (2 mM) was substituted with *t*BuOOH (5 mM), demonstrated that incubation in the presence of *t*BuOOH resulted in less oxidative protein damage than incubation in the presence of H_2O_2.

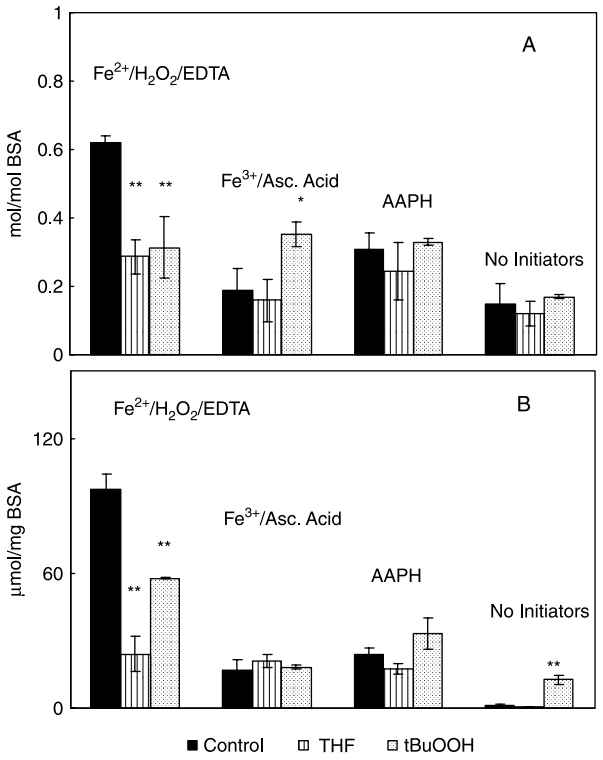


Figure 1. Protein modification (A) protein carbonyl groups, (B) protein bound hydroperoxides on BSA (10 mg/ml) and after incubation with different initiators for 3 h at 37 °C in phosphate buffer pH 7.4 and with: filled bars: control, stripped bars: in the presence of 1% THF and dotted bars: in the presence of 5 mM *t*BuOOH. With $\star p < 0.05$ and $\star\star p < 0.01$ comparing the control with the presence of THF or *t*BuOOH.

ESR spectroscopy

Investigations using ESR spectroscopy allowed the detection of the free radicals formed during oxidation

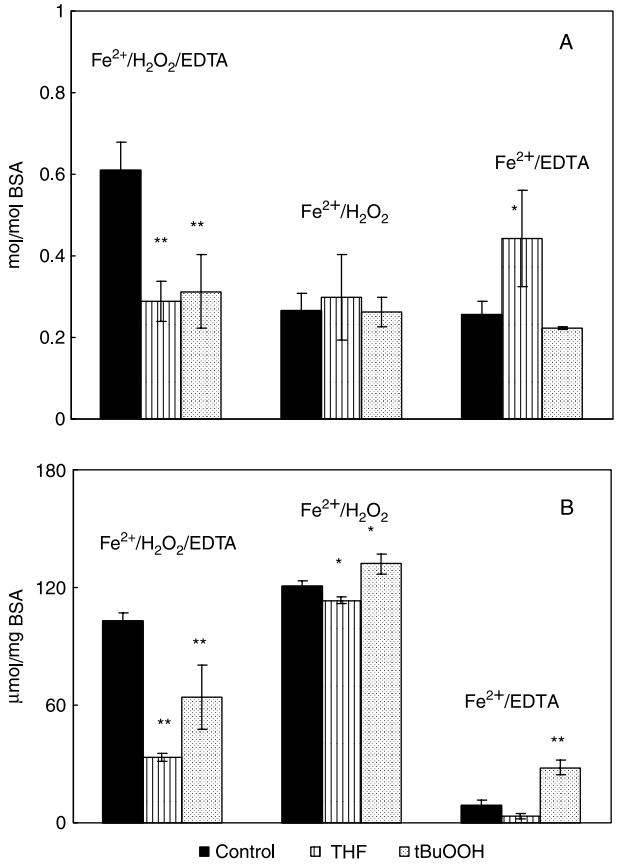


Figure 2. Protein modification (A) protein carbonyl groups, (B) protein bound hydroperoxides on BSA (10 mg/ml) after incubation with the different combination of Fenton initiators for 3 h at 37 °C in phosphate buffer pH 7.4 and with filled bars: control, stripped bars: in the presence of 1% THF and dotted bars: in the presence of 5 mM *t*BuOOH. With $\star p < 0.05$ and $\star\star p < 0.01$ comparing the control with the presence of THF or *t*BuOOH.

of BSA when Fenton chemistry was applied in the presence of different spin traps.

