Carcinogenesis vol.15 no.11 pp.2637–2643, 1994

The metabolism of 17β-estradiol by lactoperoxidase: a possible source of oxidative stress in breast cancer

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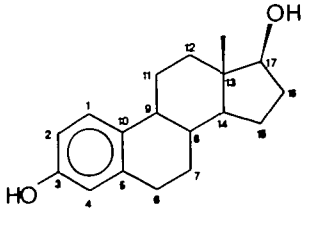
Electron spin resonance (ESR) spectroscopy and oxygen consumption measurements using a Clark-type oxygen electrode have been used to study the metabolism of the estrogen 17β-estradiol by lactoperoxidase. Evidence for a one-electron oxidation of estradiol to its reactive phenoxyl radical intermediate is presented. The phenoxyl radical metabolite abstracts hydrogen from reduced glutathione generating the glutathione thiyl radical, which is spin trapped by 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) and subsequently detected by ESR spectroscopy. In the absence of DMPO, molecular oxygen is consumed by a sequence of reactions initiated by the glutathione thiyl radical. Similarly, the estradiol phenoxyl radical abstracts hydrogen from reduced β-nicotinamide-adenine dinucleotide (NADH) to generate the NAD· radical. The NAD· radical is not spin trapped by DMPO, but instead reduces molecular oxygen to the superoxide radical, which is then spin-trapped by DMPO. The superoxide generated may either spontaneously dismutate to form hydrogen peroxide or react with another NADH to form NAD·, thus propagating a chain reaction leading to oxygen consumption and hydrogen peroxide accumulation. Ascorbate inhibits oxygen consumption when estradiol is metabolized in the presence of either glutathione or NADH by reducing radical intermediates back to their parent molecules and forming the relatively stable ascorbate radical. These results demonstrate that the futile metabolism of micromolar quantities of estradiol catalyzes the oxidation of much greater concentrations of biochemical reducing cofactors, such as glutathione and NADH, with hydrogen peroxide produced as a consequence. The accumulation of intracellular hydrogen peroxide could explain the hydroxyl radical-induced DNA base lesions recently reported for female breast cancer tissue.

Introduction

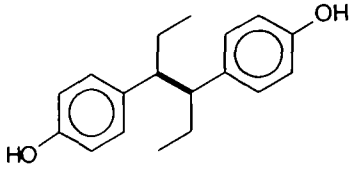
Evidence implicating reactive oxygen species in free-radical-mediated carcinogenesis has been reviewed recently (1,2). Epidemiological studies demonstrate that predominantly vegetarian populations, which presumably consume larger quantities of antioxidants in their diets, have lower incidences of breast, colon and prostate cancers, further suggesting a role for free radicals in carcinogenesis (3–5). Recent investigations of normal breast tissue and cancerous breast tissue have revealed a substantial increase in base lesions in the DNA of invasive ductal carcinoma of the female breast (6,7). The base lesions were typical of hydroxyl radical-dependent hydroxylation and ring-opening reactions of the purine bases. It is uncertain, however, if these DNA lesions are responsible for initiation and cell transformation processes in female breast cancer or if they are a consequence of the cancer, but it is clear that the cancerous cells have experienced a significant and sustained elevation in intracellular hydroxyl radical generation.

The most probable pathway for hydroxyl radical formation under physiological conditions is the Fenton reaction of ferrous (or cuprous) ions with hydrogen peroxide. Intracellular oxygen activation usually occurs by the one-electron reduction of molecular oxygen to form the superoxide anion, often mediated by other enzymatically-generated free radical metabolites, in a process known as 'futile metabolism'. In this process, large quantities of superoxide may be generated (8), which can lead to an increase in the steady-state concentration of hydrogen peroxide. In invasive ductal carcinoma of the female breast, the source of the hydrogen peroxide responsible for the observed DNA base lesions has yet to be identified.

Extensive studies on the metabolism of the synthetic estrogen and known carcinogen diethylstilbestrol (DES*, Figure 1) have been reported. It was shown that peroxidase preparations from horseradish or mouse uterus could metabolize DES in the presence of hydrogen peroxide (9). In fact, a variety of substrates containing a phenol group are readily oxidized to



17β-Estradiol



Diethylstilbestrol (DES)

Fig. 1. Molecular structures of estradiol and diethylstilbestrol (DES).

