Comparison of the Effects of Tert-Butyl Hydroperoxide and Peroxynitrite on the Oxidative Damage to Isolated Beef Heart Mitochondria

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Summary

Isolated beef heart mitochondria have been exposed to tert-butyl hydroperoxide (tBHP) and peroxynitrite (PeN) in order to model the effects of reactive oxygen and nitrogen species on mitochondria *in vivo*. The formation of malondialdehyde (MDA), protein carbonyls, lipofuscin-like pigments (LFP), and nitrotyrosine was studied during incubations with various concentrations of oxidants for up to 24 h. The oxidants differed in their ability to oxidize particular substrates. Fatty acids were more sensitive to the low concentrations of tBHP, whereas higher concentrations of PeN consumed MDA. Oxidation of proteins producing carbonyls had different kinetics and also a probable mechanism with tBHP or PeN. Diverse proteins were affected by tBHP or PeN. In both cases, prolonged incubation led to the appearance of proteins with molecular weights lower than 29 kDa bearing carbonyl groups that might have been caused by protein fragmentation. PeN induced nitration of protein tyrosines that was more intensive in the soluble proteins than in the insoluble ones. LFP, the end products of lipid peroxidation, were formed more readily by PeN. On the other hand, fluorometric and chromatographic techniques have confirmed destruction of LFP by higher PeN concentrations. This is a unique feature that has not been described so far for any oxidant.

Key words

Oxidative stress • Free radicals • Lipid peroxidation • Lipofuscin-like pigments • Nitrotyrosine

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Introduction

Reactive oxygen and nitrogen species (RONS) cause the oxidative stress under overproduction; however, at very low concentration they can function as signaling molecules (Irani *et al.* 1997, Lander 1997). Oxidative stress participates in many pathologies, and it is also considered a major factor in the ageing process (Harman 2003).

Mitochondria represent the major source of RONS in the cell. They contain nitric oxide synthase producing NO, and several pathways generating reactive oxygen species, especially superoxide (Bolisetty and Jaimes 2013, Cadenas and Davies 2000). Besides being an important source, mitochondria are also the target of RONS that results in the damage to their membrane lipids, proteins, and nucleic acids (Vaca *et al.* 1988). These modifications lead to the loss of mitochondrial function. Mitochondrial dysfunction affects the electron transport chain, inhibits oxidative phosphorylation and

releases further free radicals (Szabó *et al.* 2007, Vatassery *et al.* 2004).

Both reactive oxygen and nitrogen species initiate membrane lipid peroxidation (Korkmaz *et al.* 2009, Singh *et al.* 2007). Lipid peroxidation is the process that leads to the damage of lipid molecules in cell membranes containing unsaturated fatty acids. Secondary products of lipid peroxidation are reactive aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal. Aldehydes also react with amino groups of proteins and phospholipids. The end products of lipid peroxidation exhibit characteristic fluorescence, and they are called lipofuscin-like pigments (LFP) (Ivica and Wilhelm 2014).

Oxidative attack to the soluble and membrane proteins results in the formation of protein carbonyls that is accompanied with protein fragmentation or polymerization (Berlett and Stadtman 1997). Reactive nitrogen species produce 3-nitrotyrosine (NT) within protein structures, and it is widely used as a marker of oxidative stress induced by reactive nitrogen species (Herce-Pagliai *et al.* 1998). The principal reactive nitrogen species is peroxynitrite (PeN), which originates from the reaction between superoxide and nitric oxide (Szabó *et al.* 2007).

The decomposition of peroxynitrite to hydroxyl and nitrogen dioxide radical plays a role in the induction of lipid peroxidation and protein nitration. These reactions result in the formation of nitrotyrosine moieties in affected proteins (Beckman and Koppenol 1996, Szabó *et al.* 2007).

Tert-butyl hydroperoxide (tBHP) is an organic oxidative compound that is used as a source of free radicals in *in vitro* studies (Ayala *et al.* 2014). It has similar metabolic effects as hydrogen peroxide (Drahota *et al.* 2005, Křiváková *et al.* 2007).

In order to evaluate the specific effects of reactive oxygen species and reactive nitrogen species on mitochondria, we incubated isolated beef heart mitochondria with either tBHP or PeN *in vitro* and analyzed the products, especially reactive aldehydes, protein carbonyls, protein nitrotyrosine, and lipofuscin-like pigments during a 24 h period.

Material and Methods

Isolation of mitochondria

Beef hearts were obtained fresh from the local slaughter house. Mitochondria from beef heart were isolated by the method of Haas and Elliott (1963) with some modifications. Mitochondria were isolated by differential centrifugation. The ground muscle was homogenized in a solution containing 0.05 M Tris, 0.001 M EDTA and 0.9 % NaCl, adjusted to pH 7.4. The homogenate was centrifuged twice at 2 000 g for 10 min at 0 °C, and the precipitate was thrown away. The supernatant, which was being collected, was centrifuged at 16 000 g for 10 min at 0 °C, and mitochondria were sedimented. Mitochondrial sediment was resuspended in homogenization buffer, and once more centrifuged at 16 000 g for 10 min at 0 °C. The sediment was again resuspended in the Tris buffer. A small amount of mitochondria was taken for protein assay using Lowry's method (Lowry *et al.* 1951). Mitochondria were then frozen and kept at −70 °C until use.