POBN. The spin trap POBN was used in a series of experiments where the effect of various components in the system was studied (Figure 3). The ESR spectra from the POBN experiments without THF or *t*BuOOH could all be satisfactorily fitted to a single simulated spectrum assigned to a spin adduct of POBN (**I**) with the hydroxyl radical, POBN/•OH (Table I) [23]. A quartet signal (**II**) also appeared in some of the ESR spectra from these experiments. This signal was assigned to the *t*-butylaminoxyl radical ($Me_3CNHO\bullet$), most likely formed by decomposition of either the spin trap or the POBN/OH spin adduct.

The experiments reveal that the addition of THF to the reaction mixtures increased the formation of spin adducts considerably, and the combination of EDTA and THF in unison gave the highest levels of spin adducts. However, addition of THF to the reaction mixtures changed the ESR spectra of the spin adducts.

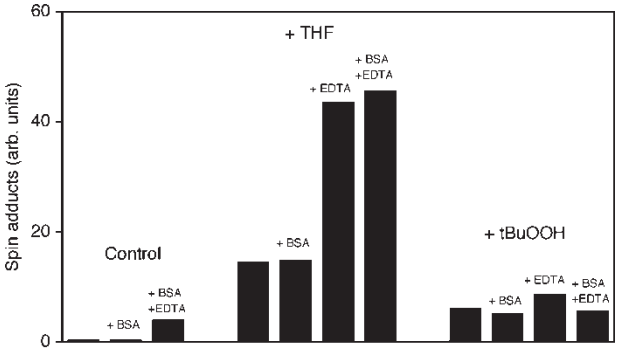


Figure 3. POBN spin adducts detected using ESR spectroscopy with Fenton oxidant ($FeCl_2$:1 mM and H_2O_2 2 mM) in 50 mM phosphate buffer pH 7.4 in the presence or in the absence of BSA (10 mg/ml), EDTA (1 mM), THF (1%) or *t*BuOOH (5 mM).

A mixture of two ESR spectra with different hyperfine coupling constants, **III** and **IV**, is needed to fit the experimental ESR spectra (Table I). The fitting reveals practically identical ratios for the two spin adducts in all experiments of 65–68% for **III** and 32–35% for **IV**. The hyperfine coupling constant values suggest that the two adducts are most likely formed by trapping alkyl radical [23]. Present in reaction mixtures only containing THF indicates that these radicals are derived from the trihydrofuran-2-yl radical obtained from THF by hydrogen abstraction. Radical trapping of POBN gave spin adducts with two chiral carbon atoms, and two sets of enantiomeric pairs with distinct hyperfine coupling constants (Figure 4). Different diastereomers of spin adducts have been separated and detected by HPLC/MS in the reaction of 1-hydroxyethyl radicals with POBN [24].

The addition of *t*BuOOH also increased the levels of spin adducts when compared to control experiments, however, the effect was much smaller than observed when adding THF. The ESR spectra of POBN spin adducts detected in reaction mixtures with *t*BuOOH were identical, and all could be fitted to a single spectrum that was assigned to spin adducts (**V**) formed by trapped alkyl radicals based on the values of the coupling constants (Table I) [23].

MNP. Similarly, incubation using MNP as a spin trap also revealed the presence of two spin adducts. The main adduct (**VI**) representing 57% of the ESR signal, could be assigned to the THF radical. Addition of EDTA to the reaction mixture significantly enhanced the production of the THF adduct which subsequently represented 84% (Figure 5).

DMPO. POBN and MNP mainly trap carbon centered radicals, therefore the ability of the spin trap DMPO to form stable spin adducts with oxygen

Table I. ESR signals and assignment of spin adducts.

