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DNA strand breaks and base modifications induced by cholesterol hydroperoxides

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Abstract

Cholesterol (Ch) can be oxidized by reactive oxygen species, forming oxidized products such as Ch hydroperoxides (ChOOH). These hydroperoxides can disseminate the peroxidative stress to other cell compartments. In this work, the ability of ChOOH to induce strand breaks and/or base modifications in a plasmid DNA model was evaluated. In addition, HPLC/MS/MS analyses were performed to investigate the formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo) after the incubation of 2'-deoxyguanosine (dGuo) with ChOOH and Cu^{2+}. In the presence of copper ions, ChOOH induced DNA strand breaks in time and concentration-dependent manners. Purine and pyrimidine base modifications were also observed, as assessed respectively by the treatment with Fpg and Endo III repair enzymes. The detection of 8-oxodGuo by HPLC/MS/MS is in agreement with the dGuo oxidation in plasmid DNA. ChOOH-derived DNA damage adds further support to the role of lipid peroxidation in inducing DNA modifications and mutation.

Keywords: *Cholesterol hydroperoxides, DNA damage, 8-oxo-7,8-dihydro-2'-deoxyguanosine, HPLC/MS/MS.*

Introduction

Cholesterol (Ch) is an essential lipid constituent of biological membranes. However, it can be oxidized under a variety of conditions, forming oxidized products such as Ch hydroperoxides (ChOOH). Lipid hydroperoxides (LOOH) are prominent intermediates of peroxidative reactions induced by activated oxygen species such as hydroxyl radical, lipid alkoxyl or peroxyl radicals, singlet molecular oxygen ($^1O_2$) and peroxynitrite [1,2]. Once formed, they can be decomposed by heating, exposure to UV light (in some cases) or by the addition of transition metals [3]. Decomposition of peroxides can generate alkoxyl and peroxyl radicals that may be able to oxidize other molecules such as proteins and DNA.

The effects of DNA adducts generated by aldehydes derived from lipid peroxidation have been extensively studied [4–7]. In addition, many efforts have been made to evaluate the role of LOOH in the induction of DNA damage, since they are implicated in the aetiology of human cancers and mutations [2,8]. For example, it has been shown that linoleic [9–11] and arachidonic acid [12,13] hydroperoxides are able to induce strand breaks and base modifications. In this sense, ChOOH are of special interest, since they were shown to be able to translocate among LDL and membranes of cell, nuclei and organelles faster than the parent Ch [14]. Moreover, unlike other LOOH, ChOOH are completely resistant to classical GSH peroxidase (GPx-1) catalysed reduction. Phospholipid hydroperoxide GSH peroxidase (GPx-4) is the only known enzyme able to detoxify ChOOH, although more slowly than other hydroperoxides [15]. These characteristics make ChOOH potentially more dangerous since they may be able to disseminate peroxidative stress.

Despite the potential role of ChOOH in propagating peroxidative stress through different cell

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compartments, the ability of these hydroperoxides to induce DNA damage has not been evaluated so far. In addressing this issue, we have examined the ability of ChOOH to induce strand breaks and base modifications in plasmid DNA. Moreover, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo) was detected by HPLC/MS/MS analyses, in agreement with 2'-deoxyguanosine (dGuo) oxidation in plasmid DNA.

Materials and methods

Materials

Cholesterol, dGuo and all the other chemicals were from Sigma (St. Louis, MO) and the solvents were from Merck (Darmstadt, Germany). Formamidopyrimidine-DNA glycosylase (Fpg) and Endonuclease III (Endo III) were purchased from New England BioLabs (Beverly, MA). All the solutions were prepared with distilled water purified with a Millipore Milli-Q system (Bedford, MA).

Cholesterol hydroperoxides synthesis, purification and quantification

ChOOH were synthesized by photo-oxidation using methylene blue as a sensitizer. Typically, 100 mg of Ch were dissolved in 50 mL of chloroform containing 0.2 mM of methylene blue and irradiated with a tungsten lamp (500 W) for 3 h. Irradiation was conducted in an ice-bath under a continuous flux of oxygen. The methylene blue was removed and ChOOH were isolated by silica gel column chromatography. Briefly, the mixture was placed in the column and isolated according to the following *n*-hexane (H) diethyl ether (E) gradient: 10 mL H; 20 mL H:E (80:20); 50 mL H:E (70:30); 50 mL H:E (65:35); 30 mL H:E (60:40); 30 mL H:E (55:45); 30 mL H:E (50:50). The concentration of ChOOH was determined by iodometry [16].

Cholesterol hydroperoxides analysis by HPLC/MS

Cholesterol hydroperoxides were analysed by HPLC (Shimadzu, Kyoto, Japan) coupled to a Quattro II Micromass mass spectrometer (Manchester, UK), with an atmospheric pressure chemical ionization (APCI) source in the positive ion mode. The ChOOH were eluted using a 250 mm × 4.6 mm (particle size 5 μm) Luna C-18 reverse-phase column (Phenomenex, Torrance, CA, USA) using 95% methanol and 5% water at a flow rate of 1 mL/min. The UV detector was set at 210 nm. For MS analyses, a small fraction of the eluant was directed into the mass spectrometer at a flow rate of 100 μL/min. The source and desolvation temperatures were held at 150°C and 300°C, respectively. The flow rates of drying and nebulizing gas were optimized at 300 and 15 L/h, respectively. The cone voltage was set at 20 V and the corona potential at 3.4 kV. Full scan data were acquired over a mass range of 100–700 *m/z*.

Cholesterol hydroperoxides analysis by NMR

NMR analyses were recorded in DPX300 instrument (Bruker-Biopsin, Rheinstetten, Germany). ChOOH were identified as 3β-hydroxycholest-5-ene-7α-hydroperoxide (7α-OOH), 3β-5α-cholest-6-ene-5-hydroperoxide (5α-OOH), 3β-hydroxycholest-4-ene-6α-hydroperoxide (6α-OOH) and 3β-hydroxycholest-4-ene-6β-hydroperoxide (6β-OOH) comparing chemical shifts with previous works [17–19]. 7α-OOH – ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 5.72 (dd, $\mathcal{J}$ = 4.94, 1.74 Hz, 1H), 4.16 (dt, $\mathcal{J}$ = 4.64, 4.60, 1.72 Hz, 1H), 3.68–3.56 (m, 1H). 5α-OOH – ^{1}H NMR (300 MHz, C_6D_6) δ ppm 5.70 (dd, $\mathcal{J}$ = 10.01, 0.91 Hz, 1H), 5.55 (dd, $\mathcal{J}$ = 9.97, 2.03 Hz, 1H), 4.19–4.02 (m, 1H). 6α-OOH – ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 5.59 (q, $\mathcal{J}$ = 1.69, 1.69, 1.69 Hz, 1H), 4.49 (tdd, $\mathcal{J}$ = 12.28, 4.63, 1.79, 1.79 Hz, 1H), 4.26–4.13 (m, 1H). 6β-OOH – ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 5.66 (t, $\mathcal{J}$ = 1.69, 1.69 Hz, 1H), 4.33 (dd, $\mathcal{J}$ = 3.99, 2.26 Hz, 1H), 4.25–4.13 (m, 1H).