*Abbreviations: DES, diethylstilbestrol; GSH, reduced glutathione; NADH, β-nicotinamide-adenine dinucleotide; SOD, superoxide dismutase; DTPA, diethylenetriaminepentaacetic acid; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; ESR, electron spin resonance.

their phenoxyl radical intermediates by peroxidases. Thus, phenoxyl radical intermediates have been detected during or implicated in the peroxidase-catalyzed metabolism of phenol (10), *p*-aminophenol (11), acetaminophen (12,13), tyrosine (14) and substituted phenol food antioxidants (15). Furthermore, the peroxidase-catalyzed futile metabolism of phenol substrates can generate significant quantities of superoxide in the presence of a suitable reductant, such as the reduced form of glutathione (GSH), or β-nicotinamide-adenine dinucleotide (NADH) (8). Elevated levels of 8-hydroxydeoxyguanosine in liver and kidney DNA of DES-induced cancers in Syrian hamsters were attributed to iron-catalyzed free radicals generated as a consequence of DES metabolism (16). The natural estrogen 17β-estradiol (henceforth referred to simply as estradiol) did not induce increased hydroxylation of guanine in either kidney or liver (16), though other evidence for estradiol-dependent oxidative stress has been reported (17,18).

Lactoperoxidase, found in milk, saliva, tears and the mammary glands, is in many ways similar to the well-studied peroxidase horseradish peroxidase. Lactoperoxidase is activated by hydrogen peroxide to its compound I enzyme intermediate, which can catalyze the oxidation of a phenolic group to its free radical intermediate, the corresponding phenoxyl radical (19). Since estradiol has a phenolic group and both estradiol and lactoperoxidase are likely to be present at the site of breast tumor formation, we have undertaken *in vitro* studies to investigate the futile metabolism of estradiol, with the concomitant production of superoxide and hydrogen peroxide. The implications of the results reported here to the development of breast cancer are discussed.

Materials and methods

Chemicals and biochemicals

Ascorbic acid was Analytical Reagent grade from J.T.Baker, Inc. (Philipsburg, NJ). Catalase (from beef liver, 65 000 U/mg crystalline suspension in water) and superoxide dismutase [SOD; from bovine erythrocytes, 3000 U/mg lyophilizate according to the method of McCord and Fridovich (20)] were purchased from Boehringer-Mannheim (Indianapolis, IN) and were used as received. Diethylenetriaminepentaacetic acid (DTPA), 17β-estradiol, water soluble 17β-estradiol (by solubilization with 2-hydroxypropyl-β-cyclodextrin), GSH, NADH and lactoperoxidase (140 U/mg protein) were purchased from Sigma Chemical Company (St Louis, MO) and were used as received. The spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), also purchased from Sigma, was vacuum-distilled twice at ambient temperature and stored at −70°C until use. The supporting buffer for all reactions was 100 mM enzyme grade tris(hydroxymethyl)-aminomethane (Bethesda Research Laboratories, Bethesda, MD) adjusted to pH 7.4 with hydrochloric acid (Tris−HCl). The Tris−HCl buffer was treated with Chelex 100 resin (Bio-Rad, Hercules, CA) to remove adventitious transition metal ions and contained 50 μM DTPA as a further precaution against trace metal catalysis.

Electron spin resonance (ESR) experiments

Room temperature ESR spectroscopy was used to detect and identify free radical intermediates formed during reaction. Although the free radicals in this experiment are too short-lived to be directly detected under the present experimental conditions, certain radical intermediates were detected using the spin trapping technique. Thus, the DMPO spin trap was present in all ESR experiments (unless otherwise stated), which reacts with the short-lived free radical intermediate to form a relatively long-lived DMPO radical adduct. The original free radical intermediate is then identified by the resulting ESR hyperfine coupling constants of its DMPO adduct.

ESR measurements were made using a Bruker ESP-300 electron spin resonance spectrometer operating at a microwave frequency near 9.77 GHz and a magnetic field of approximately 3480 G, with magnetic field modulation at 100 kHz providing the usual first derivative display of the ESR spectrum. Samples were aspirated into a quartz flat cell prepositioned in a Bruker TM-110 cavity for ESR detection (21). Spectra were recorded on a Bruker Aspect 3000 computer and transferred to an IBM-compatible computer for analysis and plotting using software developed locally (22–24). ESR figures were prepared using the Corel Corporation CorelDRAW™ software.

Oxygen consumption experiments

Oxygen consumption measurements were made using a Clarke-type oxygen electrode, fitted to a 1.8 ml Gilson sample cell and monitored by a Yellow Springs Instrument Company Model 53 oxygen monitor. Oxygen concentration in the samples as a function of time was recorded by a 100 mV strip-chart recorder or by a personal computer interfaced to the oxygen monitor with a Data Translation DT2801 data acquisition board. The oxygen consumption data were plotted by importing the ASCII data files into the Origin™ program (MicroCal Software, Inc.).

Results

Estradiol metabolism in the presence of glutathione

Glutathione thiyl free radical generation as a consequence of the lactoperoxidase-catalyzed metabolism of estradiol was demonstrated by spin trapping with DMPO. The four-line ESR spectrum of the DMPO−glutathione thiyl radical adduct was detected in a reaction medium containing estradiol, GSH, hydrogen peroxide, lactoperoxidase and DMPO at pH 7.4 (Figure 2A). The characteristic ESR spectrum, corresponding to hyperfine coupling constants $a^N = 15.1$ and $a_\beta^H = 16.2$ G, was in close agreement with that reported earlier (25,26). When estradiol was omitted from the reaction, the same signal was observed at a greatly reduced intensity, due to direct oxidation of GSH by lactoperoxidase (25,26) (Figure 2B). No signal was observed in the absence of glutathione, DMPO or lactoperoxidase, and only a trace signal was observed in the absence of hydrogen peroxide. These ESR results suggest that the estradiol phenoxyl metabolite was formed, which subsequently oxidized GSH.

