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Cytoprotective Actions of Estrogens against *tert*-Butyl Hydroperoxide-induced Toxicity in Hepatocytes

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ABSTRACT. Estrogens are effective antioxidants in diverse biological systems. Despite their antioxidant activities, it is not known yet whether estrogens prevent or alleviate liver toxicity induced by oxidative stress. In the present work, we studied this possibility by examining *in vitro* the protective potential of different estrogen compounds (17β-estradiol, 2-hydroxyestradiol, and diethylstilbestrol) against *tert*-butyl hydroperoxide-induced hepatocyte damage. Various parameters such as cell viability, lipid peroxidation, adenine nucleotide content, and thiol status were measured as an index of cytotoxicity. The protective effects of estrogens were compared to those of the iron chelator deferoxamine. The molecules tested prevented oxidant-induced cell death differently, showing variable degrees of protection. Deferoxamine was the most potent agent, followed by diethylstilbestrol and 2-hydroxyestradiol, 17β-estradiol being the least efficient. The inhibitory effects on lipid and thiol oxidations paralleled the effects on cell viability. The molecules also reduced the oxidant-induced ATP depletion, except for 17β-estradiol which had no effect on the decreased ATP levels. Our results suggest that the mechanisms of the preventive actions of estrogens may be related not only to their antioxidant activity against free radicals, but also and to a lesser extent to the maintenance of the normal redox status of the cell, which partially recovers the intracellular GSH levels. BIOCHEM PHARMACOL **56**;11:1463–1469, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. estrogens; redox state; lipid peroxidation; thiol status; adenine nucleotide content; rat hepatocyte

Oxidative stress has been shown to be linked to liver diseases such as hepatotoxicity and other liver pathological conditions [1–4]. Free radicals derived from chemicals or drugs exposed to liver cells seem to mediate liver injury, although the mechanisms of free radical toxicity are not well understood [5]. Different mechanisms have been proposed to be involved in cellular injury by oxidative stress, such as the glutathione redox cycle [6–8] or the formation of potent oxidizing radical species, capable of initiating lipid peroxidation [9, 10]. The killing of isolated hepatocytes by TBHP† has often been used as a model to study the mechanisms of irreversible cell injury resulting from acute oxidative stress. TBHP has been reported to induce hepatocyte death by two different mechanisms. On the one hand, TBHP is metabolized by GSH peroxidase, resulting in the formation of *tert*-butanol and GSSG. GSSG is reduced back to GSH by GSSG reductase, resulting in the oxidation of pyridine nucleotides [11, 12]. Depletion of GSH and pyridine nucleotide oxidation are associated with altered Ca^{2+} homeostasis which is a critical event in the formation of blebs on plasma membrane, thus leading to cellular injury [7, 13–15]. Alternatively, TBHP can be metabolized to free radicals (*tert*-butoxyl radicals) by cytochrome P450, which in turn can initiate lipid peroxidation [9, 16]. The peroxidative decomposition of membrane lipids has relevant implications in hydroperoxide-induced toxicity.

Natural and synthetic estrogens have been widely reported to be effective antioxidant agents in diverse biological systems [17–21]. They inhibit lipid peroxidation by different degrees, depending on the structure of the estrogen molecule, i.e. the number of hydroxyl groups contained in their aromatic benzene moiety. Despite their antioxidant activities, it is not known whether estrogens prevent or alleviate liver toxicity induced by oxidative stress. In the present work, we examined *in vitro* the protective potential of different estrogen compounds against TBHP-induced hepatocyte damage. Various parameters such as cell viability, lipid peroxidation, adenine nucleotide content, and thiol status were measured as an index of cytotoxicity. The molecules tested prevented oxidant-induced cell death differently, showing variable degrees of protection. The possible mechanisms of the preventive actions of estrogens are also discussed.

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† *Abbreviations:* DES, diethylstilbestrol; DF, deferoxamine; E_2, 17β-estradiol; $2OHE_2$, 2-hydroxyestradiol; LDH, lactate dehydrogenase; MDA, malondialdehyde; TBARS, thiobarbituric acid reactive substances; and TBHP, *tert*-butyl hydroperoxide.