Incubation of plasmid pBSK-II DNA with ChOOH in the presence of Cu^{2+} followed by incubation with DNA glycosylases

Supercoiled plasmid pBSK-II DNA was prepared according to standard methods and purified using QIAGEN® kit (Valencia, CA). DNA reactions with ChOOH consist of 1.4 μg of DNA, 0.5–10 mM of ChOOH and 10 μM of Cu^{2+} in 30 mM of sodium phosphate buffer pH 7.4 (final volume 100 μL). Control experiments were run replacing ChOOH solution by ethanol. For solubility reasons, all reactions contain 30% ethanol. Reaction mixtures were incubated for selected times at 37°C under agitation (conditions are depicted in figure legends). After that, ChOOH were extracted from the reaction mixtures with 300 μl of diethyl ether (repeated twice). Subsequently, 10 μL of the reaction containing the DNA were incubated with 0.8 U of Fpg (10 mM Bis Tris Propane-HCl (pH 7.0), 10 mM $MgCl_2$, 1 mM dithiothreitol and 0.1 mg/mL bovine serum albumin) or 1 U of Endo III (20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol and 0.1 mg/mL bovine serum albumin). Control experiments were done replacing enzyme solution by water. Incubations were carried out at 37°C for 30 min. After that, DNA was mixed with 2 μL of loading buffer (0.25% bromophenol blue and 15% Ficoll

type 400) and loaded onto a 1% agarose gel containing 0.3 μg/mL ethidium bromide. The gels were run at a constant voltage of 100 mV for 120 min in 90 mM Tris/borate 2 mM EDTA buffer (pH 8.0). The gels were photographed and quantified using an Epi Chemi II Darkroom (Bio Imaging Systems, Upland, CA, USA) and a Lab Works^{TM} Image Acquisition and Analysis Software (UVP, Upland, CA). Results represent the average and SD of, at least, three independent determinations.

Incubation of 2'-deoxyguanosine with ChOOH

Reactions of dGuo with ChOOH consisted of 1 mM dGuo, 10 mM ChOOH and 100 μM Cu^{2+} (final volume 100 μL). Control experiments were run replacing ChOOH solution by ethanol. In order to maximize the interactions between dGuo and ChOOH, the incubations contained 70% of ethanol. The samples were incubated for selected times at 37°C under agitation. After that, 7 pmol of [M+5] isotopically labelled 8-oxodGuo were added to the samples as an internal standard. Then, ChOOH were extracted from the reactions with 300 μl of diethyl ether (repeated three times). Subsequently, extracted samples were dried under a N_2 atmosphere, re-suspended in water and injected into the HPLC/MS/MS system.

Determination of 8-oxo-7,8-dihydro-2'-deoxyguanosine by HPLC/MS/MS

Samples resulting from dGuo reactions with ChOOH were analysed by HPLC/MS/MS (Shimadzu HPLC coupled to Quattro II Micromass mass spectrometer). The reaction products were separated in an LC-18 column (Phenomenex, 150 × 2.1 mm, 5 μm) using an isocratic gradient of ammonium formate (10 mM, pH 3.7): acetonitrile (96:4) at a flow rate of 0.1 mL/min. The eluent was monitored by electrospray ionization (ESI-MS) in the positive ion mode. The source and desolvation temperatures were held at 100°C and 150°C, respectively. The flow rates of drying and nebulizing gas were optimized at 300 and 15 L/h, respectively. The cone voltage was set at 15 V and the collision energy was set at 15 eV. Full scan data were acquired over a mass range of 100–500 *m/z*. The quantification was performed in the selected reaction monitoring mode (SRM), by selecting the mass transition of the 284 to 168 for 8-oxodGuo and 289 to 173 for the internal standard ([M+5] isotopically labelled 8-oxodGuo). Isotopically labelled 8-oxodGuo was a gift of Dr. Jean-Luc Ravanat.

Results

Cholesterol hydroperoxides analysis by HPLC/MS

After synthesis, ChOOH were purified from the parent Ch and other oxidized products, with purity and identities checked by HPLC/MS and NMR analyses. Figure 1 shows four product peaks identified in order of elution as 7α-OOH, 5α-OOH, 6α-OOH and 6β-OOH. Chloroform was used as the solvent in the photosensitization. This fact accounts for the lower yield of 5α-OOH compared to 7α-OOH, since it is well known that 5α-OOH re-arranges to 7α-OOH in non-polar solvents [17,20]. A MS spectrum from 5α-OOH, representative for all isomers, is displayed in Figure 1C. Protonated molecule (*m/z* 419) is not seen in the MS spectrum. However, the detection of fragments derived from protonated molecules indicates that the loss of the first H_2O molecule is a favoured reaction. In fact, the characteristic fragmentation pattern of ChOOH is the consecutive loss of H_2O molecules, yielding cationic fragments at *m/z* 401, 383 and 367. These results are in agreement with previous analyses for ChOOH [21,22]. A mixture of the four ChOOH isomers was used to test the possibility of DNA damage generation.

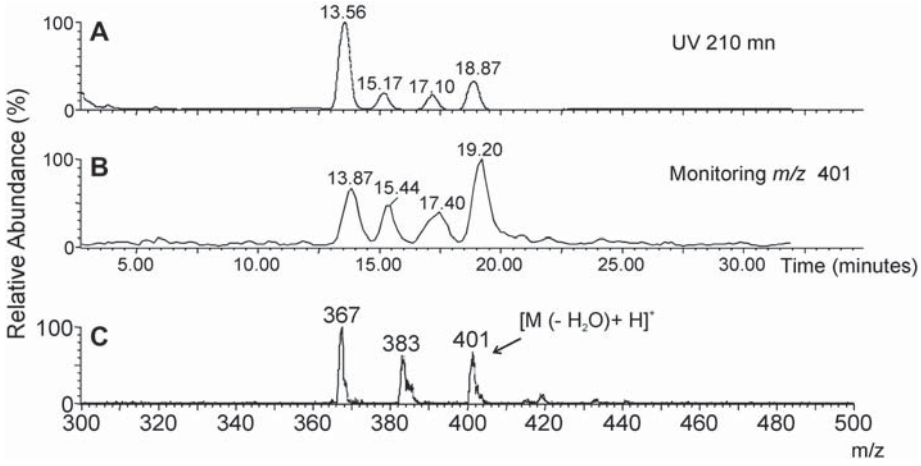

Figure 1. HPLC/APCI/MS analyses of ChOOH. (A) UV detection at 210 nm. (B) MS chromatogram selection m/z 401. (C) Mass spectrum at 15.44 min. Numbers over the peaks in (A) and (B) correspond to the retention times (min) in order of elution for 7α-OOH, 5α-OOH, 6α-OOH and 6β-OOH.