Synthesis of peroxynitrite

Peroxynitrite was synthesized in a quenched-flow apparatus according to the procedure described by Koppenol *et al.* (1996). The resulting solution contained 50-80 mM of peroxynitrite determined by absorbance at 302 nm ($\varepsilon = 1670$ mol^{-1} dm^3 cm^{-1}).

In vitro peroxidation of mitochondria

The mitochondrial suspension was divided into two parts. Both were buffered with 0.1 M phosphate buffer, at pH 7.8. Peroxidation was initiated by addition of tBHP and PeN to mitochondrial suspension containing 5 mg protein/ml. The following concentrations of oxidizing agents were used: 0.05 mM, 0.2 mM, 0.5 mM, 1 mM, 2 mM, and 3 mM. Samples for analysis were taken immediately after adding the oxidizing agent (labeled as time 0 h), and then after 1 h and 24 h of incubation at 37 °C. Aliquots were stored at −70 °C.

Quantitative assay of malondialdehyde

MDA was assayed by the method of Ohkawa *et al.* (1979) with modifications. 200 µl of sample was denatured with 200 µl of 8.1 % SDS and mixed. Then 400 µl of 20 mM EDTA and 200 µl of 40 mM hydroquinone were added. Finally, 1.5 ml of 20 % acetic acid buffered with sodium hydroxide to pH 3.5 and 1.5 ml of 0.8 % thiobarbituric acid were added. The test tubes were placed in a water bath at 96 °C for 1 h. Then, they were cooled by a stream of cold water for 10 min and 4 ml of butanol was added. The mixture was vigorously stirred and centrifuged at 500 g for 10 min. The pink organic layer was measured at 532 nm, and

1, 1, 3, 3-tetramethoxypropane was used to prepare a calibration curve. All the assays were run in triplicates, error bars in the figures represent SD.

Quantitative assay of protein carbonyls

Protein carbonyls were assayed by the method of Levine *et al.* (1990) with modifications. 200 µl of the sample containing 5 mg protein/ml was pipetted into two microtubes. The first was used for the assay, and the second was used as a control. For the assay, 800 µl of 10 mM 2, 4-dinitrophenylhydrazine was added, and for the control, 800 µl of 2.5 M HCl was added. Then, 1 ml of 20 % trichloroacetic acid was added, and the tubes were centrifuged at 10 000 g for 10 min. The pellet was resuspended three times in 1 ml of ethanol-ethyl acetate mixture (1:1, v/v) and centrifuged at 10 000 g for 10 min. The pellet was solubilized in 6 M guanidine-HCl (buffered with trifluoroacetic acid to pH 2.3) and centrifuged at 10 000 g for 10 min. The supernatant was measured at 370 nm, and the concentration of protein carbonyls was calculated from $\varepsilon = 22\,000$ mol^{-1} dm^3 cm^{-1}. All the assays were run in triplicates, error bars in the figures represent SD.

Fluorescence analysis

The technique described by Goldstein and McDonagh (1976), as modified by Wilhelm and Herget (1999), was used for the analysis of LFP in the heart mitochondria. 0.2 ml of mitochondrial suspension was transferred into a glass-stoppered test tube containing 4 ml of chloroform-methanol mixture (2:1, v/v). After 1 h extraction on a motor-driven shaker, 2 ml of double-distilled water was added; then, the sample was agitated, and the ensuing mixture was centrifuged (400 g, 10 min). After centrifugation, the lower chloroform phase was separated and used for measurements.

Fluorescence excitation spectra were measured on an Aminco-Bowman series 2 spectrofluorometer and recorded and analyzed using AB-2 computer program that also organized the spectra into tridimensional spectral arrays. The excitation spectra were measured in the range of 250-400 nm for emission adjusted between 380 and 540 nm in steps of 10 nm. All the assays were run in triplicates, error bars in the figures represent SD.

HPLC analysis

Mitochondria chloroform extracts were evaporated under the stream of nitrogen. The evaporated sample was then dissolved in approximately 0.5 ml of 80 % acetonitrile in water. 50 µl of this solution was applied on C-18 column (4x250 mm, 5 µm) attached to a JASCO HPLC instrument equipped with fluorescence detector. The detector was set at the excitation of 357 nm and the emission of 430 nm. The chromatography was run in the linear gradient mode between 20-80 % acetonitrile in water during 40 min. The flow rate was 0.7 ml min^{-1}, and the column was thermostated at 30 °C.

Quantitative assay of nitrotyrosine by ELISA

The content of NT in the mitochondrial proteins after various treatments was determined by ELISA with monoclonal antibody against NT developed in our laboratory (Herget *et al.* 2000). The modified procedure of Wilhelmová *et al.* (2006) was employed for preparation of samples. Briefly, samples after treatment were centrifuged at 20 000 g, and the supernatant was used as a source of soluble proteins. Sediments were resuspended in 1 M NaOH and hydrolyzed for 150 min at 60 °C. After cooling, the mixture was neutralized by a solution of 1 M HCl and 100 mM Tris. After centrifugation at 20 000 g, the mixture was then analyzed, and supernatants were referred to as insoluble proteins. The concentration of proteins in samples was determined by commercial BCA Protein Assay Kit (Pierce). All the assays were run in triplicates, error bars in the figures represent SD.

