Selected paper

Can estrogenic radicals, generated by lactoperoxidase, be involved in the molecular mechanism of breast carcinogenesis?

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Mutations of regulatory genes, which perturb the mechanism of cell replication resulting in abnormal cell proliferation, are the main cause of cancer. Many endogenous and exogenous chemicals (including estrogenic hormones) are known to represent a major carcinogenic risk for humans. 2-OH- and 4-OH-derivatives of estrogenic molecules have been shown to form stable adducts with purine DNA bases and act as 'depurinating' agents, thus altering gene transcription (Cavalieri EL, Stack DE, Devanesan PD *et al. Proc Natl Acad Sci USA* 1997; **94**: 10937–10942). Lactoperoxidase (LPO), which is produced by mammary glands, is likely to be involved in breast carcinogenesis, because of its ability to interact with estrogenic hormones and oxidise them through two one-electron reaction steps. We investigated the reactivity of LPO towards five molecules: 17-β-estradiol (a natural hormone), diethylstilbestrol (a synthetic drug, supplied to pregnant women for preventing spontaneous abortion), exestrol (a synthetic antigonadotropic estrogen), 2-OH- and 4-OH-estradiol (catabolic products of estradiol). Enzymatically generated radical derivatives of such molecules were stabilized by spin-trapping or by chelation of a diamagnetic metal ion and characterized with EPR spectroscopy. A kinetic study of the oxidation process was carried out using EPR and UV-visible spectroscopy.

INTRODUCTION

Mutation of critical regulatory genes is the event that initiates cancer and it can be triggered by different types of molecules, which are generally identified as carcinogens. Many exogenous and endogenous estrogenic molecules are known to represent a major carcinogenic risk to humans. Hormonal carcinogenesis has been widely investigated and a mechanism has been proposed[1] to explain how estrogens can act as tumor initiators, by promoting a genotoxic event. A reaction sequence has been proposed (Scheme 1).

According to this scheme, catecholic estrogens (which are metabolites of monohydroxylated estrogens) can be oxidised to quinones by peroxidases in the presence of hydrogen peroxide. Oxidation takes place through two

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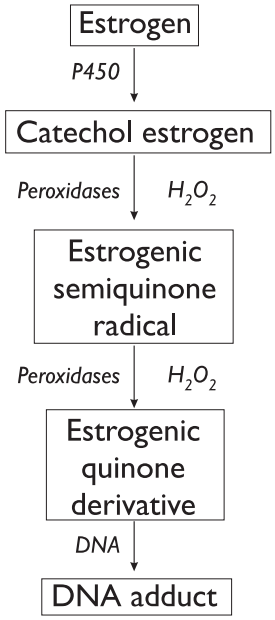

Scheme 1 Proposed reaction sequence to explain how estrogens can act as tumor initiators, by promoting a genotoxic event.

one-electron steps: first, a semiquinone radical is generated, which can be further oxidised to quinone. According to Cavalieri's hypothesis,[1] estrogenic quinones are capable of interacting with DNA molecules and form adducts with them. Certain quinone derivatives of estrogens, namely 4-hydroxyestradiol, can act as depurinating agents: they coordinate to a purine base of the DNA moiety and the resulting adduct can be cleaved, thus leaving an apurine site, which can generate mutations that initiate cancer. Other quinones, like 2-hydroxyestradiol, are thought to form stable adducts with DNA. Such complexes remain on the nucleic acid molecule unless repaired. They result in less toxicity than the depurinating adducts described above.

The mechanism proposed by Cavalieri *et al.*[1] assumes that the only toxic species is the final oxidation product (a quinone), in spite of the fact that a radical species is formed as a reaction intermediate.

We decided to investigate the possibility that estrogenic radicals are involved in DNA damage because of the well-known toxicity of radical species.