			Coupling constants		
		Spin trap	a_N (G)	a_H (G)	Assignment
I	–	POBN	15.0	1.7	POBN/•OH
II	–	POBN	14.5	14.3	Me_3CNHO•
III	THF	POBN	15.7	2.4	THF radical adduct
IV	THF	POBN	15.5	3.1	THF radical adduct
V	*t*BuOOH	POBN	15.9	2.7	Alkyl radical adduct
VI	THF	MNP	15.5	1.7	THF radical adduct
VII	THF	DMPO	14.9	14.8	DMPO/•OH
VIII	THF	DMPO	15.8	23.2	Alkyl radical adduct
IX	THF	DMPO	15.6	19.6	Acyl radical adduct
X	*t*BuOOH	DMPO	14.9	15.6	Mixture of DMPO/•OH and DMPO/tBuO·
XI	*t*BuOOH	DMPO	16.3	23.2	DMPO/•CH_3
XII	*t*BuOOH	DMPO	15.0	22.3	Alkyl radical adduct

centered radicals was used in order to detect possible precursor radicals to those detected by POBN.

In the THF experiments, the individual components of the ESR spectra could be identified by fitting of the experimental spectra to simulated spectra (Figure 6). Results showed that in the presence of EDTA the signal intensity was significantly enhanced when compared with signals obtained in the absence of EDTA. Series of spectra were recorded and the presence of THF and EDTA were varied. In all cases the fitting of the ESR spectra gave a mixture of three species **VII**, **VIII** and **IX** (Table I). Based on the values of the coupling constants the spin adduct **VII** was assigned to the hydroxyl radical adduct DMPO/•OH, while **VIII** and **IX** were assigned to spin adducts of carbon-centered radicals and acyl radicals, respectively [23,25]. The DMPO/•OH adduct (**VII**) was generally the most abundant of the three spin adducts, except in the reaction mixture containing BSA, Fe^{2+}, EDTA, H_2O_2 and THF (Figure 7). The amount of spin adducts increased considerably when EDTA was added to the reaction mixtures (Figure 7).

Similar results were obtained in a series of spin trapping experiments where *t*BuOOH was added to the reaction mixtures instead of THF. Investigation with DMPO reveals that the experimental ESR spectra can be simulated as mixtures of three species. Addition of EDTA to the reaction also enhanced the intensity of the signal (Figure 6). The spin adduct **X** had coupling constants suggesting an unresolved mixture of DMPO/•OH adduct and an adduct with a *t*butoxyl radical, whereas the two spin adducts **XI** and **XII** were assigned to trapping of carbon-centered radicals (Table I) [23,25]. The coupling constants for spin adduct **XI** were identical to the coupling constants for the DMPO spin adduct with CH_3· radicals. This adduct was the most abundant of the two alkyl radical spin adducts (Figure 7). *t*-Butoxyl radicals, formed by the Fenton reaction between *t*BuOOH and Fe^{2+}, are expected to produce methyl radicals by β-scission. The presence of EDTA increased the amount of spin adducts, where **X** and **XI** were formed in almost equal amounts in the presence of EDTA (Figure 7). High amounts of spin adducts were also formed when hydrogen peroxide was absent.

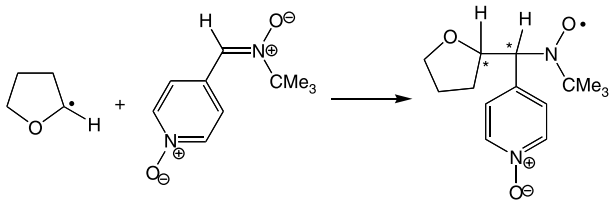


Figure 4. Trapping of the trihydrofuran-2-yl radical by POBN producing spin adducts with two chiral carbon atoms (marked by *) giving 4 stereoisomers that can be divided into 2 enantiomeric pairs (**III** and **IV**).