In addition to the generation of the glutathione thiyl radical, oxygen was consumed during the lactoperoxidase-dependent metabolism of estradiol in the presence of GSH. Thus, a

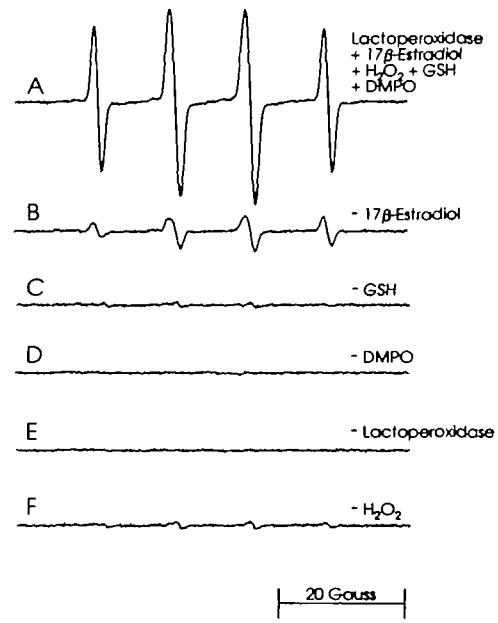


Fig. 2. ESR spectrum of the DMPO−glutathione thiyl radical adduct detected in a solution containing GSH, estradiol, hydrogen peroxide and lactoperoxidase. (**A**) Complete system containing 10 μM estradiol, 16 μM hydrogen peroxide, 1 mM GSH, 13 μg/ml lactoperoxidase and 100 mM DMPO in 100 mM Tris−HCl buffer, pH 7.4. The sample also contained about 1% ethanol from the estradiol stock solution. (**B**) As in (A), but without addition of estradiol. (**C**) As in (A), but without glutathione. (**D**) As in (A), but without DMPO. (**E**) As in (A), but without lactoperoxidase. (**F**) As in (A), but without hydrogen peroxide. The instrumental conditions were as follows: 20 mW microwave power, 1 G modulation amplitude, 41 ms time constant and a scan rate of 1.90 G/s.

solution containing GSH, estradiol and hydrogen peroxide consumed molecular oxygen when the metabolism of estradiol was initiated by the addition of lactoperoxidase (Figure 3A). After ~2 min, the oxygen consumption noticeably slowed. In the absence of estradiol, the rate of oxygen consumption was dramatically decreased (Figure 3B), corresponding to a large decrease in the amount of the glutathione thiyl radical spin trapped (Figure 2B). The small initial evolution of oxygen in the absence of estradiol was due to the low catalase-like activity of lactoperoxidase in the absence of a suitable substrate

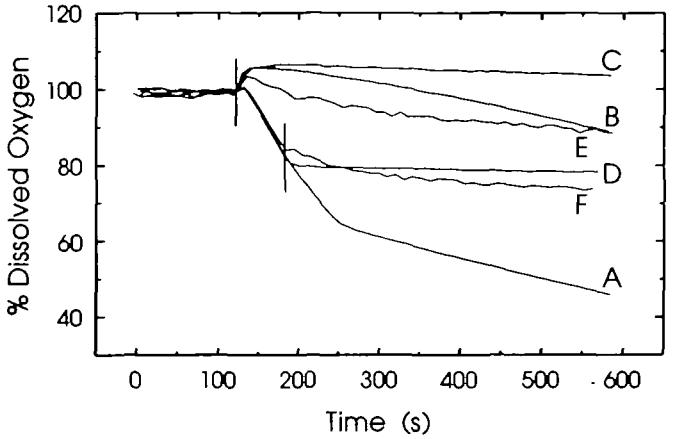


Fig. 3. Effect of ascorbate and DMPO on the rate of oxygen consumption during the oxidation of estradiol by lactoperoxidase in the presence of GSH. The two vertical bars mark the time of reaction initiation by lactoperoxidase addition and subsequent additions (if any) 1 min later. (**A**) Complete system containing 10 mM reduced glutathione, 100 μM estradiol, 200 μM H_2O_2 and 60 μg/ml lactoperoxidase in 100 mM Tris–HCl buffer, pH 7.4, in a final volume of 1.8 ml. The reaction was initiated by the addition of lactoperoxidase. (**B**) As in (A), but in the absence of estradiol. (**C**) As in (A), but with 1 mM ascorbate added before initiation. (**D**) As in (A), but with 1 mM ascorbate added after initiation. (**E**) As in (A), but with 100 mM DMPO added 1 min before initiation. (**F**) As in (A), but with 100 mM DMPO added 1 min after initiation.