Therefore, the reactivity of 5 estrogenic substrates, either endogenous or exogenous, in the presence of a peroxidase was studied. Lactoperoxidase was chosen as the enzymatic system, since it is particularly abundant in milk and, therefore, it is likely to be involved in breast carcinogenesis due to its ability to interact with estrogenic hormones. The substrates were: 17-β-estradiol (a natural hormone), diethylstilbestrol (a synthetic drug, supplied to pregnant woman for preventing spontaneous abortion), exestrol (a synthetic antigonadotropic estrogen), and 2-OH- and 4-OH-estradiol (catabolic products of estradiol). Only two molecules out of five are catechols, the others are monohydroxylated estrogens, which are known to be converted to catecholic species by catabolic enzymes. Enzymatically generated radical derivatives of these molecules were stabilized by spin-trapping or by chelation of a diamagnetic metal ion and characterized by EPR spectroscopy. A kinetic study of both one-electron steps of the oxidation reaction was carried out by means of EPR and UV-visible spectroscopy.

Materials and Methods

LPO was purified from raw milk according to the protocol described elsewhere.[2] Exestrol, 2-hydroxy-estradiol, 4-hydroxy-estradiol, 3-methyl-2-benzothiazolinone hydrazone (MBTH), 3-dimethylaminobenzoic acid (DMAB), the spin label 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO) and the spin-trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) were purchased from Sigma. 17-β-estradiol and diethylstilbestrol were purchased from Aldrich. *p*-Cresol was purchased from Carlo Erba. Dimethylsulphoxide (DMSO) and hydrogen peroxide were purchased from Fluka.

Estrogenic molecules were first solubilized in DMSO and subsequently diluted with 0.1 M phosphate buffer pH 6.8. The final DMSO concentration was 25–30% v/v.

Molar extinction coefficients of 17-β-estradiol (1401 $M^{-1}cm^{-1}$ at 279 nm), diethylstilbestrol (312 $M^{-1}cm^{-1}$ at 258 nm) and exestrol (450 $M^{-1}cm^{-1}$ at 277 nm) were determined from the slope of a calibration curve obtained by plotting the absorbance of the estrogen solutions *versus* their concentration. Solutions were obtained by dissolving weighed amounts of solute in 2 ml of solvent mixture (30% DMSO in 0.1 M phosphate buffer pH 6.8).

Specific LPO activity either in 30% DMSO/0.1 M phosphate buffer pH 4.3 or in 0.1 M phosphate buffer pH 4.3 was determined by following the oxidation of the MBTH/DMAB system in the presence of hydrogen peroxide.[3] The oxidation product absorbs at 590 nm (ε = 47600 $M^{-1}cm^{-1}$). 2.0 ml of 30% DMSO/0.1 phosphate buffer pH 4.3 containing DMAB (1.9 mM), MBTH (0.04 mM) and LPO (40 nM) were added with H_2O_2 up to a concentration of 0.24 mM. Specific activity was expressed as μmol product/min/mg enzyme.

Spectrophotometric measurements were done with a Kontron Uvikon930 double-beam spectrophotometer, with 10 mm-path length cells. Cell compartments were equipped with a magnetic stirrer and a temperature control device. All optical measurements were recorded at 25°C, using either quartz or PMMA/UV-grade cells (Kartell).

Spin-trapping experiments were done at 298 K, by adding a large excess of DMPO (20 mM) to a reaction mixture containing either 17-β-estradiol (EST), diethylstilbestrol (DES) or exestrol (EXE) – all capable of giving rise to a radical species – (0.6 mM), LPO (0.68 μM) and hydrogen peroxide (0.5 M) dissolved in 30% DMSO/0.1 M phosphate buffer pH 6.8. Samples were introduced into capillary tubes and measured immediately after mixing. EPR instrumental settings were as follows: 9.76 GHz microwave frequency, 5 mW microwave power, 1 G modulation amplitude, 60 scans.

Spin-stabilization experiments were done at room temperature by mixing 2-hydroxyestradiol (2-OHE) or 4-hydroxyestradiol (4-OHE) (9.5 mM), LPO (100 μM) and hydrogen peroxide (3.3 mM) dissolved in 30% DMSO/0.5 M magnesium acetate pH 6.8. Samples were introduced into capillary tubes and measured immediately after mixing. EPR instrumental settings were as follows: 9.76 GHz microwave frequency, 5 mW microwave power, 0.16 G modulation amplitude, 10 scans.