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MATERIAL AND METHODS

Materials

DF, DES, E_2, TBHP, albumin bovine (essentially fatty acid free), and thiobarbituric acid were purchased from Sigma Chemical. $2OHE_2$ was from Steraloids. Collagenase A, GSH, and nicotinamide adenine dinucleotides (reduced and oxidized forms) were from Boehringer Mannheim.

Isolation and Incubation of Hepatocytes

Liver cells were isolated from male Sprague–Dawley rats (180 g) by collagenase perfusion as described previously [22]. Cell viability after isolation, estimated by the trypan blue exclusion test, was typically greater than 90%. Hepatocytes (2×10^6 cells/mL) were resuspended in Dulbecco's minimum Essential medium, pH = 7.4, plus 10 mM of HEPES and 2% BSA. Incubations were performed in stoppered Erlenmeyer flasks at 37° under an atmosphere of 95% O_2/5% CO_2. After 20 min equilibration, reactions were started by the addition of 1 mM of TBHP. Estrogens dissolved in DES (0.1% final concentration) and DF dissolved in water were added to cell suspensions 10 min before the addition of TBHP. Controls with either DMSO or water additions were also performed. At timed intervals, aliquots were removed for determinations of the different parameters. Other experimental details are given in the legends to the figures. Controls indicated that DMSO additions had no effect on the indices examined.

Biochemical Assays

Viability of hepatocytes was determined by measuring the leakage of LDH from the cells into the medium. At the times indicated, two 1-mL aliquots of the cell suspensions were removed. Cells were separated from the medium by centrifugation at low speed, treated with distilled water, and frozen at −20° before the enzymatic analysis. After thawing, the samples were clarified by centrifugation. The supernatant fractions and the previously separated incubation medium were then assayed individually for LDH as described [23].

Lipid peroxidation was measured in isolated hepatocytes by the thiobarbituric acid method [24]. Cells were deproteinized with 10% trichloroacetic acid and then centrifuged. The supernatant was assayed for lipoperoxidation. It was expressed as nanomoles of MDA per million cells, using the MDA extinction coefficient of 156 mM^{-1} cm^{-1} at 532 nm. Control experiments showed that none of the molecules tested interfered with the thiobarbituric acid assay.

Spectrophotometric enzyme assays were used to measure the intracellular concentrations of AMP, ADP [25], and ATP [26]. At the indicated times, aliquots of the cell suspensions (18×10^6 cells) were removed. Cells were deproteinized with 0.05 N perchloric acid and centrifuged (1200 *g*, 4 min). The supernatant was neutralized with 2 M of K_2 CO_3 and 0.5 M of triethanolamine and further centrifuged. The supernatant fraction was frozen and stored at −80° until assayed for nucleotide content. The energy charge was calculated by the expression: (ATP + 1/2 ADP)/(ATP + ADP + AMP).

Protein thiol groups were determined using Ellman's reagent, according to the method of Albano [27]. After incubation, cells were separated from the medium by centrifugation and washed in albumin-free fresh medium. The cell pellet was treated with 1 mL of 5% trichloroacetic acid, with 5 mM of EDTA, and the protein precipitate was washed twice with the same trichloroacetic acid-EDTA solution. Protein was redissolved in 0.1 M of Tris–HCl buffer, pH 7.4, containing 5 mM EDTA and 0.5% SDS. Aliquots of this solution were reacted with Ellman's reagent. Values are expressed as nanomoles of SH equivalents/mg of protein using GSH as a standard. Proteins were assayed by Peterson [28], using BSA as standard. One million hepatocytes corresponded to 1.46 ± 0.34 mg of protein. The supernatants obtained after centrifuging the trichloroacetic acid-deproteinized cells were used for the GSH assay. GSH levels were measured as acid-soluble thiols using Ellman's reagent [27].

Statistical Analysis

All values were expressed as means ± SEM. The data were analyzed by either Student's *t*-test for nonpaired data or by two-way analysis of variance (ANOVA). Significance was judged as $P < 0.05$.