SDS-PAGE

The sample was dissolved in the reducing buffer (0.0625 M Tris, 10 % glycerol, 0.23 % SDS, 0.15 % bromophenol blue, 0.05 % 2-mercaptoethanol) and boiled for 3 min. Samples were applied to 4 % focusing gel. Separating gel was 12 %. Separation was carried out for 6 h at 8 °C (180 V, 80 mA) until the blue dye front reached the end of the gel.

Western blot analysis for nitrotyrosine and protein carbonyls

After SDS-PAGE proteins were blotted (transfer buffer: 0.015 M $Na_2B_4O_7$, 0.1 M H_3BO_3, 0.1 % SDS, pH 8.4) on a nitrocellulose membrane and probed with the anti-nitrotyrosine antibody prepared in our laboratory (Herget *et al.* 2000). Membranes were blocked with 5 % non-fat dry milk in 0.1 % Tween/phosphate buffered saline. The secondary antibody (polyclonal rabbit anti-mouse immunoglobulins/HRP from Dako; dilution 1:10 000) was labeled with peroxidase and the spots were visualized by enhanced chemiluminescence reaction

(1.25 mM luminol, 0.05 M *p*-coumaric acid, 0.01 % H_2O_2).

The commercial kit for immunochemical detection of protein carbonylation (OxyBlot from Merck Millipore) was used according to its protocol. The signal was obtained by enhanced chemiluminescence reaction and imaged for further analysis using a CCD camera (Kodak Image Station 4000R).

Statistical analysis

Statistical evaluations were performed using ANOVA with Scheffe *post-hoc* test. Values are expressed as means ± standard deviation (SD).

Results

For experiments frozen-thawed mitochondrial suspension was used. Free radical attack on membranes generates MDA from membrane fatty acids and produces carbonyl groups in affected proteins. Production of MDA

and protein carbonyls after incubation with tBHP and PeN is shown in Figure 1. Figure 1A summarizes the effects of tBHP. Immediately after the addition of 0.05 mM tBHP, the level of MDA increased about three-fold. Higher concentrations of tBHP up to 2 mM did not increase the level of MDA further. Incubation for 24 h did not produce marked changes in MDA levels. Autoxidation for 24 h resulted in MDA increase that was about half of that achieved with tBHP.

Figure 1B shows the production of MDA induced by PeN. Similarly to tBHP, 0.05 mM PeN induced the increase in MDA level immediately after the addition; however, 1 mM PeN had a two-fold higher effect, and 2 mM PeN produced a significantly lower level of MDA than 1 mM PeN. Incubation for 24 h did not produce any change in MDA level; only autoxidation resulted in significant increase in MDA concentration that did not reach the level obtained with PeN. A significantly lower MDA concentration was found at the end of incubation with 2 mM PeN compared to 1 mM PeN.
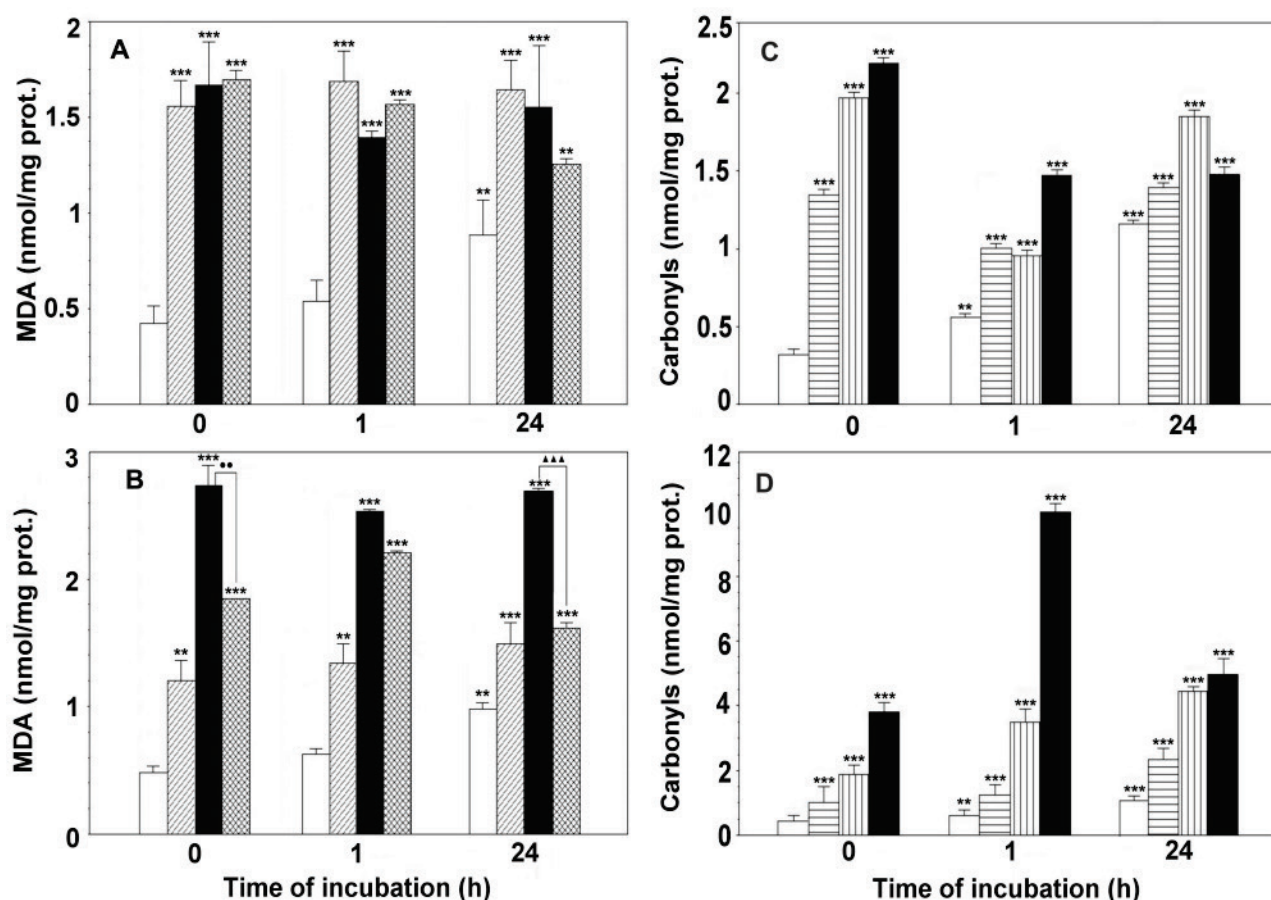