EPR kinetic measurements were done by rapidly mixing hydrogen peroxide (final concentration 0.5 mM) with a reaction mixture containing either EST, DES, EXE, 2-OHE or 4-OHE (0.6 mM), LPO (0.68 μM), DMPO (20 mM) dissolved in 30% DMSO/0.1 M phosphate buffer pH 6.8. The mixture was quickly introduced into a capillary tube and a series of 90 EPR spectra was recorded, 40 s apart from each other. In order to reduce the

recording-time as much as possible, only one absorption out of the whole EPR spectrum was registered. A kinetic profile was obtained by plotting the double integration of the resulting signal against time. From this plot a kinetic constant was calculated by numerical fitting.

Radical species' concentration was extrapolated from a calibration curve made by plotting the areas of the EPR signal of different solutions of TEMPO spin-label *versus* their concentrations. EPR instrumental settings were as follows: 9.76 GHz microwave frequency, 16 mW, microwave power, 2 G modulation amplitude, sweep width 12 G.

UV-visible kinetic measurements were done by rapidly mixing hydrogen peroxide (final concentration 0.7 mM) with a reaction mixture containing the estrogenic substrate (0.6 mM), LPO (0.68 μM), DMPO (20 mM) dissolved in 30% DMSO/0.1 M phosphate buffer pH 6.8 and by following the absorbance change at $\lambda = 315$ nm for EST and EXE, at 400 nm for DES, at 414 nm for 2-OHE and at 440 nm for 4-OHE. Kinetic values were obtained from plots of the absorbance *versus* time by numerical fitting.

Results and Discussion

In this study, we investigated the reactivity of the LPO/H_2O_2 system towards five estrogenic molecules (Fig. 1): 17-β-estradiol (EST), diethylstilbestrol (DES), exestrol (EXE) and two hydroxy-derivatives of 17-β-estradiol, 2-hydroxy- (2-OHE) and 4-hydroxy-estradiol (4-OHE). Because the solubility of estrogens in aqueous media is low, it was necessary to find an organic solvent able to dissolve these substrates without disrupting the enzyme. Some solubility assays have been made with different mixtures of organic solvents and phosphate buffer pH 6.8. The most effective solvent mixture was 30% v/v DMSO in 0.1 M phosphate buffer pH 6.8. The effect of DMSO on the LPO properties was checked by measuring the specific activity of the enzyme in 0.1 M phosphate buffer, with or without DMSO. LPO activity in the presence of the organic solvent was lowered to 24% of that measured in phosphate buffer only. No relevant modifications of the EPR spectral pattern of LPO (recorded at 4 K) was seen when the enzyme was dissolved in phosphate buffer pH 6.8, in the presence of increasing amounts of DMSO (up to 30% v/v: data not shown). This indicates that the organic solvent does not disrupt the enzyme catalytic site, although it affects its function.

EST, DES and EXE are oxidised by the LPO/H_2O_2 system through two one-electron steps. This process implies formation of a radical intermediate, which can be trapped with DMPO, according to the mechanism reported in Figure 2A. The EPR spectra of such species are shown in Figure 3. Hyperfine couplings are sensitive to the nature of the trapped radical and thus resulted in specific

Table 1. EPR splitting constants relative to the adducts of EST, DES and EXE with DMPO and to the spin-stabilized 2-OHE and 4-OHE semiquinone radicals

Adduct	a_1	a_2	a_3	a_4
EST–DMPO	14.12 G	10.45 G	–	–
DES–DMPO	14.16 G	10.33 G	–	–
EXE–DMPO	14.12 G	10.41 G		
2-OHE-*o*-semiquinone–Mg^{2+}	9.13 G	8.29 G	5.53 G	0.39 G
4-OHE-*o*-semiquinone–Mg^{2+}	7.03 G	4.56 G	0.50 G	0.38 G

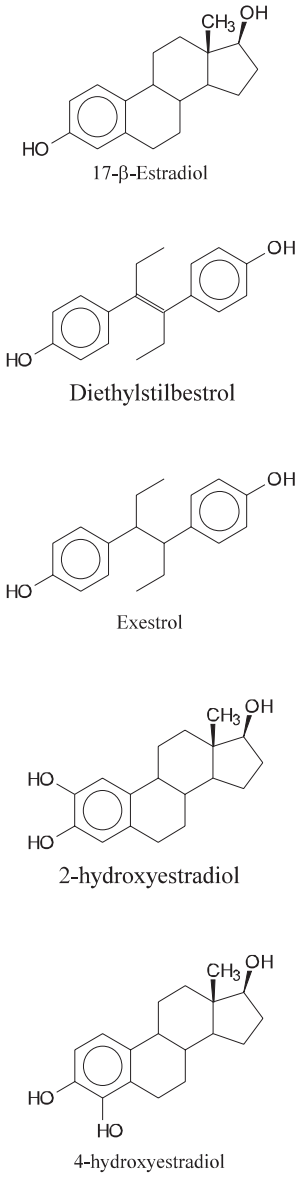

Fig. 1. Structures of the estrogenic substrates investigated in this study.