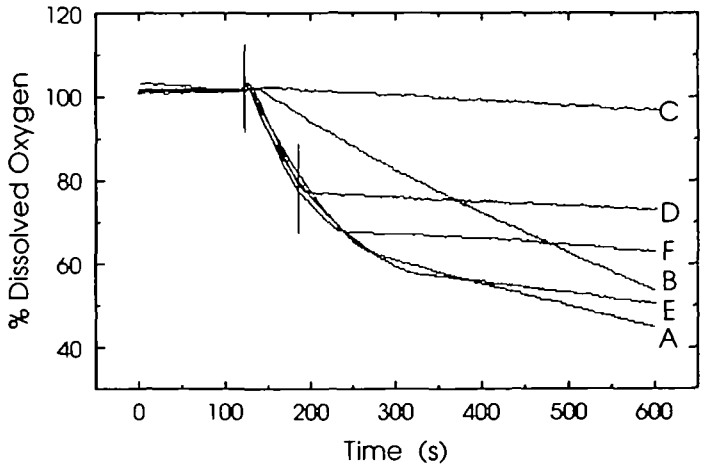


Fig. 4. Effect of catalase and SOD on the rate of oxygen consumption during the oxidation of estradiol by lactoperoxidase in the presence of GSH. The two vertical bars mark the time of reaction initiation by lactoperoxidase addition and subsequent additions (if any) 1 min later. (**A**) Complete system as in Figure 3A. The reaction was initiated by the addition of lactoperoxidase. (**B**) As in (A), but without H_2O_2 (same conditions as Figure 3B). (**C**) As in (A), but with 50 μg/ml (3250 U/ml) catalase added before initiation. (**D**) As in (A), but with 50 μg/ml (3250 U/ml) catalase added 1 min after initiation. (**E**) As in (A), but with 450 U/ml SOD added before initiation. (**F**) As in (A), but with 450 U/ml SOD added 1 min after initiation.

(27). The addition of ascorbate to the reaction mixture prior to (Figure 3C) or 1 min following (Figure 3D) initiation by lactoperoxidase completely prevented the consumption of oxygen, probably by preventing superoxide generation by reducing the estradiol phenoxyl radical back to the parent molecule. Similarly, the addition of DMPO to the reaction solution prior to (Figure 3E) or 1 min following (Figure 3F) initiation decreased the rate of oxygen consumption by spin trapping the glutathione thiyl radical intermediate, thus preventing subsequent reactions with molecular oxygen.

Hydrogen peroxide was essential for the activation of the lactoperoxidase and the initiation of oxygen consumption. The relatively slow consumption of oxygen in the absence of added hydrogen peroxide was due to trace amounts of hydrogen peroxide already in the system (Figure 4B), possibly as a result of GSH auto-oxidation, since no oxygen consumption was detected when catalase was added prior to the lactoperoxidase (Figure 4C). Catalase also halted oxygen consumption when added to the complete system 1 min after initiation by lactoperoxidase (Figure 4D). The addition of 450 U/ml SOD prior to the lactoperoxidase did not significantly affect the rate of oxygen consumption by estradiol and lactoperoxidase in the presence of GSH (Figure 4E). If the SOD was added 1 min after the reaction was initiated with lactoperoxidase, no inhibition occurred initially. After a lag period of ~90 s, however, oxygen consumption noticeably slowed (Figure 4F). The origin of this delayed inhibition by SOD is unclear.

Estradiol metabolism in the presence of NADH

The characteristic ESR spectrum of the DMPO–superoxide radical adduct was observed in a reaction containing NADH, estradiol, lactoperoxidase and DMPO (Figure 5A). The solution also contained 2% ethanol, since the estradiol was solubilized in absolute ethanol. The hyperfine coupling constants a^N = 14.2, a_β^H = 11.3 and a_γ^H = 1.2 G are in close agreement with previously published data (28,29). Similar results were obtained if the estradiol was solubilized in aqueous solution by 2-hydroxypropyl-β-cyclodextrin instead of in ethanol (data not shown). When estradiol was omitted from the reaction, the ESR signal was greatly decreased (Figure 5B). No signal was detected in the absence of either NADH (Figure 5C) or lactoperoxidase (Figure 5D). The concentration-dependent inhibition by catalase demonstrates that trace amounts of hydrogen peroxide were present and necessary for the reaction to proceed (Figure 5E and F). The hydrogen peroxide necessary for the initial activation of lactoperoxidase was probably a result of the auto-oxidation of NADH.