Fig. 1. Quantitative assay of MDA and protein carbonyl assay during incubation with tBHP and PeN. **A**: tBHP; **B**: PeN. Empty columns – control, lines up to the right – 0.05 mM, black – 1 mM, crosshatched – 2 mM. **C**: tBHP, **D**: PeN. Empty columns – control, horizontally striped – 0.2 mM, vertically striped – 0.5 mM, black – 1 mM. Significantly different from control (time 0): ** $P<0.01$, *** $P<0.001$; significantly different from 1 mM PeN: •• $P<0.01$, ▲▲▲ $P<0.001$. Addition of 2 mM PeN led to significant decrease of MDA compared with 1 mM PeN. Higher concentration of PeN led to consumption of MDA.

Figure 1C shows that the sensitivity of carbonyl formation is lower than that of MDA, as the first significant increase was observed after the addition of 0.2 mM tBHP. The highest increase was observed after the addition of 1 mM tBHP. Further incubation up to 24 h led to decreased levels of protein carbonyls. The autoxidation significantly increased protein carbonyls already after 1 h of incubation; after 24 h, the protein carbonyls further increased and reached the level induced by tBHP.

Figure 1D shows that PeN influenced protein carbonyls in a different manner. Maximum production was obtained with 1 mM PeN after 1 h incubation. This level was about four-fold higher than the maximum value obtained after incubation with tBHP. While the carbonyl levels induced by lower PeN concentrations slightly increased after 24 h incubation, the level induced by 1 mM PeN decreased to one half after 24 h in relation to 1 h incubation. This level was about the same as that induced by 0.5 mM PeN.
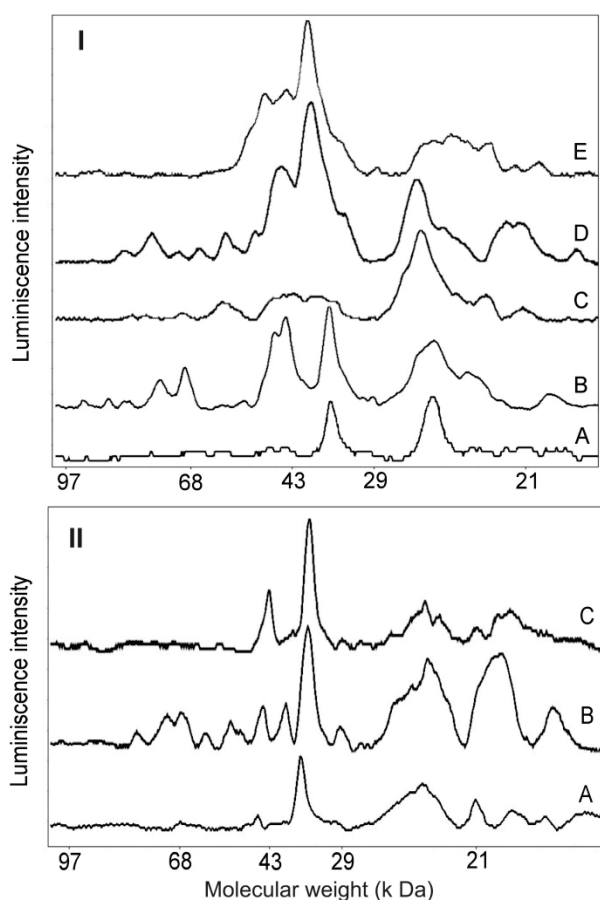
The distribution of carbonyls in individual proteins was visualized with the help of specific antibodies using the Oxyblot kit. Figure 2, panel I presents densitogram traces comparing controls and the effects of 2 mM tBHP and 2 mM PeN added either immediately or after 24 h of incubation. The traces have not been calibrated with respect to the carbonyl quantity; their aim was to show