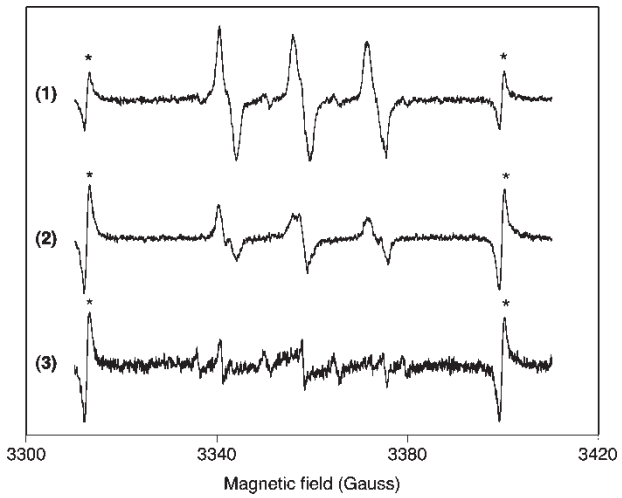


Figure 5. ESR spectra of radical adducts of MNP (20 mM) formed 2 min after incubation of BSA (10 mg/ml), Fe^{2+} (1 mM), and H_2O_2 (2 mM) in 50 mM phosphate buffer pH 7.4 with either **(1)** EDTA (1 mM) and 1% (v/v) THF or **(2)** 1% (v/v) THF or **(3)** EDTA (1 mM).

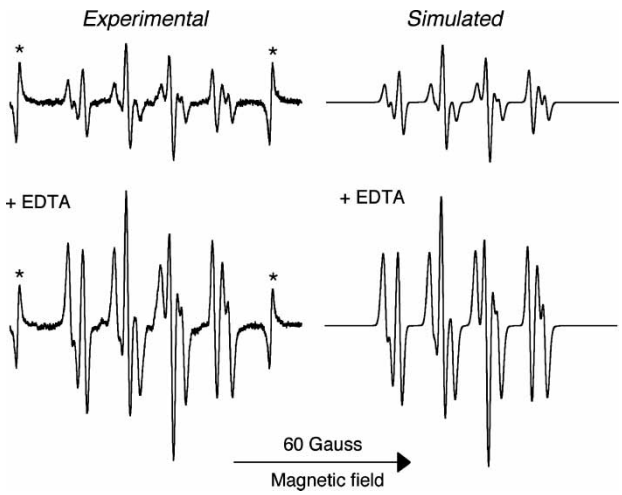


Figure 6. Experimental and simulated ESR spectra of DMPO spin adducts observed in a system with BSA, THF, H_2O_2 and Fe^{2+}. The lines marked by ⋆ indicates the Mn^{2+} marker signal. The simulated spectra are based on mixtures of spin adducts **X**, **XI** and **XII**.

Discussion

Oxidation is a process, normally occurring at low levels in cells and tissues triggering activation of cell signaling pathways [26]. However, under some conditions, oxidation may become uncontrolled resulting in the accumulation of reactive oxygen species, able to induce damage to macromolecules *in vivo* [27]. For many years lipid oxidation has been in the focus of investigation, but due to their relatively high abundance it is now recognized that proteins are the main targets for oxidants [28,29]. Highly reactive oxidants present in biological systems are able to generate reactive oxygen species, inducing protein damage. The hydroxyl radical is one of the most reactive radicals in biological systems and the rate constants for reaction with macromolecules are close to diffusion rate [30]. Proteins can scavenge 50–75% of hydroxyl radicals produced within a cell and the highest level of carbonyl groups on proteins are obtained with hydroxyl radical generating systems [28]. Production of hydroxyl radicals in model systems is often performed using Fenton oxidant, consisting of iron (II) reacting with hydrogen peroxide and sometimes in the presence of the chelating agent EDTA. EDTA can either stimulate oxidation or inhibit oxidation depending on the iron chelator ratio, causing inhibition of oxidation with ratios higher than 1:1 and stimulation of oxidation at lower ratios [30]. In contrast others have found that lower concentration of EDTA to iron (1/2:1) can inhibit the development of oxidation in foods [31]. In the present system, addition of EDTA in a ratio 1:1 with iron resulted in stimulation of protein oxidation in agreement with the proposed metal ligand ratio of 1:1 being prooxidative. From the three different initiators tested Fenton oxidants gave the highest level of protein carbonyl groups and peroxides. This confirms that hydroxyl radicals are the most reactive species and result in the highest levels of protein modification. Davies argued that the severity of the damage is important with selective oxidants [28], but this was not investigated in this study. Conditions present *in vivo* are difficult to simulate and obtained results show that protein oxidation depends to some extent on the type of oxidant used [32,33].