Induction of strand breaks upon exposure of plasmid DNA to ChOOH in the presence of Cu^{2+}

Induction of strand breaks was assessed by agarose gel electrophoresis. In each lane, the upper band corresponds to the open-circular DNA (OC), the intermediate band (if present) to linear DNA (L) and the lower band to supercoiled DNA (SC). For each lane, the SC and OC forms were summed up and the extent of DNA oxidative damage was expressed as the percentage of DNA SC form. A correction factor of 1.4 was applied to the value of SC forms to account for their fluorescence quantum yield lower than the OC form [23]. All reaction mixtures contained 30% ethanol, to allow ChOOH solubilization. The exposure of plasmid DNA to increasing concentrations (0.5–10 mM) of ChOOH in the presence of 10 μM of Cu^{2+} led to an increase in DNA damage, as shown by the decrease in the SC DNA form (Figure $2A_1$). A plot of these results is shown in Figure 2B. For all experiments, SC amount found in control incubation was considered as 100% and the results were reported as a control percentage. In this way, after 1 h of incubation with 0.5 mM of ChOOH, the SC DNA percentage had only a slight decrease to 97%. However, increasing ChOOH concentration enhances the strand breaks level, with the SC DNA percentage decreasing to 82% upon exposure to 10 mM of ChOOH. Control experiments using Cu^{2+} alone (Figure $2A_1$, lane 1) or ChOOH in the absence of Cu^{2+} (data not shown) have shown no significant strand scission.

Besides strand breaks, ChOOH-decomposition products could also lead to base oxidation, resulting in products which are potential substrates for base excision repair (BER) enzymes. In order to test this possibility, we use two BER enzymes, Fpg and Endo III. These proteins possess both DNA glycosylase and apurinic/apyrimidinic (AP) lyase activities, creating additional breaks at sites of oxidized bases. Some of the damaged bases recognized by Fpg are 8-oxodGuo, 2,6-diamino-4-hydroxy-formaminopyrimidine and a broad spectrum of modified purines [24,25]. On the other hand, Endo III recognizes and removes a number of thymine- and cytosine-derived lesions in DNA [26,27].

Treatment of plasmid DNA with Fpg after 1 h of ChOOH exposure led to a significant decrease in SC DNA form, consistent with purine bases oxidation (Figure $2A_2$). Upon exposure to 0.5 mM of ChOOH, SC DNA percentage decreased only to 90%, although upon exposure to 10 mM of ChOOH the same percentage decreased to 71% (Figure 2B). Interestingly, even untreated DNA contains a small amount of modified bases (data not shown). This observation is in agreement with a previous work [28]. Neither the treatment of plasmid DNA with Cu^{2+} alone (Figure $2A_2$, lane 1) nor the treatment with ChOOH in the absence of Cu^{2+} (data not shown) were able to significantly increase DNA damage as compared with untreated DNA. Contrasting with Fpg treatment, incubation of plasmid DNA with Endo III leads just to a slight increase in the pre-existing damage on the ribose phosphate

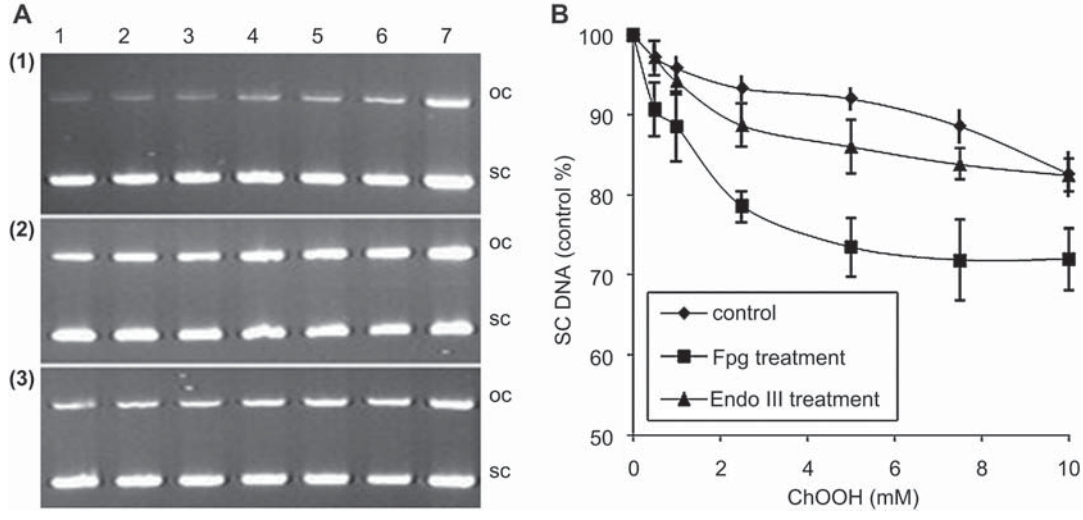

Figure 2. Induction of strand breaks upon exposure of pBSK-II plasmid DNA to ChOOH (0.5–10 mM) and 10 μM of Cu^{2+}. Reactions mixtures were incubated for 1 h at 37°C in phosphate buffer (pH 7.4, with 30% ethanol, to allow ChOOH solubilization). Subsequently, ChOOH were extracted and DNA was subjected to a second incubation with DNA glycosylases for 30 min at 37°C. (A) Agarose gel electrophoresis. (1) Incubation in the absence of enzymes; (2) Incubation in the presence of 0.8 U of Fpg; (3) Incubation in the presence of 1 U of Endo III. Lane 1: control reaction in the absence of ChOOH; Lanes 2–7: incubation with 0.5, 1.0, 2.5, 5.0, 7.5 and 10 mM of ChOOH, respectively. Plasmid DNA forms: OC: open circular; SC: supercoiled. (B) Control percentage of supercoiled DNA. The SC percentage was calculated considering the SC amount of control incubation as 100%. Results represent the average and SD of at least three independent determinations.

backbone (Figures 2A$_3$ and B), showing that pyrimidine bases are less susceptible than purines to ChOOH-derived oxidation.

Time-dependent induction of strand breaks upon exposure of plasmid DNA to 10 mM of ChOOH in the presence of Cu^{2}+

In order to better assess the DNA damage caused by ChOOH-derived oxidation products, time course experiments were carried out using 10 mM of ChOOH. As showed in Figures 3A$_1$ and B, the strand breaks increase with the time of DNA exposure. BER enzymes treatment follows the same time-dependent pattern, as showed upon incubation of the reactions with Fpg (Figure 3A$_2$) or Endo III (Figure 3A$_3$). However, the extension of damage is quite different. At all times of reaction, post-treatment with FPG increased ~ 20% the pre-existing damage caused by attack on the ribose phosphate backbone, while the post-treatment with Endo III was able to increase only ~ 10% the pre-existing damage. Taken together, these results show that ChOOH-decomposition products lead to base oxidations, preferentially at Fpg-sensitive lesions.