the molecular masses of affected proteins. In relation to controls, 2 mM tBHP generated carbonyls in proteins with molecular weights higher than 29 kDa immediately after addition; after 24 h of incubation, carbonyls in proteins with molecular weights higher than 29 kDa were reduced and stayed in proteins with molecular weights lower than 29 kDa. The situation after the treatment with 2 mM PeN was similar: only the range of affected proteins differed a bit from the effects of tBHP.
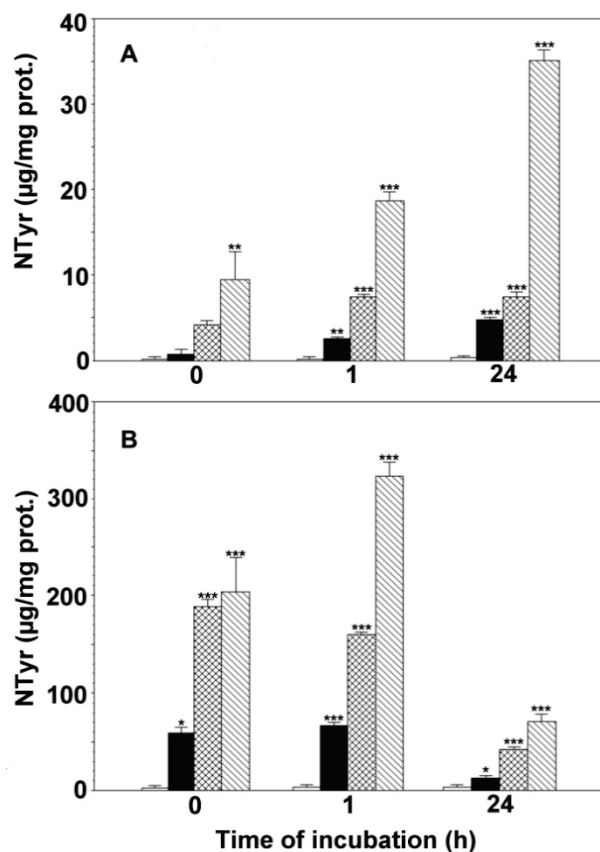

Fig. 2. Densitogram of carbonylated and nitrated proteins. **Panel I** (carbonylated proteins): Line A: control, line B: tBHP 2 mM 0 h, line C: tBHP 2 mM 24 h, line D: PeN 2 mM 0 h, line E: PeN 2 mM 24 h. **Panel II** (nitrated proteins): Line A: control, line B: PeN 2 mM 0 h, line C: PeN 2 mM 24 h

Fig. 3. ELISA – quantitative assay of nitrotyrosine in insoluble and soluble fraction during incubation with PeN. **A**: insoluble fraction; **B**: soluble fraction. Empty columns – control, black – 1 mM, crosshatched – 2 mM, lines up to the left – 3 mM. Statistical significance related to control, time 0. * P<0.05, ** P<0.01, *** P<0.001.

Panel II shows protein nitration detected using specific antibodies against NT on the blots. The very low

levels of nitrated proteins were found among proteins with lower molecular weights in the controls. The addition of 2 mM PeN resulted in nitration of proteins with molecular weights higher than 29 kDa; after 24 h of incubation, these proteins disappeared, and protein nitration was observed among proteins with molecular weights lower than 29 kDa.

The effects of the incubation with PeN on the formation of NT assayed by ELISA are illustrated in Figure 3. Incubation with tBHP did not produce any changes in very low basic levels of NT. Figure 3A shows

the situation in the non-soluble protein fraction. Only 3 mM PeN induced significant increase in NT concentration immediately after addition. Upon further incubation, NT concentration rose starting at 1 mM PeN.