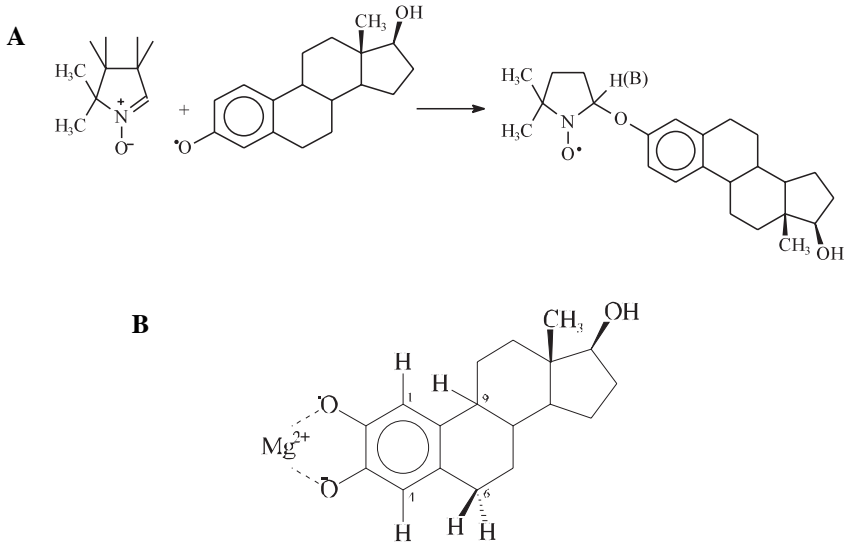

Fig. 2. (**A**) Spin-trapping mechanism involving DMPO. (**B**) Spin-stabilized catecholic radical.

lines splitting for each one of the above mentioned adducts. Computer simulation of their patterns allowed assignment of the splitting constants, whose values are summarized in Table 1. In all cases, a_1 (with the typical value around 14 G) reflects coupling to the nitrogen atom, while a_2 (which is ~10 G) is due to the β-proton on the spin-trap moiety.[4] In the absence of LPO, substrate or hydrogen peroxide, no EPR signals were seen, thus confirming that radical generation is not a parasite reaction and cannot take place in the absence of the enzymatic system.

The *o*-semiquinone nature of the enzymatically-generated 2-OHE and 4-OHE radical derivatives allowed utilization of the spin-stabilization technique (Fig. 2B). It implies chelation of a diamagnetic metallic ion (Mg^{2+} in this case) by the oxygen atoms of two hydroxyl functions lying on adjacent positions of a benzene ring. The LPO/H_2O_2 system was effective towards both substrates and two different EPR spectra were recorded. Assignment of the splitting constants was done by means of computer simulation and by comparison with previously published spectra of substituted estrogenic cathecols.[5] The EPR spectrum of the 2-OHE semiquinone radical (Fig. 3) was characterized by 16 lines and 4 splitting constants (Table 1). Only 4 out of 5 protons which are expected to couple with the unpaired electron gave rise to detectable splittings. a_1 and a_2 account for coupling with the H-9 and an H-6 proton (Fig. 2B). a_3 is assigned to the other H-6 proton and a_4 to an aromatic proton (either H-1 or H-4). Splitting due to the fifth proton, which is bound to an aromatic carbon atom, is too small to be detected.

The 4-OHE semiquinone radical gave rise to a 12-line EPR spectrum, with 4 splitting constants. We assign the two major splittings to coupling with H-9 and H-1; H-6 is responsible for a_3, while a_4 is assigned to the aromatic proton H-2. No splitting due to the other H-6 proton was detected.