The factors governing the interaction between protein and other molecules present *in vivo* is unclear, it has been reported that proteins can transfer their radicals to other molecules [34], conversely, it is also believed that proteins can trap radicals and so act as antioxidants [35]. It is generally accepted lipid oxidation breakdown products such as peroxides and that secondary oxidation products such as reactive aldehydes interact with proteins resulting in protein modification [10,11,36]. Peroxyl radicals, generated in systems undergoing lipid peroxidation, are believed to damage protein [37,38]. Surprisingly the addition of *t*BUOOH or THF, likely to generate reactive peroxyl and alkoxyl radicals, to the reaction mixture resulted in significantly less protein damage when added in combination with the Fenton oxidant. Furthermore, addition of lipid to the reaction mixture, containing *t*BuOOH, did not affect the level of protein oxidation (data not shown). ESR investigation revealed that peroxyl and alkoxyl radical species are formed, but do not induce any significant or detectable protein damage. *t*BuOOH and THF are often used in model systems, respectively to simulate the hydrophobic lipid hydroperoxide [16,17] or as solvent for non water soluble antioxidants [19,20]. Scheme 1 shows the different radicals formed in the reaction mixture demonstrating that easily oxidized small molecules can prevent or retard damage to macromolecules in the presence of Fenton oxidant.

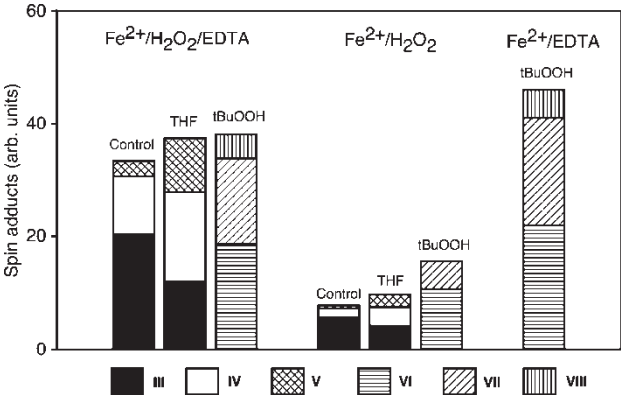
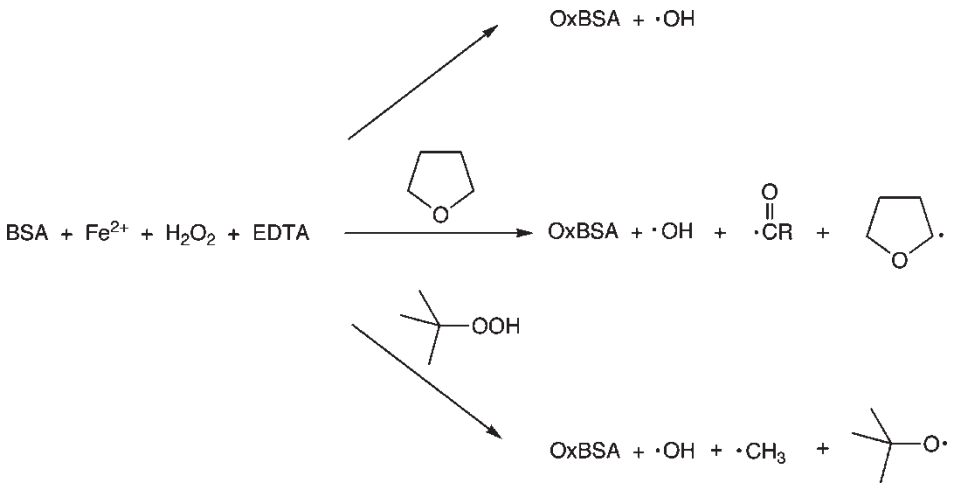


Figure 7. DMPO spin adducts detected using ESR spectroscopy during incubation of BSA (10 mg/ml) in 50 mM phosphate buffer pH 7.4 with Fenton oxidant ($FeCl_2$, 1 mM; EDTA, 1 mM; H_2O_2, 2 mM) in the presence or in the absence of THF (1%) or *t*BuOOH (5 mM).