Figure 3B shows the effects of PeN in protein soluble fraction. Generally, NT levels achieved in soluble proteins were about 10-fold higher than in the insoluble fraction. A significant increase was found with all tested PeN concentrations immediately after the addition. Incubation for 24 h led to a significant decrease in NT concentration at all concentrations of PeN.
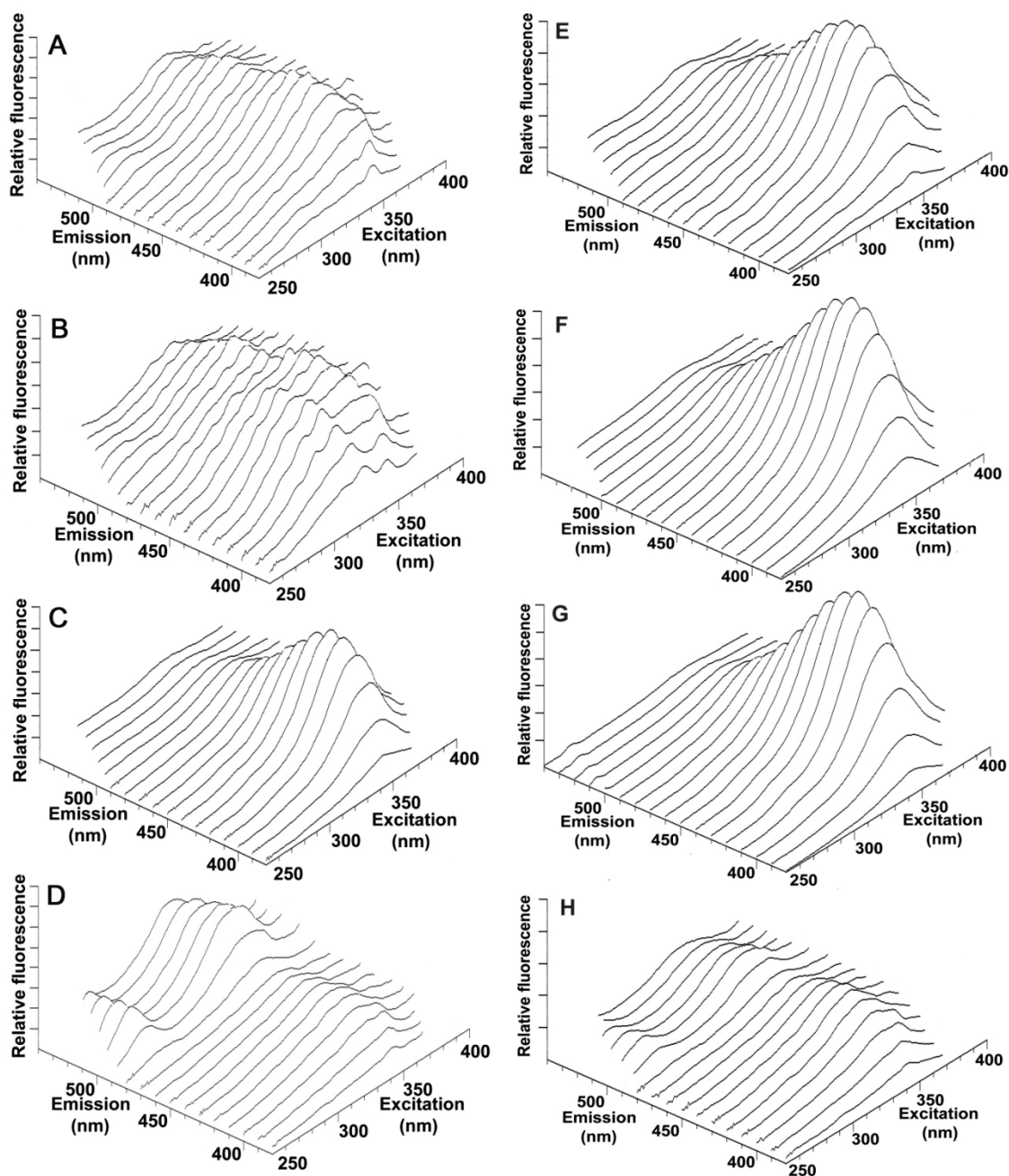

Fig. 4. 3D spectral arrays of LFP at the beginning and after 24 h of the incubations. **Panel A**: control, **panel B**: 4 mM tBHP 0 h, **panel C**: 1 mM PeN 0 h, **panel D**: 3 mM PeN 0 h. **Panel E**: control, **panel F**: 4 mM tBHP 24 h, **panel G**: 1 mM PeN 24 h, **panel H**: 3 mM PeN 24 h

LFP are the lipophilic fluorescent end products of lipid peroxidation composed of many fluorophores. 3D fluorescence spectral arrays show all the fluorophores present in the measured sample. The effects of tBHP and PeN concentrations are illustrated in Figure 4. Chloroform extracts of control mitochondria contained basic levels of LFP (panel A). The addition of 4 mM tBHP produced only minor changes in the spectral array (panel B); however, typical LFP fluorophores have been formed immediately after the addition of 1 mM PeN (panel C). After injection of 3 mM PeN, the major LFP fluorophores have been destroyed, and degradation products with emission around 500 nm appeared in the spectra (panel D).

It is apparent that 24 h of autoxidation without any addition (panel E), incubation with 4 mM tBHP (panel F) and 1 mM PeN (panel G) produced similar fluorophores. Incubation with 3 mM PeN led to the destruction of all fluorophores (panel H).

Based on the 3D spectral arrays, we have chosen fluorophores for quantitative assay. The results are summarized in Figure 5. Three fluorophores were employed to characterize the incubation with tBHP: F357430 (excitation 357 nm, emission 430 nm), F365440 and F380460, and three fluorophores observed during incubation with PeN: F357430, F360450, and F385500, respectively.