RESULTS

The toxicity induced by 1 mM of TBHP in rat hepatocytes was investigated by measuring the time-course of LDH release, TBARS formation, and the early changes in ATP levels and thiol status (Fig. 1). The oxidant produced a progressive increase in TBARS, reaching a plateau at nearly 10 min of incubation. TBHP also decreased the GSH content beginning at the first 2.5 min assayed, and further depleted ATP (58% depletion at 7.5 min). A subsequent sharp cell damage, measured as LDH leakage (45% viability at 15 min), was also observed (Fig. 1). The free protein thiols, however, did not change during this period.

The actions of E_2, $2OHE_2$, DES, and DF on the cytotoxicity induced by TBHP were then investigated. The molecules were tested at different concentrations (10–200 μM) at a fixed incubation time of 30 min with the oxidant. Figure 2 shows the cell viability results. In the absence of additions, over 60% of the cells died as a result of the TBHP exposure. The estrogens and DF, in a dose-dependent manner, reduced hepatocyte damage by different degrees. DF was found to be the most potent protector (81% viability at the concentration of 10 μM), followed by DES and $2OHE_2$ (50 and 43%, respectively, at 10 μM). These estrogens at the concentration of 100 μM pre-

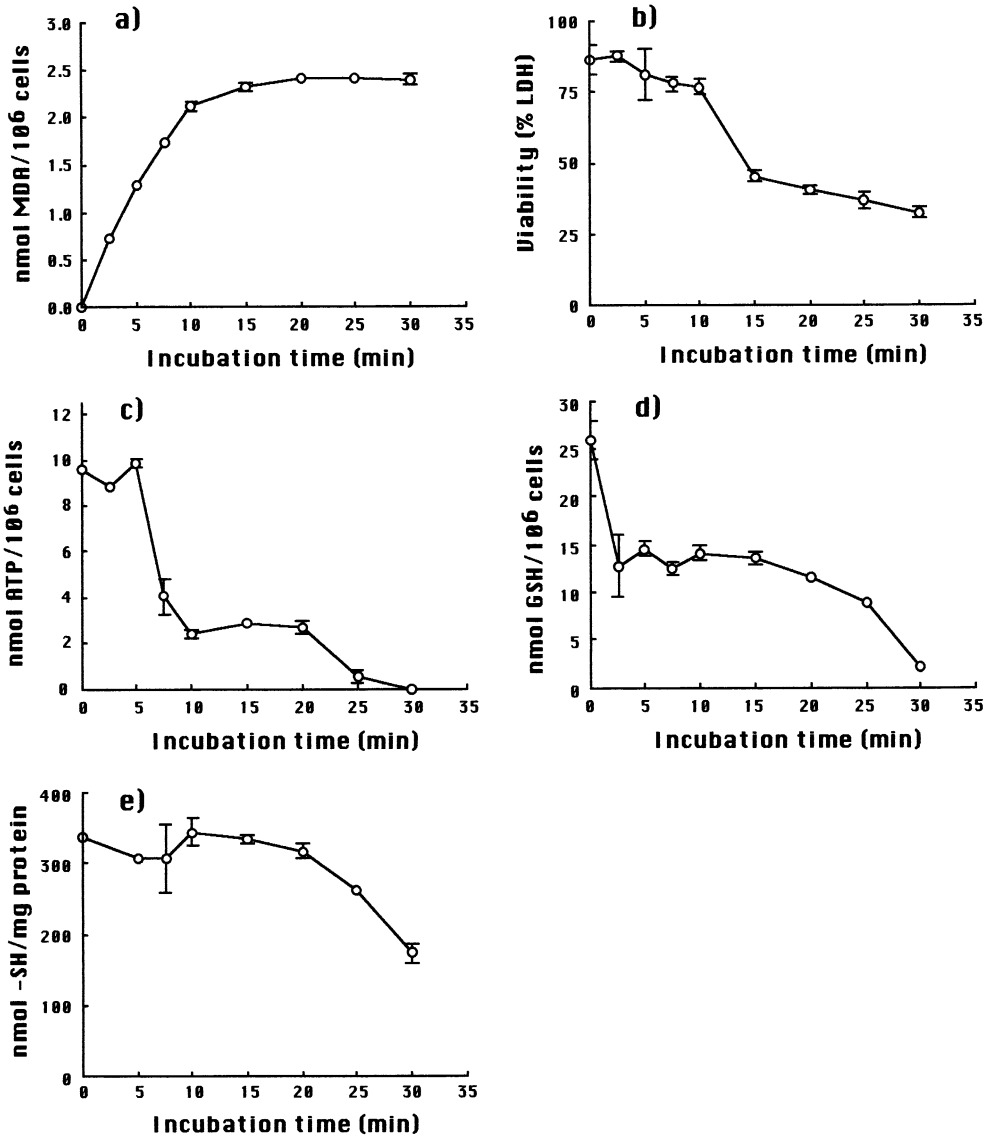

FIG. 1. Effect of TBHP on (a) lipid peroxidation, (b) cell viability, (c) ATP content, (d) protein sulphydryl groups, and (e) GSH of hepatocytes. Hepatocytes (2×10^6 cells/mL) were incubated in Dulbecco's minimum Essential medium, pH = 7.4, plus 10 mM of HEPES and 2% BSA in the presence of 1 mM of TBHP at 37°. Results are from one typical experiment out of three.