These assignments have been made by considering the spin-density lying on aromatic and aliphatic carbon atoms in estrogenic substrates. Hyperconjugative effect accounts for the high spin density on C-9 and determines the a_1 value. A similar mechanism concerns the H-6 protons, although their positions on the ring make them more sensitive to steric effects. This could explain the remarkably different 'a' values relative to H-6, observed in 2-OHE and 4-OHE. Finally, aromatic protons adjacent to oxygen atoms are characterized by lower spin density with respect to those in a *meta* position. The coupling mechanism which involves aromatic protons is based on spin-polarization.[5–7]

Once the ability of the LPO/H_2O_2 system to generate radicals in estrogenic molecules was ascertained, we decided to look at the quantitative aspects of this process *in vitro*. An attempt to measure the formation rate of the radical species using the spin-trapping technique associated with EPR spectroscopy was made. We obtained a series of first-order kinetic profiles and a kinetic constant value was extrapolated from each of them, by numerical fitting (Table 2). We also measured the steady-state concentration of the spin-trapped radical species, whose values were extremely low (10^{-6}–10^{-7} M: Table 2). The kinetic constants were similar for all substrates (10^{-3} s^{-1}). This fact, together with the low steady-state concentrations of the trapped radical, made us conclude that the spin-trapping process was not competitive with the second one-electron step of substrate oxidation, the latter being too kinetically favoured. Further evidence supporting our conclusion was obtained by measuring the oxidation rate of *p*-cresol in the presence of DMPO: LPO is known to be extremely reactive towards this substrate. Once again, the order of magnitude of the kinetic constant was the same as those previ-

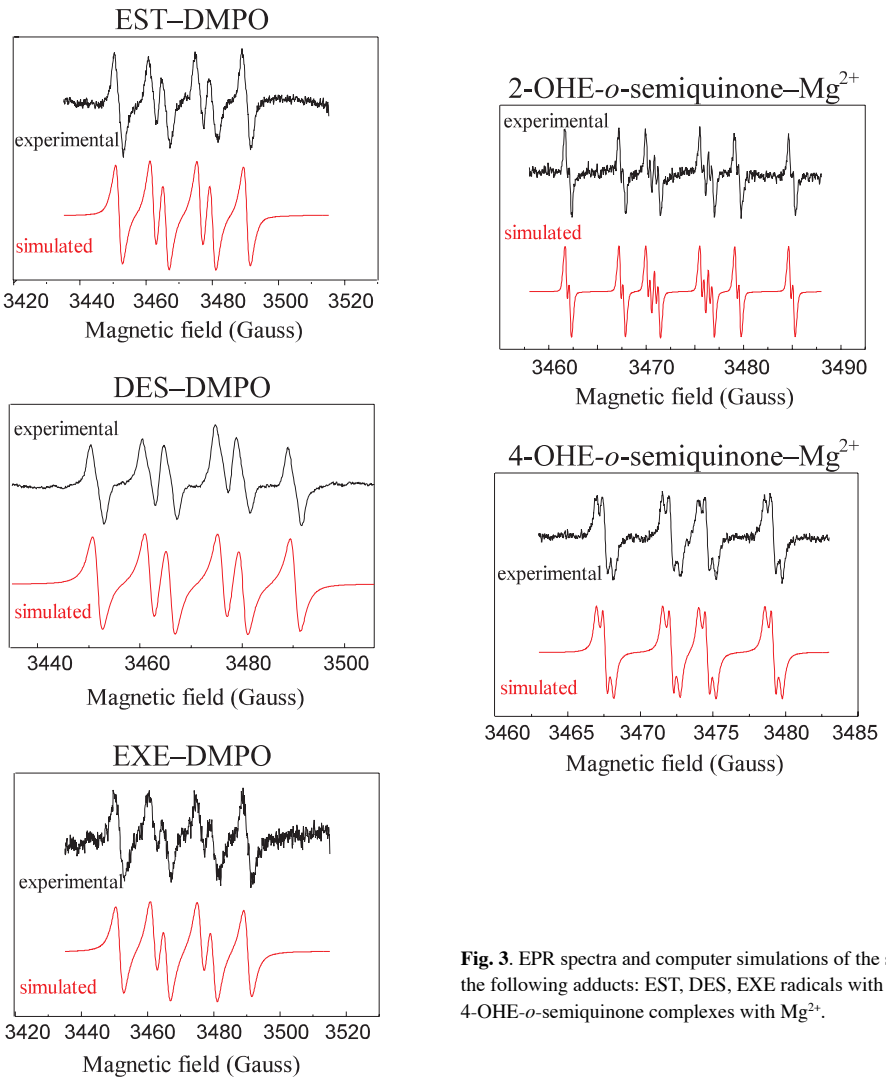

Fig. 3. EPR spectra and computer simulations of the spectral patterns of the following adducts: EST, DES, EXE radicals with DMPO; 2-OHE- and 4-OHE-*o*-semiquinone complexes with Mg^{2+}.

