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MODE OF ACTION OF BUTYLATED HYDROXYANISOLE (BHA) AND OTHER PHENOLS IN PREVENTING LOSS OF 11β-HYDROXYLASE ACTIVITY IN CULTURED BOVINE ADRENOCORTICAL CELLS

PETER J. HORNSBY,* KATHY A. ALDERN and SANDRA E. HARRIS
Department of Medicine, University of California, San Diego, School of Medicine, La Jolla, CA 92093, U.S.A.

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Abstract—When cultured bovine adrenocortical cells are incubated with cortisol, or other steroids that are pseudosubstrates for 11β-hydroxylase (cytochrome P-450$_{11\beta}$), the activity of the enzyme decreases. In previous experiments, three substances were shown to protect 11β-hydroxylase against loss of enzymatic activity in the presence of pseudosubstrates: BHA (butylated hydroxyanisole, 2(3)-*tert*-butyl-4-methoxyphenol), dimethyl sulfoxide (DMSO), and metyrapone. The present experiments examine the protective effects of several phenolic analogs of BHA in this system, and compare their activities to that of DMSO and metyrapone. When a variety of analogs of BHA were tested for their abilities to prevent loss of 11β-hydroxylase activity in cultured adrenocortical cells incubated with 50 μM cortisol for 24 hr, phenol itself was found to be about equipotent with BHA. Addition of methyl, methoxy and benzyl groups to phenol did not diminish protective activity of the compound, but addition of one and particularly two *tert*-butyl groups greatly diminished activity. Thus, BHT (2,6-di-*t*-butyl-4-methylphenol) was inactive, in contrast to BHA. The hydroxy group of phenol was essential since benzene and fluorobenzene were inactive. Compounds with multiple hydroxyl groups were not as active as phenol itself, with the exception of catechol. No products of phenol formed during incubations of cells with cortisol were detected by high performance liquid chromatography. Estimated EC_{50} values for protection of 11β-hydroxylase by phenols were about 100 μM, whereas the EC_{50} values for dimethyl sulfoxide and metyrapone were 10 mM and 300 nM respectively. On a semilogarithmic plot, the dose–response curves for all these compounds were approximately parallel. To aid in determining the mechanism of protection of 11β-hydroxylase, phenols and DMSO were tested for prevention of loss of 11β-hydroxylase activity at three different oxygen concentrations (2, 5, and 19% O_2). Lowering the oxygen concentration itself resulted in a small diminution of the loss of 11β-hydroxylase. Phenols and dimethyl sulfoxide were more effective at low oxygen and less effective in air. Because the cytochrome P-450 inhibitor metyrapone was found previously to be very effective in protecting 11β-hydroxylase against loss of activity, we examined whether phenols and dimethyl sulfoxide may act by directly inhibiting 11β-hydroxylase activity. In a 1-hr incubation with cells, BHA, phenol, and dimethyl sulfoxide all inhibited 11β-hydroxylase, but at concentrations that ranged from 4- to >100-fold higher than those required for protection. In contrast, for metyrapone, the EC_{50} values for protection and inhibition were very similar. These results indicate that it is unlikely that phenols act simply as inhibitors but may need to bind close to the active site of the enzyme. The observed synergism with lowered oxygen suggests an involvement, in the loss of 11β-hydroxylase activity, of oxygen-centered radicals that may be reactants for protective phenols.

When cultured bovine adrenocortical fasciculata-reticularis cells are incubated with cortisol or several other adrenal steroids, the activity of 11β-hydroxylase (cytochrome P-450$_{11\beta}$) decreases [1]. The half-life of the enzyme activity is reduced from 40 hr to 9 hr in the presence of 30 μM cortisol [1]. All the steroids found to decrease the half-life of 11β-hydroxylase have an 11β-hydroxy group or no substituent at position 11 of the steroid nucleus [1]. Steroids with an 11α-hydroxy group or an 11-keto group are ineffective. Thus, it has appeared to be necessary for steroids to interact with the enzyme substrate site in order to cause loss of activity. Products of the enzyme were hypothesized to act as pseudosubstrates [1, 2]. Pseudosubstrates are compounds that bind to the enzyme substrate site and cause enzyme reduction and oxygen binding but cannot be hydroxylated by the enzyme. Because the loss of 11β-hydroxylase activity when adrenocortical cells are incubated with cortisol is reduced when the concentration of oxygen is lowered or when antioxidants are included in the medium, an oxygen radical produced by the enzyme, resulting from interaction with pseudosubstrates, appeared likely to cause the loss of activity [1, 2]. We have reviewed elsewhere the possible mechanisms of generation of such radicals in biological systems generally and by cytochrome P-450 specifically [3]. Two substances were identified as offering substantial protection against loss of enzymatic activity: BHA (butylated hydroxyanisole, 2(3)-*tert*-butyl-4-methoxyphenol) and dimethyl sulfoxide (DMSO). It was suggested that BHA acts to inhibit lipid peroxidation (i.e. reacts with lipoperoxy radicals) and that DMSO scavenges hydroxyl radicals, formed as a secondary product of O_2^- released from P-450$_{11\beta}$ [1–3]. The present experiments investigated in more detail the