Oxygen was consumed in an incubation of NADH, lactoperoxidase and estradiol (Figure 6A). When estradiol was excluded from the experiment, oxygen was consumed at a greatly decreased rate, confirming the stimulatory effect of estradiol metabolism on the rate of oxygen consumption in this system (Figure 6B). The addition of ascorbate to the reaction mixture prior to lactoperoxidase prevented oxygen consumption (Figure 6C), while the addition of ascorbate 2 min after the reaction was initiated immediately quenched oxygen consumption (Figure 6D). Likewise, the addition of DMPO prior to (Figure 6E) or 2 min after (Figure 6F) the addition of lactoperoxidase decreased the rate of oxygen consumption. DMPO was less efficient than ascorbate in quenching the rate of oxygen consumption in this reaction, perhaps because the observed rate of reaction of DMPO with superoxide is only ~30 $M^{-1}s^{-1}$ at pH 7.4 (28,30).

Unlike the experiments with GSH, the rate of oxygen

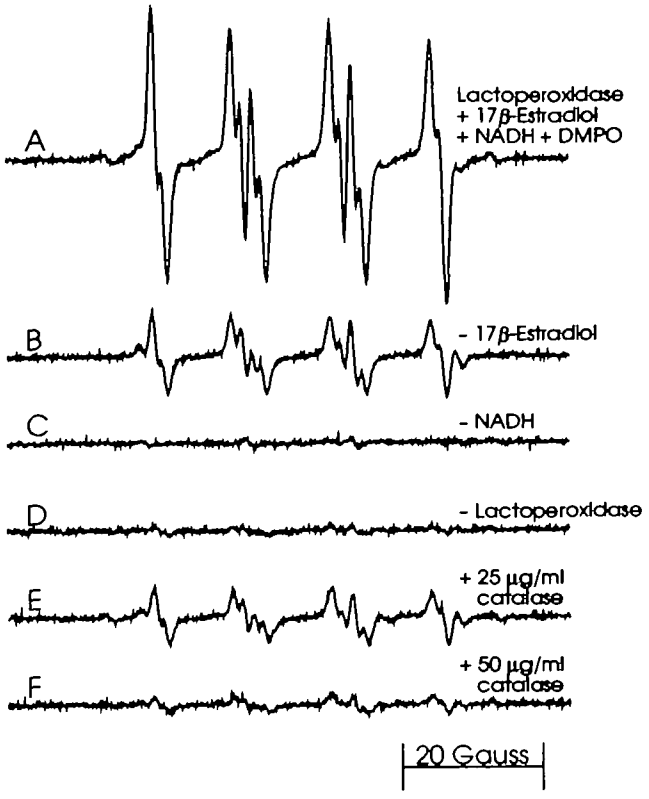


Fig. 5. ESR spectrum of the DMPO–superoxide adduct detected in a solution containing NADH, estradiol and lactoperoxidase. (**A**) Complete system containing 10 μM estradiol, 4 mM NADH, 13 μg/ml lactoperoxidase and 100 mM DMPO in 100 mM Tris–HCl buffer, pH 7.4. The incubation also contained about 2% ethanol from the estradiol stock solution. (**B**) As in (A), but without addition of estradiol. (**C**) As in (A), but without NADH. (**D**) As in (A), but without lactoperoxidase. (**E**) As in (A), but with 25 μg/ml (1625 U/ml) catalase added prior to lactoperoxidase. (**F**) As in (A), but with 50 μg/ml (3250 U/ml) catalase added prior to lactoperoxidase. The instrument conditions were as follows: 20 mW microwave power, 1 G modulation amplitude, 20.5 ms time constant and a scan rate of 1.90 G/s.

consumption in the experiment containing estradiol, lactoperoxidase and NADH was extremely sensitive to the presence of SOD. As shown in Figure 7, 0.45 U/ml SOD was sufficient to completely inhibit oxygen consumption, with 0.075 U/ml SOD having a noticeable effect. The addition of 0.45 U/ml SOD 2 min after the reaction was initiated with lactoperoxidase strongly inhibited the rate of oxygen consumption (Figure 7F).

No oxygen consumption was observed in a solution of estradiol and lactoperoxidase if NADH was excluded from the reaction. When NADH was present, catalase effectively inhibited oxygen consumption when added prior to or following reaction initiation by lactoperoxidase (data not shown). These results are consistent with the ESR results in Figure 5F.