Panel I shows the effects of tBHP. It is apparent that a significant increase in the concentration of all fluorophores was found for tBHP concentrations in the range of 0.05-1 mM only after 24 h incubation. Autoxidation did not produce a significant increase in LFP fluorophores quantity.
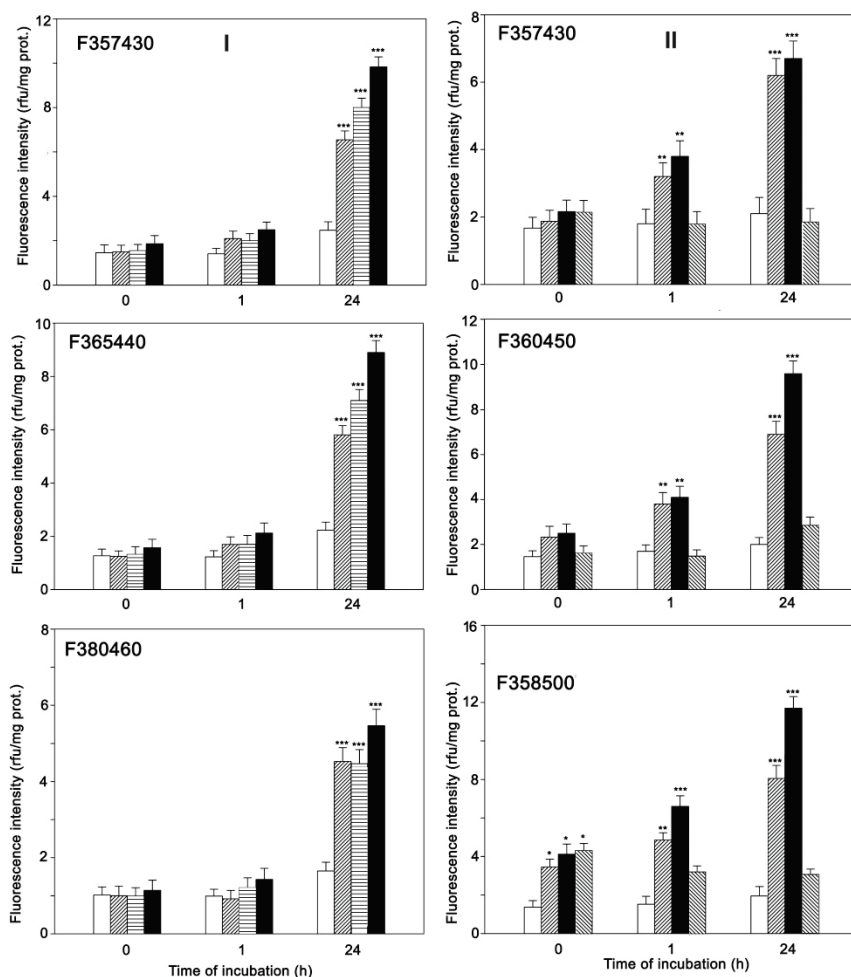

Fig. 5. Quantitative assay of three fluorophores during incubation with tBHP and PeN. **Panel I:** tBHP. Empty columns – control, lines up to the right – 0.05 mM, horizontally striped – 0.2 mM, black – 1 mM. **Panel II:** PeN. Empty columns – control, lines up to the right – 0.05 mM, black – 1 mM, lines up to the left – 3 mM. Statistical significance related to control, time 0. * P<0.05, ** P<0.01, *** P<0.001

The effects of the incubation with PeN are summarized in panel II. Immediately after the addition of 0.05-3 mM PeN, a statistically significant increase in F385500 concentration was observed. The other two fluorophores did not rise significantly. All fluorophores increased after the incubation with 0.05 mM and 1 mM PeN for 1 h. Incubation with 3 mM PeN did not produce any increase after 1 h nor after the incubation for 24 h.

The 24 h incubation with 0.05 mM and 1 mM PeN led to a further increase in all fluorophores' concentrations.

Spectrally characterized fluorophores can be further resolved by HPLC. The examples of analyses of the fluorophore F357430 are shown in Figure 6. It is apparent that one major chromatographically distinct species is formed with a retention time around 4.2 min during autoxidation (panel A) and after incubation for 24 h with 1 mM tBHP (panel B) or 1 mM PeN (panel C). Incubation with 3 mM tBHP leads to formation of a new fraction with retention time around 1.5 min (panel D), whilst the incubation with 3 mM PeN destroys all the fluorophores (panel E).
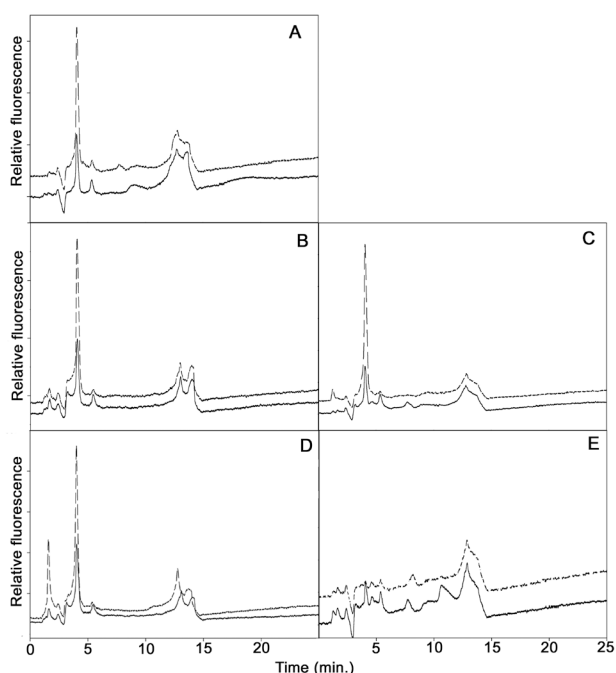