* Author to whom all correspondence should be addressed.

mechanism of action of BHA and similar compounds as protectors for 11β-hydroxylase in bovine adrenocortical cells incubated with cortisol. The activities of several phenolic analogs of BHA were compared with the activities of dimethyl sulfoxide and metyrapone. It was demonstrated that BHA probably does not act as an antioxidant as such, because the order of potencies of the protective phenolic compounds did not correspond to the known order of reactivities of these substances with peroxy radicals. In previous experiments, we showed that the cytochrome P-450 inhibitor metyrapone is effective at low concentrations in preventing loss of 11β-hydroxylase activity in adrenocortical fasciculata-reticularis cells [4] and in preventing loss of activity in both 11β-hydroxylase and corticosterone methyl oxidase (cytochrome P-450_{CMO}) in adrenocortical glomerulosa cells [5, 6]. The latter enzyme, which converts corticosterone to aldosterone, probably by a double hydroxylation at C-18 [2], has been shown to be subject to a similar process of inactivation in the presence of pseudosubstrates [2, 5, 6]. Although we previously demonstrated that BHA and DMSO do not significantly inhibit 11β-hydroxylase at concentrations that offer protection against loss of 11β-hydroxylase activity, it appeared important to recheck whether protective substances may act as 11β-hydroxylase inhibitors.

MATERIALS AND METHODS

Preparation of primary bovine adrenocortical fasciculata-reticularis cultures. Primary cultures of bovine adrenocortical cells were prepared as follows. Cells dispersed from the inner zones (fasciculata and reticularis, excluding the aldosterone-secreting glomerulosa) of the bovine adrenal cortex tissue were prepared as previously described [7] and stored frozen in 5% DMSO until required for experiments. After cells had been thawed, they were plated in 10% fetal bovine serum in a 1:1 mixture of Ham's F-12 medium and Dulbecco's modified Eagle's medium, which was shown to be optimal for adrenocortical cell growth [8]. Cells were plated in 24-well multiwell dishes, which had been coated with 0.1 mg/ml poly-L-lysine to improve cell attachment [9], at a concentration of 10,000 cells/well. The dishes were then incubated at 37° in a humidified atmosphere of 5% O_2, 85% N_2 and 10% CO_2 in a Queue Systems (Parkersburg, WV) three-gas incubator.

Incubation of cultures with cortisol and 11β-hydroxylase assay. After 48 hr, cultures were changed to serum-free medium formulated for adrenocortical cells [8] with omission of low density lipoprotein (LDL), thrombin, ascorbic acid, selenite, and vitamin E. After an additional 24 hr, the medium was replaced with serum-free medium containing 50 μM cortisol, added from a 1000-fold concentrate in acetone. This concentration was shown previously to be maximally effective in causing loss of 11β-hydroxylase activity in a 24-hr period [1]. Additionally, the medium contained various concentrations of compounds being assessed as protective for 11β-hydroxylase. Water-soluble compounds were added from 100-fold concentrates in water; non-water-soluble compounds were added from 1000-fold concentrates in acetone. This concentration of acetone had no effect on 11β-hydroxylase or on protective effects. Prior to use, compounds were tested for effects on cellular growth rate over a 3-day period. Except as noted in the text, compounds were used only at concentrations that had no effect on the cell growth rate, a sensitive indicator of nonspecific toxicity. The incubation with cortisol and potential protective compounds was performed at 5% O_2, except when the effect of varying the oxygen concentration was being assessed. After 24 hr, the medium was replaced with serum-free medium only, and the cells were incubated for 1 hr to remove treatment substances. The medium was then replaced with serum-free medium, containing 10 μM deoxycorticosterone (DOC) as 11β-hydroxylase substrate, and incubated for an additional 1 hr. DOC conversion to corticosterone was used to assay 11β-hydroxylase rather than deoxycortisol conversion to cortisol to avoid inclusion of cortisol carried over from the pretreatment in assessment of amount of product; however, control experiments demonstrated that negligible amounts of cortisol were carried over. Previous experiments have shown that 10 μM DOC is close to the K_m for 11β-hydroxylase in this system [6]. Cell numbers were measured using a Coulter Counter (Coulter Electronics, Hialeah, FL) as previously described [10].