Estradiol metabolism in the presence of ascorbate

When ascorbate undergoes a one-electron oxidation, the relatively stable ascorbate anion radical may be detected directly with ESR spectroscopy. A strong ESR spectrum of the ascorbate radical was observed in a reaction containing 1 mM ascorbate, 10 μM estradiol, 3 μg/ml lactoperoxidase and 16 μM hydrogen peroxide (Figure 8A). The two-line ESR spectrum had the characteristic hyperfine coupling constant $a^H = 1.79$ G, in close agreement with that reported previously (31). Although the ascorbate radical has two additional, smaller hyperfine coupling constants [$a^H = 0.19$ G and $a^H = 0.07$ G (31)], obser-

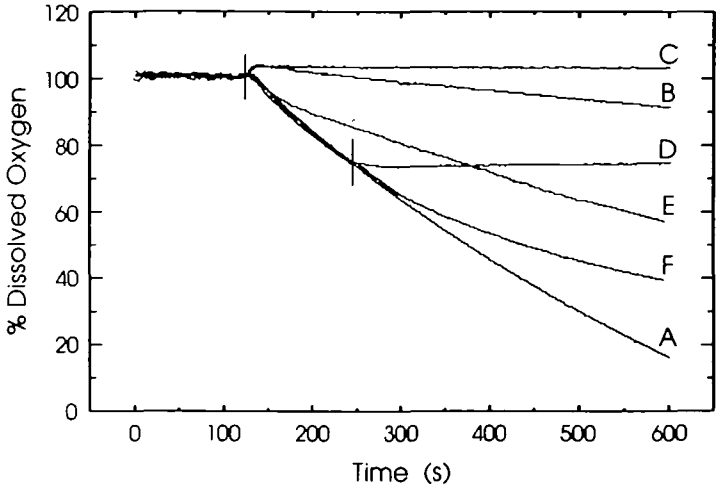


Fig. 6. Effect of ascorbate and DMPO on the rate of oxygen consumption during the oxidation of estradiol by lactoperoxidase in the presence of NADH. The two vertical bars mark the time of reaction initiation by lactoperoxidase addition and subsequent additions (if any) 2 min later. (**A**) Oxygen uptake was determined in a complete system of 3 mM NADH, 0.1 mM estradiol (as inclusion compound in cyclodextrin) and 20 μg/ml lactoperoxidase in 100 mM Tris–HCl buffer, pH 7.4, in a final volume of 1.8 ml. The reaction was initiated by the addition of lactoperoxidase. (**B**) As in (A), but in the absence of estradiol. (**C**) As in (A), but with 1 mM ascorbate added before initiation. (**D**) As in (A), but with 1 mM ascorbate added 2 min after initiation. (**E**) As in (A), but with 100 mM DMPO added before initiation. (**F**) As in (A), but with 100 mM DMPO added 2 min after initiation.

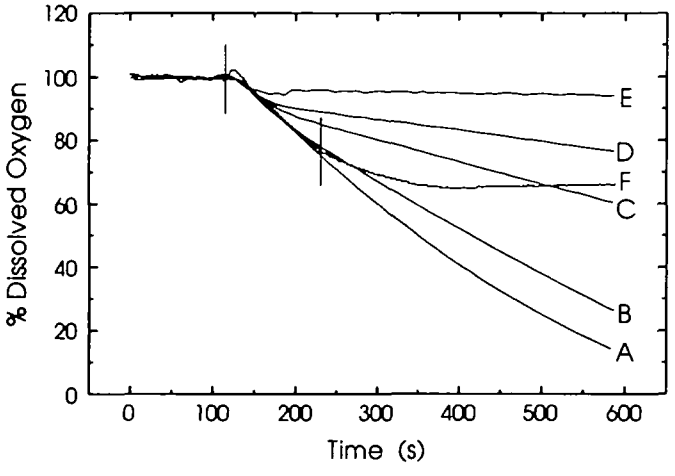


Fig. 7. Effect of SOD on the rate of oxygen consumption during the oxidation of estradiol by lactoperoxidase in the presence of NADH. The two vertical bars mark the time of reaction initiation by lactoperoxidase addition and subsequent additions (if any) 2 min later. (**A**) Complete system as in Figure 6A. (**B**) As in (A), but with 0.075 U/ml SOD added before initiation. (**C**) As in (A), but with 0.15 U/ml SOD added before initiation. (**D**) As in (A), but with 0.225 U/ml SOD added before initiation. (**E**) As in (A), but with 0.45 U/ml SOD added before initiation. (**F**) As in (A), but with 0.45 U/ml SOD added 2 min after initiation.

vations of these hyperfine couplings were precluded by the magnetic field modulation amplitude used in our observations. The presence of GSH did not affect the ESR signal intensity (Figure 8B), indicating that the estradiol phenoxyl radical reacts preferentially with ascorbate relative to GSH. The absence of estradiol led to a greatly decreased ESR signal intensity (Figure 8C). Only small residual ESR signals of the ascorbate radical were detected in the absence of lactoperoxid-