vented cell death almost completely. E_2 was the less potent protector.

The inhibitory effects on lipid peroxidation paralleled the effects on cell viability (Fig. 3). DF was the most potent antioxidant. $2OHE_2$ and DES gradually inhibited MDA formation in a similar concentration-dependent manner, while E_2 was the less potent antioxidant.

The intracellular content of adenine nucleotides is presented in Fig. 4. The hydroperoxide alone provoked a rough ATP depletion (1.2 ± 0.3 nmol/10^6 cells, at 30 min) (Fig. 4a). The iron chelator DF significantly reduced the oxidant-induced ATP depletion (9.5 ± 0.8 nmol/10^6 cells at 25 μM). $2OHE_2$ and DES partially reduced the oxidant-induced ATP depletion, while E_2 had no effect (Fig. 4a). The actions of TBHP on the hepatocyte content of ADP and AMP were less pronounced (Fig. 4, b and c). A similar pattern of action by the molecules tested could be seen for energy charge (Table 1). DF, $2OHE_2$, and DES prevented the TBHP-induced energy charge loss by different degrees, while E_2 had no effect.

TBHP decreased GSH and protein thiol groups after 30-min incubation (protein thiol groups from 336 ± 2 to 173 ± 14 nmol of −SH equivalents/mg protein, and GSH from 26.00 ± 2.00 to 2.26 ± 0.03 nmol GSH/million cells). Estrogens and DF dose dependently reduced the TBHP-induced depletion of these groups (Figs. 5, 6). It is noteworthy that at the minimum concentration of 10 μM assayed, neither $2OHE_2$ nor DES had any effect on GSH status, although they did prevent cell death significantly by 58 and 85%, respectively at that dose.