Steroid analysis. Steroids were extracted from the medium into 2 vol. of dichloromethane, and the dichloromethane was evaporated at 40°. Steroids were redissolved in 20% acetonitrile and chromatographed using an Altex 320 high performance liquid chromatograph with a Regis Hi-Chrom C_{18} silica column. A gradient of 20–100% acetonitrile over 10 min was used to separate DOC from corticosterone. The identity of u.v.-absorbing peaks was established by comparison of retention times of authentic standards. Conversion rates were calculated by comparing peak heights with peak heights of known amounts of standards, calculating percent conversion product, and converting percent conversion to pmoles from the known amount of precursor added. Conversion rates were expressed as pmoles per 10^4 cells per hr.

Metabolism of phenol and aniline. In experiments on metabolism of [^{14}C]phenol and [^{14}C]aniline, cells were incubated with 10 μM phenol (1 μCi) or 10 μM aniline (1 μCi) for 4 hr. The medium was removed and stored frozen until analysed. For analysis, the medium was passed through a 30,000 molecular weight exclusion Amicon filter and then loaded onto a C_{18} silica loop column. Material adsorbed to the loop column was then chromatographed on a C_{18} silica column using a gradient of 0–100% acetonitrile for [^{14}C]phenol and a gradient of 0–60% methanol with 0.25 M ammonium phosphate, pH 7.0, for [^{14}C]aniline. Fractions were collected over 0.1 min (within peaks) or 0.5 min (between peaks) using a programmable fraction collector (Isco, Lincoln, NE), and radioactivity was counted in 5 ml scintillation fluid [11]. Phenol was well separated from the three possible dihydroxy products, and aniline was well separated from the three possible aminophenol products using these separation systems.

Materials. Steroids were obtained from Sigma (St.

Table 1. Prevention of loss of activity of 11β-hydroxylase by BHA and other phenols*

	11β-Hydroxylase activity (pmoles/10^4 cells/hr)
No cortisol	1210 ± 134
50 μM Cortisol	166 ± 18
+ phenol	409 ± 35†
+ 2-*t*-butylphenol	311 ± 34†
+ 2,6-di-*t*-butylphenol	210 ± 23
+ 2-methylphenol	470 ± 48†
+ 4-methylphenol	435 ± 88†
+ 2-methoxyphenol	414 ± 46†
+ 3-methoxyphenol	266 ± 39†
+ 4-methoxyphenol	424 ± 67†
+ 2-*t*-butyl-4-methylphenol	274 ± 30†
+ 2,6-di-*t*-butyl-4-methylphenol (BHT)	183 ± 15
+ 2-*t*-butyl-4-methoxyphenol (BHA)	443 ± 49†
+ 2,6-di-*t*-butyl-4-methoxyphenol	131 ± 14
+ 2-benzylphenol	490 ± 54†
+ 2-(4-hydroxybenzyl)phenol	464 ± 51†
+ benzene	200 ± 29
+ 2,6-di-*t*-butyl-4-hydroxymethylphenol	187 ± 20
+ Trolox C (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylate)	212 ± 33

* Bovine adrenocortical cell cultures were incubated with 50 μM cortisol in the presence of the indicated phenols, added at a final concentration of 100 μM. After 24 hr, 11β-hydroxylase activity was measured as described in Materials and Methods. Values shown are means ± S.D. for triplicate incubations.

† Significantly different from cortisol only value ($P < 0.05$).

Louis, MO), and solvents from J. T. Baker (Phillipsburg, NJ) except dichloromethane (Fisher, Fair Lawn, NJ). Sera were purchased from Irvine Scientific (Irvine, CA). [^{14}C]Phenol and [^{14}C]aniline were from New England Nuclear (Boston, MA). Trolox C was a gift of Dr. W. E. Scott of Hoffmann-LaRoche Inc., Nutley, NJ.