Determination of 8-oxo-7,8-dihydro-2'-deoxyguanosine

Since 8-oxodGuo is the most common lesion excised by Fpg, it is worthy to confirm if ChOOH were able to provoke this lesion. In this way, experiments using isolated dGuo and HPLC/MS/MS techniques were carried out. A typical chromatogram obtained after 2 h of ChOOH incubation with dGuo in the presence of 100 μM of copper ions is shown in Figure 4. Controls were made replacing the solution of ChOOH by ethanol. In the control incubation (Figure 4A$_1$), a small amount of 8-oxodGuo was produced (area = 401). However, after 2 h of exposure to ChOOH and copper ions, the amount of 8-oxodGuo increased significantly (Figure 4B$_1$, area = 3 819). To ensure correct measurements, an isotopically labelled 8-oxodGuo was added to the samples as an internal standard. The amount of internal standard did not vary significantly among different incubations (Figures 4A$_2$ and B$_2$). Similar reactions and the respective controls were carried out for each selected time (0.5, 1, 2, 4 and 6 h). For each time, an 8-oxodGuo/8-oxodGuo-N^{15} ratio was calculated, dividing the 8-oxodGuo area by the area of the internal standard. These results are shown in Figure 5. 8-oxodGuo is produced in a time dependent-manner, reaching the maximum level after 4 h of incubation. On the other hand, control reactions did not show significant levels of this lesion. A decrease in 8-oxodGuo amount was obtained after 6 h of incubation. This result can be explained by the fact that formed 8-oxodGuo can be further oxidized by ChOOH-decomposition products, since 8-oxodGuo is far more easily oxidized than the parent dGuo [29]. For example, it has been shown that 8-oxodGuo is a better substrate for $^{1}O_2$

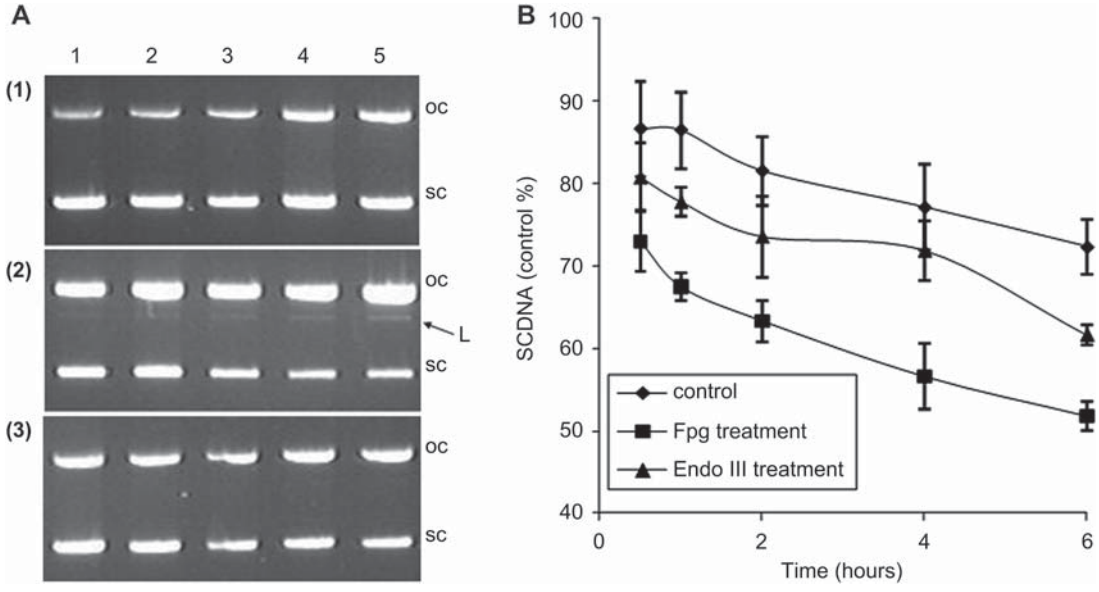

Figure 3. Time-dependent induction of strand breaks upon exposure of pBSK-II plasmid DNA to 10 mM of ChOOH and 10 μM of Cu^{2+}. Reactions mixtures were incubated for different times at 37°C in phosphate buffer (pH 7.4, with 30% ethanol, to allow ChOOH solubilization). Subsequently, ChOOH were extracted and DNA was subjected to a second incubation with DNA glycosylases for 30 min at 37°C. (A) Agarose gel electrophoresis. (1) Incubation in the absence of enzymes; (2) Incubation in the presence of 0.8 U of Fpg; (3) Incubation in the presence of 1 U of Endo III. Lanes 1–5: incubation for 0.5, 1, 2, 4 and 6 h, respectively. Plasmid DNA forms: OC: open circular; SC: supercoiled: L: linear. (B) Control percentage of supercoiled DNA. The SC percentage was calculated considering the SC amount of control incubation as 100%. Results represent the average and SD of at least three independent determinations.