Fig. 6. HPLC chromatograms of the fluorophore F357430. Solid line – time 0, dashed line – 24 h incubation. **Panel A** – autoxidation, **panel B** – 1 mM tBHP, **panel C** – 1 mM PeN, **panel D** – 3 mM tBHP, **panel E** – 3 mM PeN

Discussion

The rapid formation of MDA was its distinguishing feature. Production of MDA sharply increased in 20 s after the addition of an oxidant. This indicates that very fast reacting species were in play. Previous studies have shown that tBHP generates hydroxyl radicals by a Fenton reaction with cellular ferrous iron (Masaki *et al.* 1989). Redox cycling of an iron-containing proteins in the presence of organic hydroperoxides generates both peroxyl and alkoxyl radicals (Pryor 1966). A tertiary alkoxyl radical formed in

these reactions is unstable and undergoes fragmentation through β-scission to form the methyl radical and a ketone (Kalyanaraman *et al.* 1979). Peroxynitrite yields hydroxyl and nitrogen dioxide radicals that attack practically all biomolecules (Alvarez and Radi 2003, Korkmaz *et al.* 2009).

The hydroxyl radical thus appears to be the most probable reactant in our systems. Figure 1A shows that 0.05 mM tBHP is sufficient to oxidize all present substrate immediately after its addition, since higher concentrations of tBHP did not yield higher concentrations of MDA, and prolonged incubation did not increase MDA production any further. On the other hand, autoxidation for 24 h did not produce the same amount of MDA as tBHP, indicating the efficiency of tBHP-related oxidants. The effect of PeN differed in sensitivity to oxidant concentration (Fig. 1B). 1 mM PeN produced more MDA than 0.05 mM PeN, and a significantly lower concentration of MDA was generated with 2 mM PeN. As this effect was observed also after 24 h incubation, it might have been caused by consumption of MDA by further reactions under higher PeN concentrations.

Formation of protein carbonyls revealed a diverse set of mechanisms of action of tBHP and PeN. While PeN produced a higher concentration of protein carbonyls than tBHP immediately after the addition, incubation for 1 h resulted in further increase in carbonyl concentration induced by PeN and a decrease induced by tBHP. Besides that, different proteins were affected by tBHP and by PeN. The fractions with molecular weights lower than 29 kDa observed after oxidant treatment might represent the products of protein fragmentation.

LFP are considered to be the stable end-products of lipid peroxidation and have been used as an indicator of oxidative damage in several studies (Ivica and Wilhelm 2014). In this study we document that similar fluorophores originated from the autoxidation and the effects of tBHP or PeN. Also, HPLC analysis proved that the products were independent of the method of oxidation. Both the spectroscopic and chromatographic techniques confirmed the destruction of LFP at high PeN concentrations. This is a unique feature that has not been observed with any other oxidant so far.

From a quantitative point of view, different fluorophores respond to individual oxidants in a diverse manner. PeN was more efficient than tBHP in the generation of LFP. All the major fluorophores significantly increased after 1 h incubation with PeN,

whereas tBHP-induced LFP appeared after 24 h, and autoxidation was not sufficient to generate significant levels of LFP. The inability of autoxidation to generate LFP is in contradiction to its efficiency in generation of significant amount of MDA. It suggests that MDA must not be in the main pathway to LFP formation in mitochondria. The mechanism of autoxidation involves a redox cycling of iron ions present in trace amounts in mitochondrial preparation. The same mechanism is applied to the effect of tBHP, but the oxidant levels are higher and so is the amount of generated LFP. The fluorescence spectra of the end products after autoxidation and the incubation with tBHP indicate that the same fluorophores were formed in both of these processes; they only differed in quantity. On the other hand, 1 mM PeN generated typical LFP spectra immediately after the addition, and the fluorophore F358500 rose significantly. From it ensures that specific reactive species participate in LFP generation initiated by PeN. Nitrogen dioxide radical appears as the responsible agent, since formation of LFP is accompanied with protein nitration. The formation of 3-nitrotyrosine can proceed only by the radical mechanism, as the electrophilic substitution would yield 2- or 6-nitrotyrosine. The higher level of nitration of soluble proteins in comparison with insoluble proteins might have been caused by easier accessibility of tyrosines in the soluble proteins for the hydrophilic PeN.

Conclusions

Oxidation of proteins producing carbonyls had different kinetics and also a probable mechanism with tBHP or PeN. PeN induced nitration of protein tyrosines that was more intensive in the soluble proteins than in the insoluble ones. LFP were formed more readily by PeN. Fluorometric and chromatographic techniques have confirmed destruction of LFP by higher PeN concentrations. This is a unique feature that has not been described so far for any oxidant.

Conflict of Interest
There is no conflict of interest.

Acknowledgements

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