RESULTS

Phenols as protectors for 11β-hydroxylase in incubations of adrenocortical cells with cortisol. When a variety of phenols were compared with BHA for their abilities to prevent loss of 11β-hydroxylase activity in cultured bovine adrenocortical fasciculata-

Table 2. Effect of polyhydric phenols on loss of 11β-hydroxylase activity*

	11β-Hydroxylase activity (pmoles/10^4 cells/hr)
No cortisol	1040 ± 160
50 μM Cortisol	122 ± 17
+ phenol, 100 μM	356 ± 44†
+ phenol, 10 μM	260 ± 32†
+ 1,2-dihydroxybenzene (catechol), 100 μM	334 ± 41†
+ 1,3-dihydroxybenzene (resorcinol), 10 μM	Toxic
+ 1,4-dihydroxybenzene (hydroquinone), 10 μM	119 ± 17
+ 1,4-dihydroxy-2-*t*-butylbenzene (*t*-butylhydroquinone), 10 μM	152 ± 19
+ 1,2,3-trihydroxybenzene (pyrogallol), 100 μM	166 ± 20
+ 1,3,5-trihydroxybenzene (phloroglucinol), 10 μM	88 ± 21
+ benzoate, 100 μM	109 ± 13
+ 4-hydroxybenzoate, 100 μM	122 ± 15
+ 3,4-dihydroxybenzoate (protocatechuate), 100 μM	110 ± 13
+ 3,4,5-trihydroxybenzoate (gallate), 100 μM	139 ± 17

* Bovine adrenocortical cultures were incubated with 50 μM cortisol in the presence of the indicated phenols, added at a final concentration of 100 μM. After 24 hr, 11β-hydroxylase activity was measured as described in Materials and Methods. Values shown are means ± S.D. for triplicate incubations.

† Significantly different from cortisol only value ($P < 0.05$).

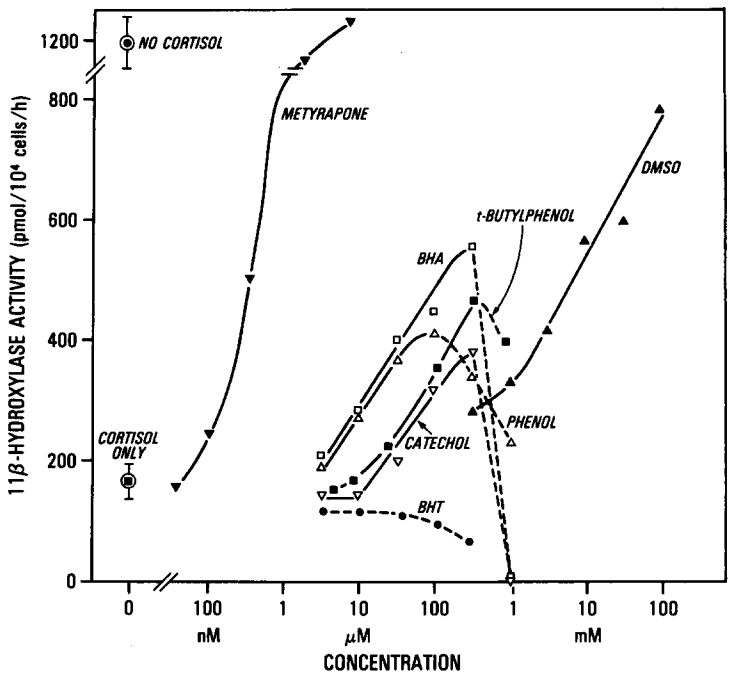

Fig. 1. Comparison of prevention of loss of 11β-hydroxylase by metyrapone, phenols and DMSO: Dose–response curves. Cortisol (50 μM) was added to primary bovine adrenocortical cells cultured in a serum-free defined medium together with various concentrations of the indicated substances. After 24 hr, compounds were removed and 11β-hydroxylase activity was measured as described in Materials and Methods.

reticularis cells incubated with the 11β-hydroxylase psdudosubstrate cortisol, phenol itself was about equipotent with BHA (Table 1). Addition of methyl, methoxy and benzyl groups to the phenol structure did not decrease its activity, but addition of one and particularly two *tert*-butyl groups greatly diminished the protective effect. Thus, BHT (2,6-di-*t*-butyl-4-methylphenol) was inactive whereas BHA was active. Additionally, aniline and flurobenzene, with an amino group and a fluoro group, respectively, in place of the hydroxy, were ineffective at non-toxic concentrations. Because the hydroxy group appeared to be the only functional group on the benzene ring essential for activity in this experiment, the effect of addition of multiple hydroxy groups to the ring was investigated (Table 2). Polyhydric phenols were not more effective than phenol in preventing loss of 11β-hydroxylase activity, and some were too toxic to test at high concentrations. Catechol was the only compound with activity close to that of phenol.