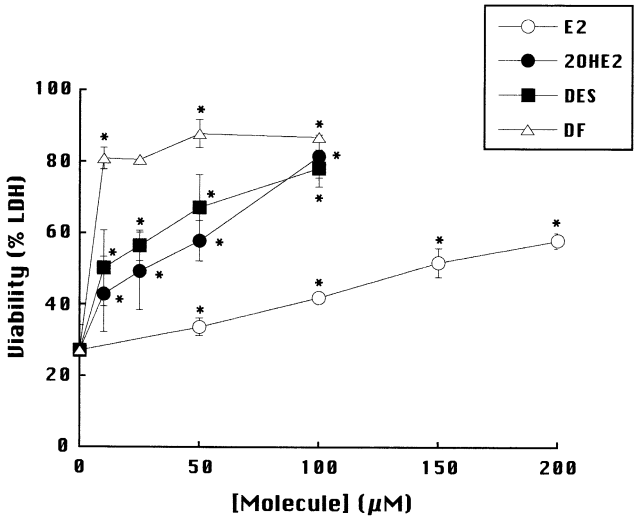

FIG. 2. Effects of E_2, $2OHE_2$, DES, and DF on TBHP-induced cell death. Hepatocytes (2×10^6 cells/mL) resuspended in Dulbecco's minimum Essential medium, pH = 7.4, plus 10 mM of HEPES and 2% BSA were incubated for 30 min in the presence of 1 mM of TBHP and the molecules assayed at the indicated final concentrations. Values are the means ± SEM of 3 separate hepatocyte preparations. *P < 0.01 (ANOVA).

DISCUSSION

TBHP is often used as a model compound to induce oxidative stress in cell systems. It has been described that low concentrations (<1 mM) of TBHP kill hepatocytes by a mechanism that depends on lipid peroxidation, while high concentrations (>1 mM) induce cell death by a lipid peroxidation-independent mechanism [9]. In the present study, we have used 1 mM of TBHP and have seen that hepatocyte injury (measured as LDH leakage) was preceded by the generation of TBARS and by intracellular GSH depletion. Both mechanisms seem to mediate TBHP-induced toxicity in hepatocytes. Depletion of intracellular

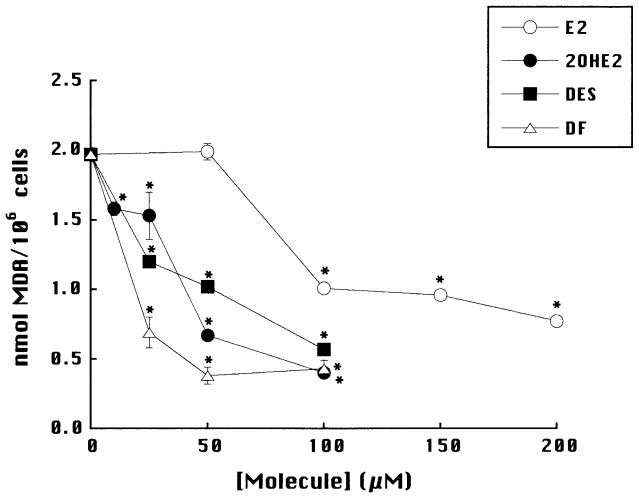

FIG. 3. Effects of E_2, $2OHE_2$, DES, and DF on TBHP-induced lipid peroxidation. The incubation conditions are described in the legend to Fig. 2. Values are the means ± SEM of 3 separate hepatocyte preparations. *P < 0.01 (ANOVA).