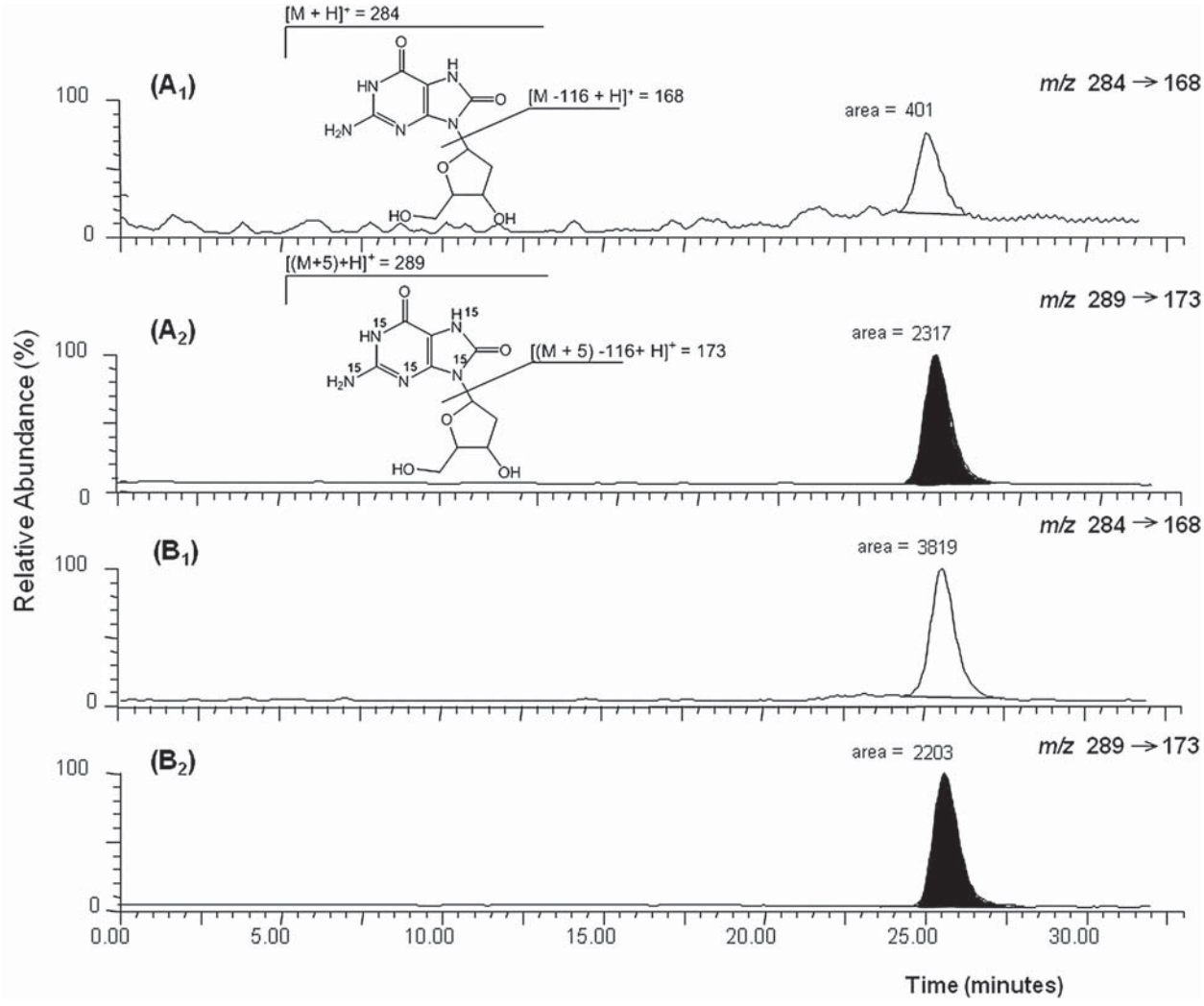

Figure 4. Typical chromatograms for the detection of 8-oxodGuo by HPLC coupled to tandem mass spectrometry in the SRM mode. Reactions mixtures were incubated for 2 h at 37°C under agitation. After that, [M+5] isotopically labelled 8-oxodGuo was added to the samples and the ChOOH were extracted from the reactions prior to injection onto HPLC/MS/MS system. (A) Control chromatograms replacing ChOOH by ethanol. (B) Incubation of 10 mM of ChOOH with 5 mM of dGuo in the presence of 100 μM of Cu^{2+}. (1) Specific mass transition for 8-oxodGuo, 284 → 168. (2) Specific mass transition for the internal standard, [M+5] isotopically labelled 8-oxodGuo, 289 → 173.

oxidation, as compared with the parent dGuo [29,30]. In the same way, the 8-oxodGuo produced in ChOOH incubations can be consumed in further oxidative reactions and the measured amount can be a balance between the amount of produced and consumed 8-oxodGuo.

Discussion

Lipid peroxidation products have been implicated in DNA damage generation. For example, linoleic acid hydroperoxides were shown to cause single and double strand breaks in supercoiled plasmid DNA in the presence of iron ions [11]. In addition, arachidonic acid peroxidation was shown to induce DNA damage and mutations [12]. Additional evidences on the role of lipid hydroperoxides in causing DNA damage were obtained in this work with ChOOH. Copper catalysed decomposition of these hydroperoxides lead to plasmid DNA strand breaks in time and concentration-dependent manners.

It has been shown that copper ions occur in the mammalian cell nucleus [31,32]. This metal ion can be bound to the phosphate groups of the DNA backbone and to certain amino acids of some proteins, including chromatin [33–36]. Indeed, decomposition of organic peroxides by transition metals can generate peroxyl and alkoxyl radicals (showed in equations (1) and (2), respectively, as Ch-derived peroxyl (ChOO•) and alkoxyl (ChO•) radicals) [37]. An alternative mechanism, involving one-electron reduction of lipid hydroperoxides by Cu^{2+}, yielding alkoxyl radicals and Cu^{3+}, has also been proposed for alkoxyl radicals generation (equation 3) [38].

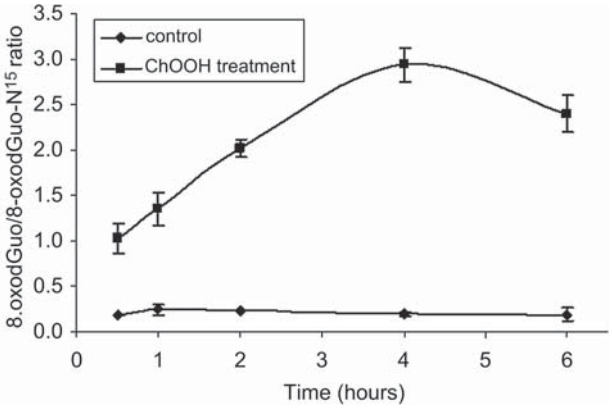

Figure 5. Time-dependent production of 8-oxodGuo upon exposure of 5 mM of dGuo to 10 mM of ChOOH and 100 μM of Cu^{2+}. Reaction mixtures were incubated for 0.5, 1, 2, 4 and 6 h at 37°C under agitation. After that, [M+5] isotopically labelled 8-oxodGuo was added to the samples and the ChOOH were extracted from the reactions prior to injection onto HPLC/MS/MS system. Control reactions were done replacing ChOOH for ethanol. For each time, the 8-oxodGuo/8-oxodGuo-N^{15} ratio was calculated dividing the 8-oxodGuo area by the area of the internal standard. Results represent the average and SD of at least three independent determinations.

$$ChOOH + Cu^{2+} \rightarrow ChOO^{\bullet} + Cu^{1+} + H^{+} \quad (1)$$
$$ChOOH + Cu^{1+} \rightarrow ChO^{\bullet} + HO^{-} + Cu^{2+} \quad (2)$$
$$ChOOH + Cu^{2+} \rightarrow ChO^{\bullet} + HO^{-} + Cu^{3+} \quad (3)$$

Furthermore, not only metal ions can be responsible for lipid-derived radicals formation. Many endogenous oxidative processes such as lipid peroxidation and protein oxidation may give rise to peroxyl radicals. Peroxyl radicals can attack a DNA molecule remote from their site of formation due to their relatively long lifetime when compared with other radicals [39]. The presence of lipid peroxyl radicals has been related to enhanced levels of 8-oxodGuo in nuclear and mitochondrial DNA [40].

Cholesterol-derived radicals can generate oxidative base modifications no matter their source. In addition, an initial damage on the nucleobases can also lead to secondary DNA strand scission [41]. The preferred pathway of DNA oxidation by ChOOH-decomposition products was base modifications, as evaluated by incubation of plasmid DNA with BER enzymes. As already mentioned, Fpg excises modified purines, while Endo III excises modified pyrimidines. Purines were shown to be favoured sites of ChOOH-derived products attack. For comparison, alkoxyl radicals, formed by photodecomposition of [4-(tert-Butyldioxycarbonyl) benzyl]triethylammonium chloride also generates a high number of Fpg-sensitive modifications and only a small amount of Endo III-sensitive lesions. Additionally, peroxyl radicals generated in arachidonic acid peroxidation were shown to preferentially oxidize guanine base and, to a minor extent, cytosine and thymine. The same study also presents evidence for the involvement of 8-oxodGuo in the oxidation of DNA by arachidonic peroxyl radicals [13]. The results obtained in our work and also in previous ones are not surprising, considering the fact that dGuo is the most easily oxidizable nucleic acid base by $^1O_2$ and one-electron transfer mechanisms in DNA [42,43]. In this way, with the exception of the unselective hydroxyl radicals, other oxygen-derived radicals react preferentially with dGuo [44].

Korytowski et al. [45] have shown that different ChOOH isomers have different ability to induce free radical lipid peroxidation in cell membranes. These authors showed that, contrasting with the results obtained for 7α-OOH and 5α-OOH, the 6β-OOH isomer was unable to initiate chain peroxidation, although all isomers were reduced by a lipophilic iron chelate and ascorbate ($Fe(HQ)_3/AH^-$) at the same rate [46]. In the same way, the contribution of each ChOOH isomer in causing the overall oxidative DNA damage is now under investigation.