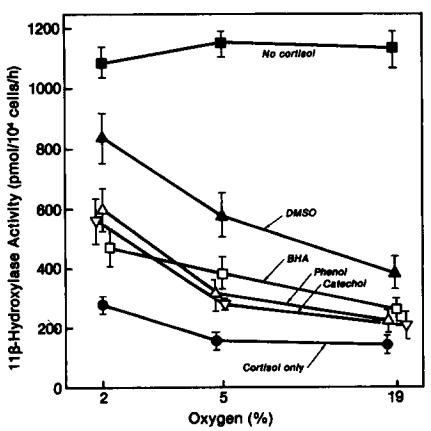

Fig. 2. Prevention of loss of 11β-hydroxylase activity by DMSO and phenols at different oxygen concentrations. Experiments were performed as in Fig. 1 except that the oxygen concentration was that indicated on the abscissa. The assays of 11β-hydroxylase were all performed at 5% O_2. Concentrations used: dimethyl sulfoxide, 100 mM, phenol, 100 μM, BHA, 100 μM, catechol, 100 μM.

Comparison of protection of 11β-hydroxylase by phenols and DMSO: Dose–response curves. Representative phenols were compared with DMSO as protectors for 11β-hydroxylase in the presence of cortisol. Additionally, the effects of the cytochrome P-450 inhibitor, metyrapone, which we showed previously to be an effective protector of 11β-hydroxylase, were compared to those of the other compounds. Substances were used over a range of concentrations until a cytotoxic level was reached (Fig. 1). Phenols were not toxic at concentrations lower than 1 mM. DMSO provided a greater absolute level of protection than the phenols, but the phenols were active at much lower concentrations. Phenols (BHA, phenol, catachol) continued to protect 11β-hydroxylase from loss of activity when used at concentrations up to about 300 μM. DMSO continued to provide increased protection up to 100 mM. Metyrapone was active at much lower concentrations, with complete protection of 11β-hydroxylase at a concentration of 1 μM. On a semilogarithmic plot, the dose–response curves for all compounds were approximately parallel.

Effect of the oxygen concentration on protection of 11β-hydroxylase by phenols and DMSO. To aid in determining the mechanism of action of phenols and DMSO in preventing loss of 11β-hydroxylase activity in the presence of cortisol, these substances were tested as protectors for 11β-hydroxylase at three different oxygen concentrations (2% and 19% O_2 as well as 5% O_2). Cultures were incubated with phenols or DMSO together with 50 μM cortisol at these three different gas phases. Each substance tested was used at its optimal concentration (Fig. 2). Figure 2 shows that lowering the oxygen concentration itself resulted in a small protective action on 11β-hydroxylase activity. Additionally, all of the tested substances were more effective in protection of 11β-hydroxylase at low oxygen and less effective in air.

Protective compounds as potential direct inhibitors of 11β-hydroxylase. Previously, we demonstrated that the 11β-hydroxylase inhibitor metyrapone protects effectively against loss of 11β-hydroxylase and corticosterone methyl oxidase activities in the presence of pseudosubstrates. However, the other two substances previously identified as effective protectors of 11β-hydroxylase, BHA and DMSO, were shown not to be direct inhibitors of 11β-hydroxylase at concentrations that prevented loss of 11β-hydroxylase activity (100 μM and 10 mM respectively) [1]. In contrast, metyrapone, at a concentration that was shown to offer protection against loss of 11β-hydroxylase activity (5 μM), showed almost complete direct inhibition of 11β-hydroxylase activity [4]. In the present experiments, the possible direct inhibitory effects of phenol, BHA, and DMSO were investigated in more detail and compared to the inhibitory effect of metyrapone (Fig. 3). Adrenocortical cells after 3 days in culture were incubated for 1 hr in serum-free medium containing the indicated concentrations of phenol, BHA, metyrapone, or DMSO. Both BHA and phenol were directly inhibitory to 11β-hydroxylase, but at higher concentrations than those required for protection of the enzyme during incubation with cortisol (for BHA, the EC_{50} for inhibition was 600 μM compared with 300 μM for protection; for phenol, the EC_{50} for inhibition was 3 mM compared with 300 μM for protection). In contrast, the EC_{50} values for protection and inhibition of 11β-hydroxylase in the case of metyrapone were approximately equal (~250 nM for both actions).