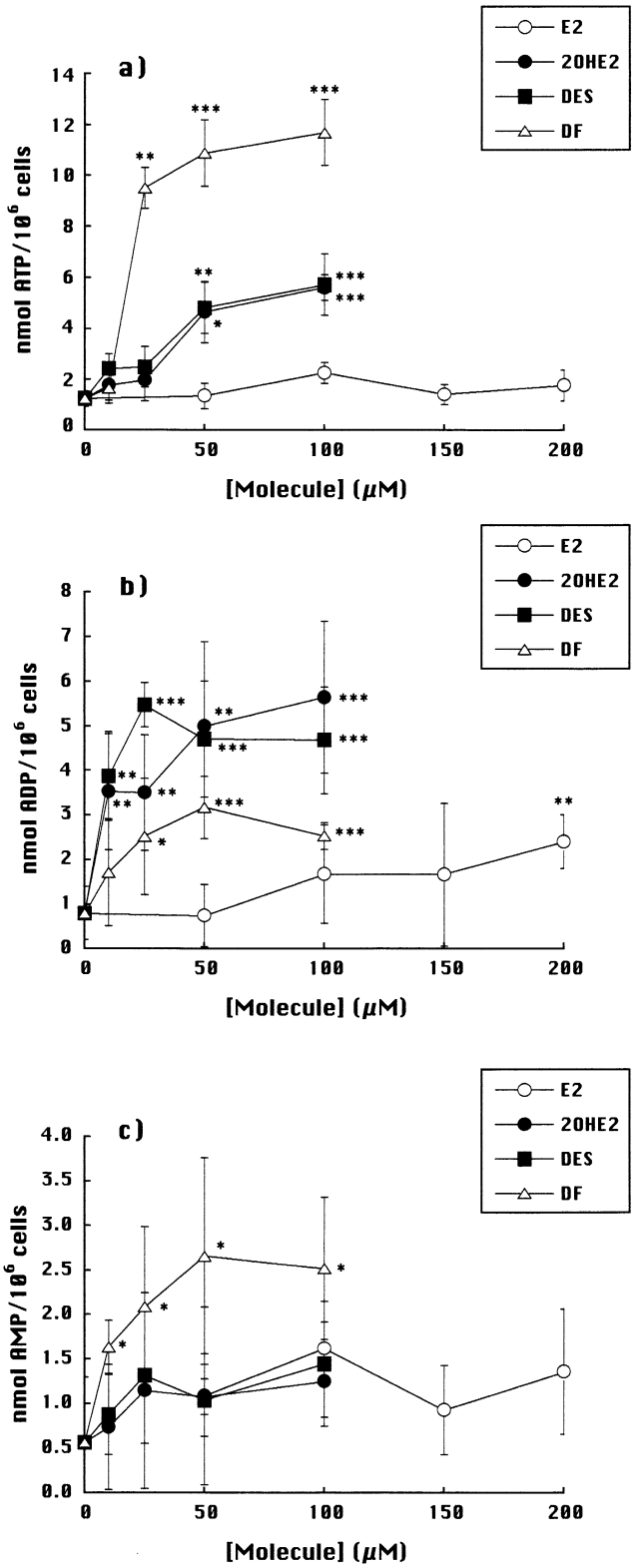

FIG. 4. Effect of E_2, $2OHE_2$, DES, and DF on the TBHP-induced depletion of (a) ATP, (b) ADP, and (c) AMP. The incubation conditions are described in the legend to Fig. 2. Data are given as means ± SEM of 3 separate hepatocyte preparations. *P < 0.05, **P < 0.025, and ***P < 0.001 (Student's *t*-test).

TABLE 1. Effects of E_2, $2OHE_2$, DES, and DF on the energy charge (ATP+1/2ADP)/(ATP+ADP+AMP) of hepatocytes exposed to 1 mM TBHP

Molecule	Energy charge
None	0.815 ± 0.01
TBHP	0.560 ± 0.02†
TBHP + E_2	0.599 ± 0.02
TBHP + $2OHE_2$	0.634 ± 0.03*
TBHP + DES	0.634 ± 0.02*
TBHP + DF	0.801 ± 0.04*

E_2, $2OHE_2$, DES, and DF were incubated for 30 min at the final concentration of 100 μM. Values are the means ± SEM of 3–4 separate hepatocyte preparations. Data were analyzed by two-way analysis of variance (ANOVA).

* $P < 0.01$ compared with the TBHP value.

† $P < 0.01$ compared with the control value (without additions).

ATP also occurred before cell death. However, no decrease in free thiol groups was found in the first 15 min, although over 45% of the cells died during this period. These findings indicate that the loss of protein thiol groups does not contribute significantly to TBHP-induced cytotoxicity.

Until now, few reports have described the beneficial effects of estrogens in intact cells. Recently, Behl *et al.* [29] reported the protective effects of high concentrations of 17β-estradiol in neuronal cells exposed to oxidative stress, with these effects ascribed to the nonreceptor-mediated antioxidant activity of the hormone [29]. The present results show that estrogens inhibit TBHP-induced hepatocyte lipid peroxidation. These results are in agreement with previous ones, observations demonstrating the antioxidant actions of these molecules in rat liver microsomes incubated with TBHP [21]. Estrogens have been shown to possess antioxidant activities under a wide variety of conditions [17–21]. In all the experimental models used, catecholestrogens were found to possess higher antioxidant capacities than phenolic estrogens [21, 30–33]. Recently, we have described that E_2 and $2OHE_2$ inhibit hepatocyte lipid peroxidation induced by ADP-chelated iron *in vitro* [34]. With this pro-oxidant system, lipid peroxidation is initiated enzymatically through the reduction of the Fe^{3+}/ADP complex by intracellular cytochrome P450 reductase.