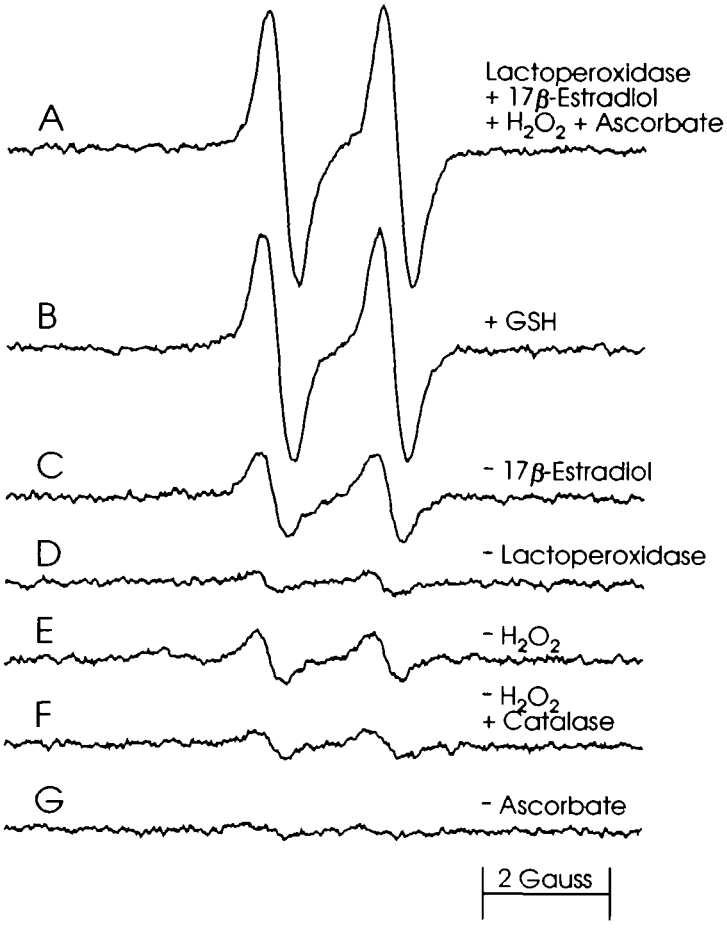


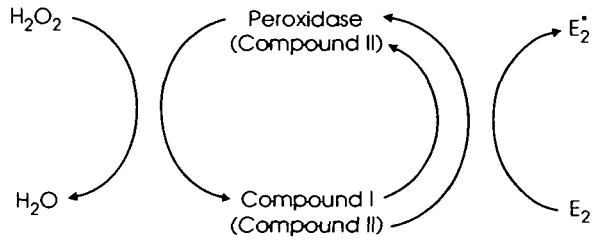
Fig. 8. ESR spectrum of the ascorbate radical generated during the oxidation of estradiol by lactoperoxidase. (**A**) Sample containing 10 μM estradiol, 1 mM ascorbate, 16 μM hydrogen peroxide and 3 μg/ml lactoperoxidase in 100 mM Tris−HCl buffer, pH 7.4. The incubation also contained about 1% ethanol from the estradiol stock solution. (**B**) As in (A), but with 1 mM glutathione added. (**C**) As in (A), but without addition of estradiol. (**D**) As in (A), but without lactoperoxidase. (**E**) As in (A), but without hydrogen peroxide. (**F**) As in (A), but without hydrogen peroxide and with 50 μg/ml (3250 U/ml) catalase added. (**G**) As in (A), but without ascorbate. The instrument conditions were as follows: 20 mW microwave power, 0.25 G modulation amplitude, 41 ms time constant and 1.91 G/s scan rate.

ase, which was further decreased by catalase (Figure 8D−F respectively).

Discussion

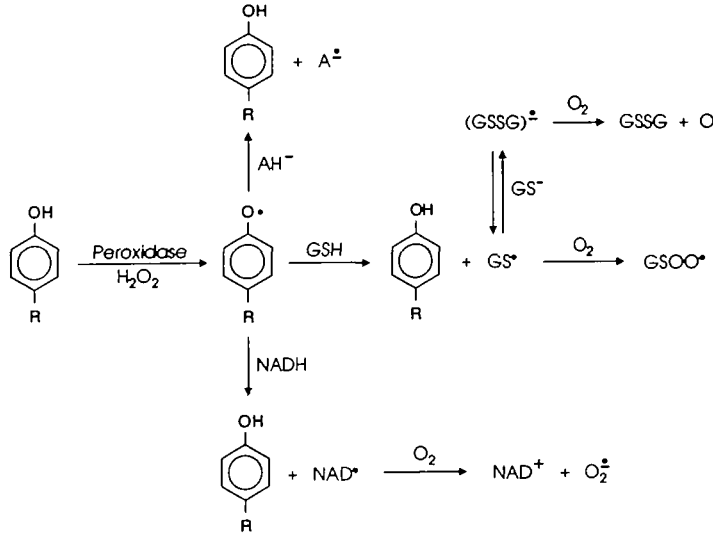
A firm link between female reproductive variables and increased risk of developing breast cancer has been established from epidemiological studies. Tables summarizing the various risk factors identified to date can be found in several reviews on the subject (32–34). For example, women who experience early menarche and late menopause have an increased risk of developing breast cancer. On the other hand, early menopause prior to age 40, surgically-induced by a bilateral ovariectomy, significantly reduces the risk of developing breast cancer. Such trends suggest the involvement of endogenous female hormones in the etiology of breast cancer, though the cancer is inherently a genetic disease.