Table 2. Kinetic constants and steady state concentrations of the oxidation products relative to the oxidation of estrogenic substrates by the LPO/H_2O_2 system measured by EPR and UV-visible spectroscopy

	EPR		UV-visible	
Substrate	k (s^{-1})	[Rad] (M)	k_1 ($s^{-1}Abs^{-1}$)*	
EST	4.32×10^{-3}	1.58×10^{-6}	0.285	
EXE	3.56×10^{-3}	6.78×10^{-7}	0.298	
			k_1 (s^{-1})	k_2 (s^{-1})
DES	1.78×10^{-3}	7.56×10^{-7}	0.2094	0.00193
2-OHE–Mg^{2+}	3.47×10^{-3}	1.05×10^{-6}	0.0742	0.00160
4-OHE–Mg^{2+}	1.69×10^{-3}	3.71×10^{-7}	0.0812	–

*k_1 is expressed in $s^{-1}Abs^{-1}$ since no ε for the reaction product is available.

ously measured (5.2×10^{-3} s^{-1}). As a consequence, we propose that these values reflect the rate of spin-trapping rather than the actual rate of formation of the radical species. It is likely that only a very small fraction of radical species is trapped, thus making the EPR kinetic measurements not significant.

Then, we decided to look at the kinetics of the overall oxidation process, by measuring the oxidation rate by

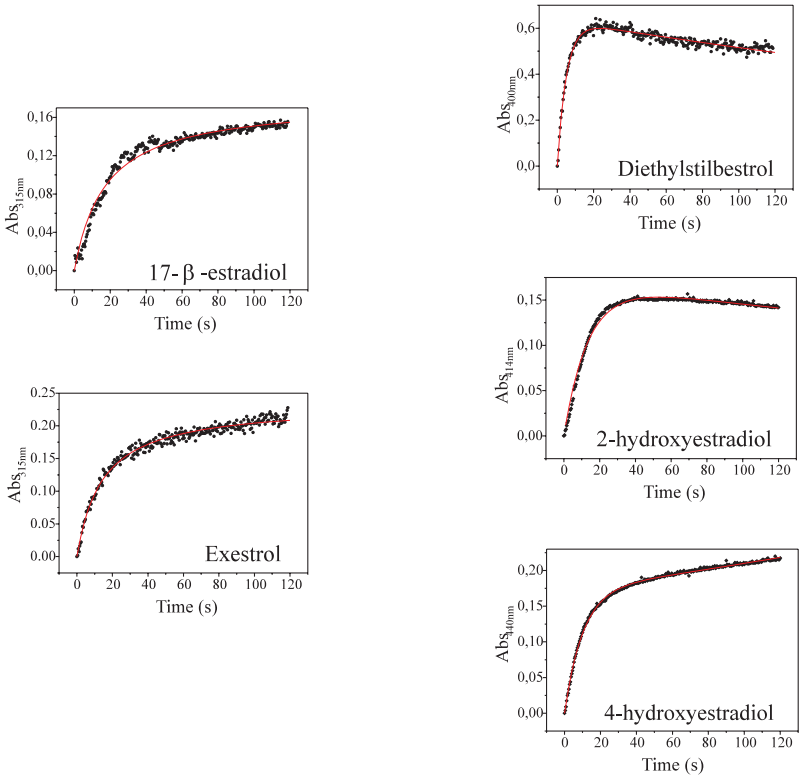

Fig. 4. Kinetic profiles relative to the oxidation of EST, EXE, DES, 2-OHE and 4-OHE by the LPO/H_2O_2 system obtained by absorption spectroscopy.