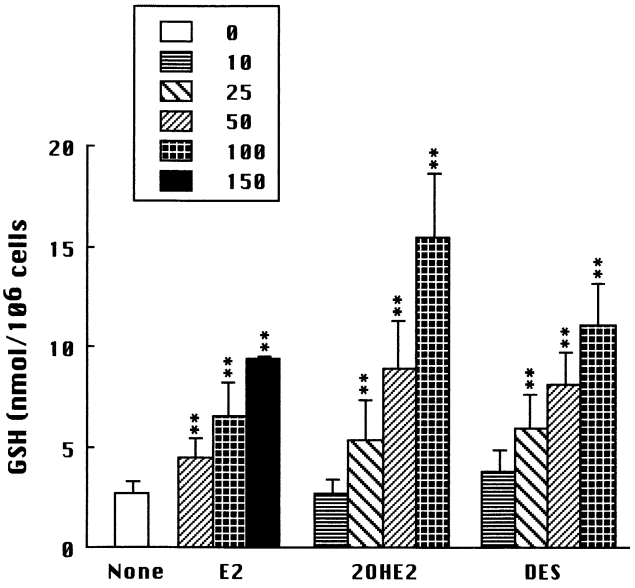

FIG. 6. Effects of E_2, $2OHE_2$, and DES on the TBHP-induced depletion of GSH. The incubation conditions are described in the legend to Fig. 2. Estrogens were incubated at the indicated μM concentrations. Data are given as means ± SEM of 3 separate hepatocyte preparations. *P < 0.05, **P < 0.01 (ANOVA).

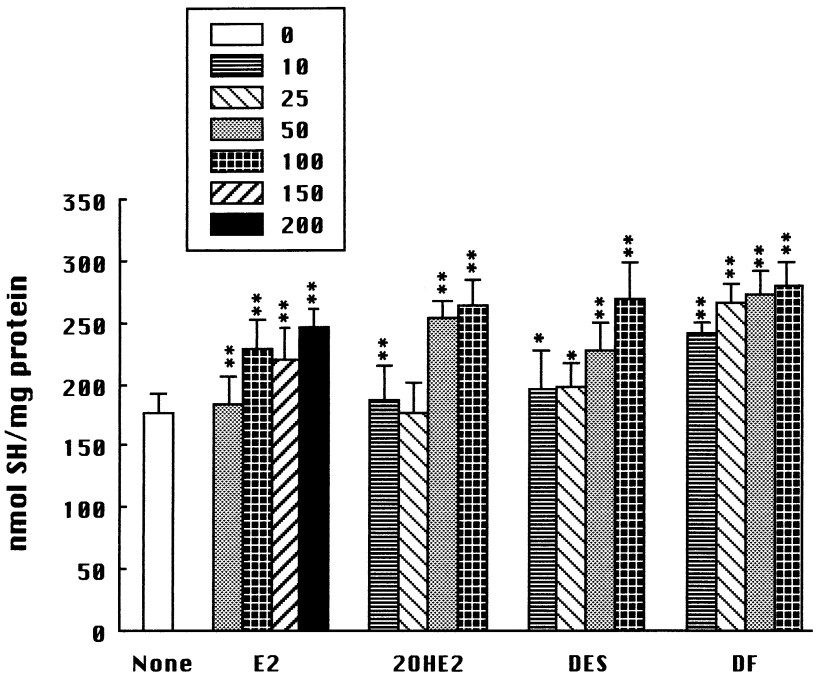

FIG. 5. Effects of E_2, $2OHE_2$, DES, and DF on the TBHP-induced decrease of protein thiols. The incubation conditions are described in the legend to Fig. 2. Estrogens and DF were incubated at the indicated μM concentrations. Data are given as means ± SEM of 3 separate hepatocyte preparations. *P < 0.05, **P < 0.01 (ANOVA).