Intra-cellularly, LOOH might move for example, from mitochondria (which are under relatively high oxidative pressure) to the nucleus, where DNA could be damaged [47]. Phospholipids and unesterified Ch are known to move from one membrane compartment to another within cells. However, it has been shown that phospholipids translocate extremely slowly on their own (i.e. without protein mediation), whereas Ch does so relatively rapidly [48]. Indeed, phospholipid hydroperoxides translocate much more rapidly than their respective parent phospholipids [49]. The same behaviour is found for ChOOH, with rate constants for ChOOH transfer far exceeding that of parent Ch [14,47].

An interesting point that should be mentioned is the unexpected high levels of oxysterols observed in human plasma, LDL and erythrocytes. In contrast to polyunsaturated fatty acids, Ch does not contain bisallylic hydrogen and the reactivity of allylic hydrogen toward peroxyl radical, a chain-carrying species in lipid peroxidation, is ~ 100 times smaller than that of bisallylic hydrogen. However, the high level of oxygenated oxysterols may be ascribed to slower metabolism and/or excretion [50]. At least three different classes of intracellular enzymes have been implicated in reductive LOOH detoxification: GSH peroxidases [51], peroxiredoxins [52] and GSH-S-transferase [53]. The former class includes the enzymes GPx-1 and GPx-4 and is considered to be more important in overall LOOH disposal [2]. However, GPx-1 cannot directly act on phospholipid hydroperoxides in membranes, unless *sn*-2 fatty acyl bonds are first hydrolysed to liberate the peroxidized fatty acids. Only GPx-4 has this ability [51,54]. More interestingly, ChOOH are completely resistant to GPx-1 detoxification and are also more resistant to GPx-4-dependent elimination

than fatty acid hydroperoxides and their esterified form [15,55].

Cholesterol is found in the membrane of all eukaryotic cells. The fact that nuclear membranes contain much less Ch than plasma membranes (~ 10% in nuclear membranes when comparing with 40% of the total lipid of plasma membranes) can be overwhelmed by the relatively rapidly translocation of ChOOH among membranes, along with their slower detoxification rates [2,56]. Moreover, oxidation of nuclear membrane cholesterol was shown to inhibit the activity of nucleoside triphosphatase (NTPase), an enzyme believed to be involved in nucleo-cytoplasmic trafficking [57]. Unspecified lipid peroxidation products were also implicated in nuclear effects. For example, it was shown that primary photosensitization reactions involving $^1O_2$ generation in membranes lead to DNA damage. This effect was probably caused by secondary reactive species derived from lipid peroxidation [58]. Therefore, ChOOH should be considered as potential effectors that can play a role in DNA oxidative damage.

In summary, this work showed that ChOOH-derived products oxidize DNA, especially at Fpg-labile sites. The results obtained with isolated dGuo and HPLC/MS/MS analyses support the dGuo oxidation in plasmid DNA. Alkoxyl and/or peroxyl radicals derived from reaction of ChOOH with copper ions should be responsible for the damage. DNA damage caused by putative hydroxyl radicals derived from secondary reactions can be ruled out, since experiments were performed employing 30% ethanol as co-solvent. Ethanol is a well known scavenger of hydroxyl radicals [3].

Despite the recognized role of catalytic metals in inducing lipid peroxidation, the exact mechanism is still not clear. It has been argued from thermodynamic calculations that LOOH are unable to reduce Cu^{2+} to Cu^{1+}, and only the last one interacts with LOOH [59]. However, another study showed that in the presence of an oxidizable substrate, LOOH are capable of reducing Cu^{2+} yielding Cu^{1+} and lipid-derived peroxyl radicals [60]. The last work proposed a reversible intermediate complex, denoted as [Cu^{2+}-LOOH], which will generate Cu^{1+} and peroxyl radical.

In this view, DNA damage generated by ChOOH in the presence of copper ions can be started by the reaction of ChOOH and Cu^{2+}, yielding ChOO$^\bullet$ and Cu^{1+} (Figure 6). Besides the equilibrium of equation (1) lies to the left, in the presence of an oxidizable substrate (in this case, DNA molecule) the thermodynamic barrier can be overcome, and ChOO$^\bullet$ and Cu^{1+} will be formed. Then, a redox cycling can be proposed. Cu^{1+} ions react with another ChOOH molecule, yielding ChO$^\bullet$ and regenerating Cu^{2+}. The ChO$^\bullet$ are more reactive than ChOO$^\bullet$, being able to react with DNA. Also, ChO$^\bullet$ can undergo cyclization,

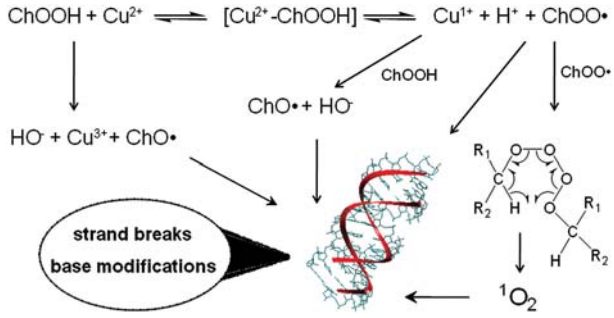

Figure 6. Possible pathways for ChOOH-derived DNA damage.

yielding carbon-centred epoxyallylic radicals (OCh$^\bullet$). The later radicals can couple with molecular oxygen, forming epoxyperoxyl radicals (OChOO$^\bullet$), further amplifying the damaging agents able to react with DNA [61]. Another possibility for ChO$^\bullet$ is β-scission reaction forming aldehydes and alkyl radicals (Ch$^\bullet$), which also couple with O_2, yielding ChOO$^\bullet$. Additionally, self-termination of peroxyl radicals will give rise to $^1O_2$, as proposed by Russell [62] and confirmed for lipid peroxides in the presence of metal ions or other oxidants [63–65]. Singlet molecular oxygen can react with dGuo base, yielding 8-oxodGuo [42]. The initial reaction of ChOOH with Cu^{2+}, yielding ChO$^\bullet$ and Cu^{3+}, must also be considered [38]. Expected cholesterol end-products are 5,6-epoxide, 7-ketone and the diols derived from decomposition of each hydroperoxide, especially the stable 7-OH [46,54,66].

ChOOH-derived DNA damage adds further support to the role of lipid peroxidation in inducing DNA modifications and mutation. Moreover, ChOOH can be considered to play a special role in the development of pathological conditions linked to DNA damage, giving their role in translocation through membranes, disseminating the peroxidative stress [67]. The results obtained here point to the importance of ChOOH as damaging agents to DNA and warranty further studies about the involvement of these species in the aetiology of some diseases.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Girotti AW. Mechanisms of lipid peroxidation. Free Radic Biol Med 1985;1:87–95.

[2] Girotti AW. Photosensitized oxidation of membrane lipids: reaction pathways, cytotoxic effects, and cytoprotective mechanisms. J Photochem Photobiol B 2001;63:103–113.

[3] Hallliwell B, Gutteridge JMC. Free radicals in biology and medicine. 4th ed. Oxford University Press, Oxford: 2007. p 851.