The ESR experiments reported here provide strong evidence for the formation of a phenoxyl radical intermediate during the peroxidase-mediated metabolism of estradiol (Scheme I),



based on the observation of glutathione thiyl, superoxide and ascorbate radical formation in Figures 2, 5 and 8, respectively. As shown in Scheme I, lactoperoxidase can potentially oxidize two molecules of estradiol during one enzymatic cycle.

Formation of phenoxyl free radical metabolites can contribute to oxidative stress in biological systems by propagating the formation of superoxide, which can lead to an accumulation of hydrogen peroxide. Thus, in the presence of glutathione or NADH, the reaction sequence outlined in Scheme II



is proposed (8). As predicted by this Scheme, estradiol enhanced both the formation of the glutathione thiyl radical ($GS^{\bullet}$, Figure 2) and the consumption of oxygen in the glutathione−lactoperoxidase−H_2O_2 system (Figure 3). Oxygen consumption occurs when the glutathione thiyl radical reacts either with molecular oxygen to produce the glutathione peroxyl radical (35,36) or with another GSH molecule to form the glutathione disulfide radical anion, which reacts rapidly with oxygen to produce the superoxide radical anion (8). No superoxide or glutathione peroxyl radicals were detected in this system using ESR spectroscopy, perhaps because their precursor radical, the glutathione thiyl radical, is efficiently scavenged by DMPO. This is demonstrated by the strong inhibitory effect of DMPO on the consumption of oxygen in the system (Figure 3E and F). In addition to the formation of hydrogen peroxide, the generation of superoxide in the presence of GSH may lead to the formation of singlet oxygen, which can cause intracellular damage (37–39).

Estradiol greatly stimulated the rate of oxygen consumption when metabolized by lactoperoxidase in the presence of NADH (Figure 6) and the resulting superoxide radical is readily spin trapped by DMPO and detected by ESR spectroscopy (Figure 5). The sensitivity of the rate of oxygen consumption to the presence of SOD in this experiment suggests a chain reaction mechanism involving superoxide. The reactions leading to the formation of superoxide are shown in Scheme II.

Following protonation, the superoxide may oxidize another molecule of NADH ($k = 1.8 \times 10^5$ per $M^{-1}s^{-1}$) to generate NAD· and thus propagate the chain reaction (40). Since the rate of oxygen consumption was quenched by as little as 0.45 U/mol SOD in the NADH–lactoperoxidase reaction, the NADH–superoxide chain reaction appears to be the dominant mechanism of oxygen consumption. The oxidation of NADH by the estradiol radical metabolite, however, initiates the NADH–superoxide chain reaction, as demonstrated by comparing Figure 6A and B. Such an effect on the rate of oxygen consumption by SOD was not observed during GSH-dependent oxygen consumption.

The complete suppression of oxygen consumption by the addition of ascorbate to either the estradiol–GSH–lactoperoxidase reaction (Figure 3) or the estradiol–NADH–lactoperoxidase reaction (Figure 6) and the estradiol-dependent increase in the ascorbate radical concentration (Figure 8) further support the formation of a phenoxyl radical intermediate during the metabolism of estradiol by lactoperoxidase. Unlike GSH and NADH, however, the ascorbate radical is relatively stable and does not contribute to further oxidative stress through reaction with oxygen. Therefore, the futile metabolism of estradiol in the presence of sufficient ascorbate does not promote oxygen-derived radical formation.

Based on the similarity in their structures to estradiol, other estrogens which possess a phenoxy group, such as estrone and estriol, would be expected to react in much the same way. When estradiol was replaced by estrone in the experiments described in this report, indistinguishable ESR and oxygen consumption results were observed (data not shown). Therefore, if breast tissue experiences an unexpectedly large estrogen flux or a significant increase in peroxidase activity, peroxidase-catalyzed metabolism may become more prevalent and lead to an accumulation of intracellular hydrogen peroxide, especially if the cellular antioxidant defense systems are compromised. Peroxidase activity in mammary tumors has been shown to be significantly higher than in normal, non-lactating mammary tissue; however, there is disagreement as to whether such an increase in activity is the result of an infiltration of eosinophils and macrophages caused by mast cell degranulation or the result of an increase in intracellular peroxidase activity of the mammary cells (41–43).

Nevertheless, an increase in intracellular hydrogen peroxide concentration would set the stage for the type of DNA base lesions observed in female breast cancer (6,7). The results reported here do not prove such a sequence of events; however, the evidence for the lactoperoxidase-catalyzed metabolism of estradiol to its phenoxyl radical intermediate *in vitro*, with subsequent superoxide and hydrogen peroxide formation, establishes a possible mechanism for estrogen-mediated oxidative stress.

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Received on April 15, 1994; revised on June 8, 1994; accepted on June 21, 1994