UV-visible spectroscopy. All five estrogen species are colourless, since they absorb in the near UV, around 280 nm (the typical absorption bands associated with phenolic rings). Their enzymatic oxidation results in substantial modifications of the absorption spectra, which are characteristic of each substrate. EXE and EST oxidation products are colourless: their generation is followed by an increase of turbidity of their solution; moreover, a shoulder around 315 nm becomes evident. This spectral feature, together with the solubility decrease which is responsible for turbidity provides reasonable evidence that a dimerization reaction takes place, a process that has often been observed in the presence of peroxidases.[8]

On the other hand, DES, 2-OHE and 4-OHE gave rise to coloured oxidation products, whose absorption spectra are typical of molecules containing conjugated double bonds and quinone functions.

In particular, an intense yellow colour appears upon oxidation of DES, which is associated with an absorption at 350 nm, typical of *para*-quinones.[9] The intensity of such absorption keeps stable for a few minutes; then it decreases and the band shifts to lower wavelength (~325 nm); this indicates that the quinone is not stable and undergoes further modifications, which have not been characterized in this study.

2-OH and 4-OH oxidation products are characterized by absorptions around 410–450 nm, which are generally associated with *ortho*-quinones.[10,11]

The kinetic profiles relative to the oxidation of all the above-mentioned substrates are shown in Figure 4. EST and EXE showed second-order kinetic patterns and the kinetic constant values were almost coincident. This is consistent with the hypothesis that both undergo a dimerization process.

Oxidation of DES, 2-OHE and 4-OHE seems to follow a more complicated pathway in the presence of LPO and hydrogen peroxide (data not shown). Since their oxidation products do not look stable with time, as already reported in the literature for analogous molecules,[12] we supposed that two consecutive irreversible first-order reactions take place in this case, according to the following scheme:

$A \rightarrow B \rightarrow C$, with k_1 and k_2 as the kinetic constants for the two consecutive reactions. We think that a quinone is formed as the first oxidation product (B) and we measured [B] *versus* time; the kinetic patterns were fitted with the following equation:

$$y = [k_1/(k_2 - k_1)] \bullet a \bullet [\exp(-k_1 \bullet x) - \exp(-k_2 \bullet x)] \quad \text{Eq. 1}$$

and both k_1 and k_2 were extrapolated.

The k_1 values for 2-OHE and 4-OHE look very similar (about 0.070 s^{-1}). 4-OHE species C, which has not been characterized, shows an absorption band in the same region as B. As a consequence, as soon as the reaction proceeds toward C the absorbance does not decrease, since B consumption is compensated by C production. This make the k_2 value not meaningful for this substrate.

The higher k_1 value observed for DES indicates that this substrate is more easily oxidised than 2-OHE and 4-OHE. The small k_2 values observed for both DES and 2-OHE indicate that quinone production is kinetically more favoured than the subsequent reaction.

EPR spectroscopy provided evidence that all substrates are initially oxidised to radicals by the LPO/H_2O_2 system. DES, 2-OHE and 4-OHE subsequently generate quinones, while EST and EXE more likely dimerize (as suggested by UV-visible spectroscopy). Dimerization or quinone formation are favoured over reaction of the radical with the spin-trap; this explains the low yield of trapped radicals detected by EPR as well as the rapid oxidation of the substrates. Since the radical–radical reaction is fast, one could expect that the ability of such radical species to interact with DNA is less favoured. Consequently, it is more likely that quinones are responsible for DNA damage through depurination (according to the mechanism proposed by Cavalieri *et al.*[1]) rather than the radical itself.

The lower toxicity of EST and EXE (with respect to the other three substrates)[1] is consistent with the fact that they undergo a dimerization process and do not generate a quinone.

Conclusions

In conclusion, this paper has shown that the LPO/H_2O_2 system is able to oxidise EST, DES, EXE, 2-OHE and 4-OHE through a radical mechanism. Our data on catecholic estrogens strongly suggest that, although these substrates can give rise to radical intermediates, their toxicity is associated with the ortho-quinones generated through two one-electron oxidations. This agrees with the hypothesis by Cavalieri *et al.*[1] on the chemical origin of breast carcinogenesis. Nevertheless, we cannot rule out the possibility that radical derivatives of monohydroxylated estrogens, generated by the LPO/H_2O_2 system, can play a carcinogenic role *in vivo*, by interacting with other chemical species present in the cellular environment, which have not been taken into account in the present context.

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