[4] Marnett LJ. Oxy radicals, lipid peroxidation and DNA damage. Toxicology 2002;181:219–222.

[5] Marnett LJ. Oxyradicals and DNA damage. Carcinogenesis 2000;21:361–370.

[6] Marnett LJ, Burcham PC. Endogenous DNA adducts: potential and paradox. Chem Res Toxicol 1993;6:771–785.

[7] Medeiros MHG. Exocyclic DNA adducts as biomarkers of lipid oxidation and predictors of disease. Challenges in developing sensitive and specific methods for clinical studies. Chem Res Toxicol 2009;22:419–425.

[8] Bartsch H, Nair J. Oxidative stress and lipid peroxidation-derived DNA-lesions in inflammation driven carcinogenesis. Cancer Detect Prev 2004;28:385–391.

[9] Adam W, Andler S, Saha-Moller CR. DNA cleavage induced by oxyl radicals generated in the photosensitized decomposition of fatty ester hydroperoxides derived from oleic and linoleic acid. Arch Biochem Biophys 1998;349:261–266.

[10] Inouye S. Site-specific cleavage of double-strand DNA by hydroperoxide of linoleic-acid. Febs Letters 1984;172: 231–234.

[11] Yang M, Schaich K. Factors affecting DNA damage caused by lipid hydroperoxides and aldehydes. Free Radic Biol Med 1996;20:225–236.

[12] Lim P, Sadre-Bazzaz K, Shurter J, Sarasin A, Termini J. DNA damage and mutations induced by arachidonic acid peroxidation. Biochemistry 2003;42:15036–15044.

[13] Lim P, Wuenschell GE, Holland V, Lee DH, Pfeifer GP, Rodriguez H, Termini J. Peroxyl radical mediated oxidative DNA base damage: implications for lipid peroxidation induced mutagenesis. Biochemistry 2004;43:15339–15348.

[14] Vila A, Korytowski W, Girotti AW. Dissemination of peroxidative stress via intermembrane transfer of lipid hydroperoxides: model studies with cholesterol hydroperoxides. Arch Biochem Biophys 2000;380:208–218.

[15] Thomas JP, Maiorino M, Ursini F, Girotti AW. Protective action of phospholipid hydroperoxide glutathione peroxidase against membrane-damaging lipid peroxidation. *In situ* reduction of phospholipid and cholesterol hydroperoxides. J Biol Chem 1990;265:454–461.

[16] Buege J, Aust S. Microsomal lipid peroxidation. Methods Enzymol 1978;52:302–310.

[17] Beckwith ALJ, Davies AG, Davison IGE, Maccoll A, Mruzek MH. The mechanisms of the rearrangements of allylic hydroperoxides - 5-α-hydroperoxy-3-β-hydroxycholest-6-ene and 7-α-hydroperoxy-3-β-hydroxycholest-5-ene. J Chem Soc, Perkin Trans 2 1989;815–824.

[18] Korytowski W, Bachowski GJ, Girotti AW. Chromatographic separation and electrochemical determination of cholesterol hydroperoxides generated by photodynamic action. Anal Biochem 1991;197:149–156.

[19] Kulig MJ, Smith LL. Sterol metabolism. XXV. Cholesterol oxidation by singlet molecular oxygen. J Org Chem 1973;38: 3639–3642.

[20] Schenck GO, Neumüller OA, Eisfeld W. Zur photosensibilisierten autoxydation der steroide: Δ^5-Steroid-7α-hydroperoxyde und -7-ketone durch allylumlagerung von Δ^6-steroid-5α-hydroperoxyden. Justus Liebigs Ann Chem 1958;618:202–210.

[21] Adachi J, Asano M, Naito T, Ueno Y, Tatsuno Y. Chemiluminescent determination of cholesterol hydroperoxides in human erythrocyte membrane. Lipids 1998;33:1235–1240.

[22] Ronsein GE, Prado FM, Mansano FV, Oliveira MCB, Medeiros MHG, Miyamoto S, Di Mascio P. Detection and characterization of cholesterol-oxidized products using HPLC coupled to dopant assisted atmospheric pressure photoionization tandem mass spectrometry. Anal Chem 2010;82:7293–7301.

[23] Lloyd RS, Haidle CW, Robberson DL. Bleomycin-specific fragmentation of double-stranded DNA. Biochemistry 1978; 17:1890–1896.

[24] Boiteux S, Gajewski E, Laval J, Dizdaroglu M. Substrate-specificity of the Escherichia-coli FPG protein (formamidopyrimidine DNA glycosylase) - excision of purine lesions in DNA produced by ionizing-radiation or photosensitization. Biochemistry 1992;31:106–110.

[25] Boiteux S, O'Connor TR, Lederer F, Gouyette A, Laval J. Homogeneous Escherichia coli FPG protein. A DNA glycosylase which excises imidazole ring-opened purines and nicks DNA at apurinic/apyrimidinic sites. J Biol Chem 1990;265: 3916–3922.

[26] Dizdaroglu M, Laval J, Boiteux S. Substrate specificity of the Escherichia coli endonuclease III: excision of thymine- and cytosine-derived lesions in DNA produced by radiation-generated free radicals. Biochemistry 1993;32:12105–12111.

[27] Hatahet Z, Kow YW, Purmal AA, Cunningham RP, Wallace SS. New substrates for old enzymes. 5-Hydroxy-2'-deoxycytidine and 5- hydroxy-2'-deoxyuridine are substrates for Escherichia coli endonuclease III and formamidopyrimidine DNA N-glycosylase, while 5- hydroxy-2'-deoxyuridine is a substrate for uracil DNA N-glycosylase. J Biol Chem 1994;269: 18814–18820.

[28] Sanchez C, Shane RA, Paul T, Ingold KU. Oxidative damage to a supercoiled DNA by water soluble peroxyl radicals characterized with DNA repair enzymes. Chem Res Toxicol 2003;16:1118–1123.

[29] Sheu C, Foote CS. Reactivity toward singlet oxygen of a 7,8-dihydro-8-oxoguanosine (8-hydroxyguanosine) formed by photooxidation of a guanosine derivative. J Am Chem Soc 1995;117:6439–6442.

[30] Sheu CM, Foote CS. Photosensitized oxygenation of a 7,8-dihydro-8-oxoguanosine derivative - formation of dioxetane and hydroperoxide intermediates. J Am Chem Soc 1995;117:474–477.

[31] Bryan SE, Vizard DL, Beary DA, Labiche RA, Hardy KJ. Partitioning of zinc and copper within sub-nuclear nucleoprotein particles. Nucleic Acids Res 1981;9:5811–5823.

[32] Gao YG, Sriram M, Wang AHJ. Crystallographic studies of metal-ion DNA interactions - different binding modes of cobalt(Ii), copper(Ii) and barium(Ii) to N-7 of guanines in Z-DNA and a drug-DNA complex. Nucleic Acids Res 1993;21:4093–4101.

[33] Phelps RA, Putnam FW, Lynn LT, Neet KE. Cupric ion catalysis of cleavage of gamma-globulin and other proteins by hydrogen peroxide. J Biol Chem 1961;236:96–105.

[34] Floyd RA. DNA-ferrous iron catalyzed hydroxyl free-radical formation from hydrogen-peroxide. Biochem Biophys Res Comm 1981;99:1209–1215.