$2OHE_2$ was also found to be a more potent inhibitor than E_2. However, this iron-induced peroxidation of hepatocyte lipids did not result in cell toxicity (measured as the loss of LDH activity). In the present study, the antioxidant capacity of estrogens, not only as efficient inhibitors of lipid peroxidation but also as good protectors of hepatocyte injury, is demonstrated. Estrogens reduced TBHP-induced cell killing associated with their effects on lipid peroxidation, and also partially decreased cell injury related to TBHP metabolization by GSH peroxidase. In this way, E_2 at the concentration of 50 μM reduced TBHP-induced cell death by 24%, without affecting lipid peroxidation and increasing the hydroperoxide-reduced GSH levels. Nevertheless, lipid peroxidation seems to greatly contribute to the hepatocyte injury induced by 1 mM of the toxicant. Thus, the more potent lipid peroxidation inhibitor $2OHE_2$ at the concentration of 10 μM reduced the TBHP-induced cell death by 58%, both decreasing lipid peroxidation and without affecting GSH depletion.

Different mechanisms of inhibition could mediate the effects of estrogens on lipid peroxidation. TBHP requires iron to initiate peroxidation, since the iron chelator DF significantly lowered TBARS production to levels found in hepatocytes at zero time or after 30-min incubation in the absence of the pro-oxidant (Fig. 3). Estrogens could act as iron chelators, as has been postulated for $2OHE_2$ [35], thus eliminating iron ions from the oxidative process. Furthermore, estrogens could also interact with cytochrome P450 [36], which in turn participates in the formation of superoxide radicals capable of mobilizing iron from ferritin, and thus inhibit the Fe^{2+}-catalyzed formation of *tert*-butoxyl radicals by a Fenton-type reaction. At the moment, it is not known whether estrogens scavenge the *tert*-butoxyl radicals derived from the hydroperoxide directly.

TBHP provoked depletion of intracellular ATP, which preceded cell death, and also decreased the energy charge. The hydroperoxide inhibits the mitochondrial respiratory chain. In cells with low mitochondrial membrane potentials, the glycolytic maintenance of ATP is an important process in preventing hepatocyte death on exposure to TBHP [37]. However, high doses of TBHP also inhibit glycolysis [37]. The 1 mM concentration of TBHP resulted in a rapid depletion of GSH (Fig. 1), which can in turn inhibit the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase, by oxidation of critical thiol groups located at the active site of the enzyme [38, 39]. In the present study, we found that $2OHE_2$ and DES prevented hepatocyte death induced by TBHP and that ATP levels were not completely depleted. These results appear to suggest that the depletion of ATP below a minimal level is a critical event in the induction of irreversible injury. E_2, however, did not restore ATP levels, although it partially prevented cytotoxicity. Therefore, hepatocyte susceptibility to TBHP injury is not dependent only on the ATP status. Cytotoxicity did not correlate with ATP levels, being more dependent on the restoration of the normal redox state of the cell. The maintenance of GSH is a critical component in protection against oxidative damage. TBHP oxidizes NAD(P)H and GSH, thus reducing the intracellular $NAD(P)H/NAD(P)^+$ ratio. In the presence of E_2, there occurred a recovery of GSH that could account for the ATP synthesis via glycolysis. E_2 increases the cellular $NADH/NAD^+$ ratio [40], probably as a result of its cytosolic metabolism by dehydrogenases, and also induces NADPH-generating systems [40, 41]. A similar mechanism of toxicity, where the maintenance of the cellular redox homeostasis rather than the ATP content is responsible for cell viability, has been described in hypoxic hepatocytes [42].

In conclusion, the results presented here demonstrate that estrogens prevent oxidant-mediated hepatocyte toxicity. These actions seem to be related to both the antioxidant activity of estrogens against free radicals (thus inhibiting lipid peroxidation) as well as the maintenance of the normal redox state of the cell.

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