Scheme 1. Oxidation products formed after oxidation of BSA by Fenton oxidant. Addition of THF or *t*BuOOH results in less protein damage and generates THF or *t*BuOOH derived radicals.

Nevertheless, it is possible that the level of reactive radicals present in the system was too low to induce any noticeable modification of the proteins. However, in a series of studies performed by Davies group, it was demonstrated that the presence of lipid can divert some radical reactions from the protein giving concomitant lipid oxidation, and resulting in limited protein modification [3,39]. These results are in agreement with our findings where both THF and *t*BuOOH result in less protein oxidation. In other systems, solvents have also been reported to behave like antioxidants, e.g. ethanol in beer [40]. Similar results were obtained with DNA [41], where less damage was observed in the presence of ethanol when initiation was performed using the Fenton oxidant.

The extent of protein oxidation using Fenton oxidant was dependent on the presence of the chelating agent EDTA indicating the importance of the iron ions in the process. Investigation performed using the Fenton system without EDTA resulted in similar levels of protein carbonyls as with other types of initiators, but in contrast gave the highest level of peroxides, independently of the presence of THF or *t*BuOOH (Figure 1). The literature discussing either pro- or anti-oxidative activity of chelators such as EDTA is confusing. For example, Manusco et al. [42] found that a combination of iron and EDTA increased the level of Tween 20 peroxide, which they explained by inhibition of peroxide decomposition resulting in accumulation as a consequence of chelation of the iron by EDTA. Addition of EDTA to the reaction mixture in the presence of THF or *t*BuOOH changed the extent of the protein damage, resulting in reduced levels of carbonyls and peroxides (Figure 2). EDTA is shown to induce the formation of more radical adducts but EDTA in combination with THF changes the ratio of the adduct lowering the levels hydroxyl radical of and increasing THF or *t*BuOOH derivative radical, which in turn resulted in reduced protein damage. EDTA decreases the level of protein oxidation in the presence of small oxidizable molecules, whereas EDTA increases the level of protein oxidation in the absence of oxidizable molecules when compared to a system with no EDTA. Proteins have positively charged residues on their surface and chelating agents may bind to their surface, therefore, a chelating agent might preferentially bind at the surface of BSA protecting it against oxidation. Yin et al. [43] report that EDTA could protect protein form oxidation but that exposed methionine residues were more difficult to protect from oxidative modification than the more buried methionine residues. Others also report that oxidative degradation of albumin by the iron EDTA complex is governed by the binding of iron EDTA complex to the protein and that damage occurs randomly at the surface of the protein caused by OH radicals generated in solution [44]. With the Fenton reaction the location of the active metals is very important. Chelators as well as residues such as His, Cys and Met are important in metal binding, localizing the iron in their vicinity and inducing local damage. The different capacity of proteins to bind metals often renders them redox inactive or conversely presents them in active form. Retention of iron by BSA is reported to be rather low when compared to other proteins at between 0.6 and 1.4 mg iron per g protein [45]. We proposed that addition of EDTA to the reaction mixture results in displacement of the iron from the BSA core thereby preventing damage to BSA but targets oxidation of smaller susceptible molecules present at the site of radical attack. It can be speculated that an effective antioxidant strategy could be designed where antioxidant and protein compete for the free radical attacks.

Conclusion

Fenton chemistry is complex and the extent of oxidation depends on many factors including nature, localization and concentration of metal ions, presence of peroxide, availability of iron binding ligands and the relative rate of different competing reactions. However, it has been shown that proteins can be protected by small molecules which are able to react with the initial radicals without inducing further damage to the protein. At present, we have no fundamental understanding of how protein interacts with endogenous molecules such as antioxidants and how protein and lipid damage are interrelated, therefore, more model system studies are needed. However, future studies must keep in mind that results obtained in such model systems are always challenged by possible side reactions, and therefore the results obtained may not always reflect the situation encountered in more complex systems especially *in vivo*.

Acknowledgements

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