[35] Floyd RA. Direct demonstration that ferrous ion complexes of diphosphate and triphosphate nucleotides catalyze hydroxyl free-radical formation from hydrogen-peroxide. Arch Biochem Biophys 1983;225:263–270.

[36] Rowley DA, Halliwell B. Superoxide-dependent and ascorbate-dependent formation of hydroxyl radicals in the presence of copper-salts - A physiologically significant reaction. Arch Biochem Biophys 1983;225:279–284.

[37] Hallliwell B, Gutteridge JMC. Free radicals in biology and medicine. 3rd ed. Oxford University Press, Oxford: 1999.

[38] Jones CM, Burkitt MJ. EPR spin-trapping evidence for the direct, one-electron reduction of tert-butylhydroperoxide to the tert-butoxyl radical by copper(ii): paradigm for a previously

overlooked reaction in the initiation of lipid peroxidation. J Am Chem Soc 2003;125:6946–6954.
[39] Pryor WA. Oxyradicals and related species - their formation, lifetimes, and reactions. Annu Rev Physiol 1986;48: 657–667.
[40] Van Remmen H, Richardson A. Oxidative damage to mitochondria and aging. Exp Gerontol 2001;36:957–968.
[41] Burrows CJ, Muller JG. Oxidative nucleobase modifications leading to strand scission. Chem Rev 1998;98:1109–1152.
[42] Steenken S, Jovanovic SV. How easily oxidizable is DNA? One-electron reduction potentials of adenosine and guanosine radicals in aqueous solution. J Am Chem Soc 1997;119: 617–618.
[43] Pratviel G, Meunier B. Guanine oxidation: one- and two-electron reactions. Chemistry 2006;12:6018–6030.
[44] Breen AP, Murphy JA. Reactions of oxyl radicals with DNA. Free Radic Biol Med 1995;18:1033–1077.
[45] Korytowski W, Schmitt JC, Girotti AW. Surprising inability of singlet oxygen-generated 6-hydroperoxycholesterol to induce damaging free radical lipid peroxidation in cell membranes. Photochem Photobiol 2010;86:747–751.
[46] Korytowski W, Girotti AW. Singlet oxygen adducts of cholesterol: photogeneration and reductive turnover in membrane systems. Photochem Photobiol 1999;70:484–489.
[47] Vila A, Korytowski W, Girotti AW. Spontaneous intermembrane transfer of various cholesterol-derived hydroperoxide species: kinetic studies with model membranes and cells. Biochemistry 2001;40:14715–14726.
[48] Phillips M, Johnson W, Rothblat G. Mechanisms and consequences of cellular cholesterol exchange and transfer. Biochim Biophys Acta 1987;906:223–276.
[49] Vila A, Korytowski W, Girotti AW. Spontaneous transfer of phospholipid and cholesterol hydroperoxides between cell membranes and low-density lipoprotein: assessment of reaction kinetics and prooxidant effects. Biochemistry 2002;41: 13705–13716.
[50] Niki E. Lipid peroxidation: physiological levels and dual biological effects. Free Radic Biol Med 2009;47:469–484.
[51] Ursini F, Maiorino M, Brigelius-Flohe R, Aumann K, Roveri A, Schomburg D, Flohe L. Diversity of glutathione peroxidases. Methods Enzymol 1995;252:38–53.
[52] Manevich Y, Shuvaeva T, Dodia C, Kazi A, Feinstein S, Fisher A. Binding of peroxiredoxin 6 to substrate determines differential phospholipid hydroperoxide peroxidase and phospholipase A(2) activities. Arch Biochem Biophys 2009;485: 139–149.
[53] Yang Y, Cheng J-Z, Singhal SS, Saini M, Pandya U, Awasthi S, Awasthi YC. Role of glutathione S-transferases in protection against lipid peroxidation. Overexpression of hGSTA2-2 in k562 cells protects against hydrogen peroxide-induced apoptosis and inhibits JNK and caspase 3 activation. J Biol Chem 2001;276:19220–19230.
[54] Girotti AW. Lipid hydroperoxide generation, turnover, and effector action in biological systems. J Lipid Res 1998;39: 1529–1542.
[55] Thomas J, Girotti A. Photooxidation of cell membranes in the presence of hematoporphyrin derivative: reactivity of phospholipid and cholesterol hydroperoxides with glutathione peroxidase. Biochim Biophys Acta 1988;962:297–307.
[56] Bloch K. Sterol. Structure and membrane function. Crit Rev Biochem Mol Biol 1983;14:47–92.
[57] Ramjiawan B, Czubryt M, Massaeli H, Gilchrist J, Pierce G. Oxidation of nuclear membrane cholesterol inhibits nucleoside triphosphatase activity. Free Radic Biol Med 1997;23: 556–562.
[58] Ouedraogo GD, Redmond RW. Secondary reactive oxygen species extend the range of photosensitization effects in cells: DNA damage produced via initial membrane photosensitization. Photochem Photobiol 2003;77:192–203.
[59] Esterbauer H, Gebicki J, Puhl H, Jürgens G. The role of lipid peroxidation and antioxidants in oxidative modification of LDL. Free Radic Biol Med 1992;13:341–390.
[60] Patel RP, Svistunenko D, Wilson MT, DarleyUsmar VM. Reduction of Cu(II) by lipid hydroperoxides: implications for the copper-dependent oxidation of low-density lipoprotein. Biochem J 1997;322:425–433.
[61] Wilcox AL, Marnett LJ. Polyunsaturated fatty-acid alkoxyl radicals exist as carbon-centered epoxyallylic radicals - a key step in hydroperoxide-amplified lipid-peroxidation. Chem Res Toxicol 1993;6:413–416.
[62] Russell GA. Deuterium-isotope effects in the autoxidation of aralkyl hydrocarbons. Mechanism of the interaction of peroxyl radicals. J Am Chem Soc 1957;79:3871–3877.
[63] Miyamoto S, Martinez G, Martins A, Medeiros MHG, Di Mascio P. ^{18}O-Labeled lipid hydroperoxides and HPLC coupled to mass spectrometry as valuable tools for studying the generation of singlet oxygen in biological system. Biofactors 2004;22:333–339.
[64] Miyamoto S, Martinez G, Medeiros MHG, Di Mascio P. Singlet molecular oxygen generated from lipid hydroperoxides by the Russell mechanism: studies using ^{18}(O)-labeled linoleic acid hydroperoxide and monomol light emission measurements. J Am Chem Soc 2003;125:6172–6179.
[65] Miyamoto S, Martinez GR, Rettori D, Augusto O, Medeiros MHG, Di Mascio P. Linoleic acid hydroperoxide reacts with hypochlorous acid, generating peroxyl radical intermediates and singlet molecular oxygen. Proc Natl Acad Sci USA 2006; 103:293–298.
[66] Murphy RC, Johnson KM. Cholesterol, reactive oxygen species, and the formation of biologically active mediators. J Biol Chem 2008;283:15521–15525.
[67] Girotti AW. Translocation as a means of disseminating lipid hydroperoxide-induced oxidative damage and effector action. Free Radic Biol Med 2008;44:956–968.

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