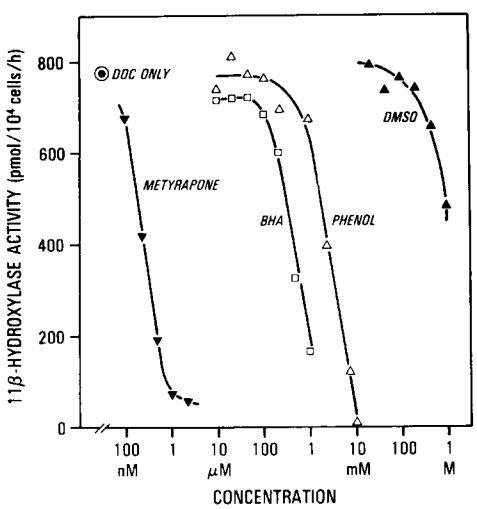

Fig. 3. Direct inhibition of 11β-hydroxylase by metyrapone, BHA, phenol and DMSO. Primary adrenocortical cells were cultured for 3 days in serum-free medium. The cells were then incubated for 1 hr in serum-free medium containing the indicated concentrations of these substances together with 10 μM DOC as 11β-hydroxylase substrate. Conversion to corticosterone was then measured as described in Materials and Methods.

Metabolism of [^{14}C]phenol by adrenocortical cells. To assess whether phenol was metabolized to a product during incubation with cultured bovine adrenocortical cells and cortisol, cultured cells were incubated for 4 hr with 10 μM [^{14}C]phenol with or without 50 μM cortisol. Incubations both with and without cortisol demonstrated limited metabolism (~1%) of phenol to one polar product eluting prior to catechol and the other dihydroxybenzenes. The extent of metabolism of phenol to this product was unaffected by the presence of cortisol. Dependence of formation of this product on intact cell function was demonstrated by showing that no product was formed in incubations of [^{14}C]phenol with serum-free medium only. Additionally, adrenocortical cells were incubated with [^{14}C]aniline, in the presence or absence of cortisol. Aniline has been shown to be metabolized by cytochrome P-450 by a free hydroxyl radical mechanism [12, 13]. As with [^{14}C]phenol, no cortisol-dependent metabolite of aniline was detected.

DISCUSSION

In bovine adrenocortical fasciculata-reticularis cells in long-term culture, the ability of ACTH or cyclic AMP to induce the activity of 11β-hydroxylase (cytochrome P-$450_{11\beta}$) declines greatly over the first 2–3 passages [1, 14, 15]. The loss of the enzyme activity is associated with accumulation in the culture medium of stimulated steroids, such as cortisol, which appear to act as pseudosubstrates for 11β-hydroxylase [1, 2]. Two substances were identified as offering protection against loss of activity of 11β-hydroxylase in the presence of pseudosubstrates: BHA and dimethyl sulfoxide. In the present experiments, we investigated in more detail the mode of action of BHA and other phenols in protecting 11β-hydroxylase activity in the presence of the pseudosubstrate cortisol. The results indicate that BHA did not react with free lipoperoxy radicals in a peroxidation chain reaction [3], because the order of potencies of the phenolic compounds as 11β-hydroxylase protectors does not correspond to the known order of reactivity of these substances with peroxy radicals. An alternative model is proposed in which a bound oxygen-centered radical is reduced by the identified protective compounds, acting as hydrogen donors.

Many cytochrome P-450s are subject to destruction or inactivation when they metabolize certain substrates or interact with certain pseudosubstrates [3]. Free radical mechanisms often appear to be involved, with release of superoxide, formation of $OH^{\cdot}$, and initiation of lipid peroxidation [3]. Loss of cytochrome P-450 appears to be a side-reaction

resulting from the peroxidase activity of cytochrome P-450; breakdown of peroxides by cytochrome P-450 results in formation of destructive radicals that attack the heme moiety. A general property of hemeproteins is the ability to act as a peroxidase [16–19]. When homolytic cleavage of the peroxide bond occurs on interaction of hemeproteins with peroxides, radicals are formed which give rise to a variety of phenomena, such as quasi-lipoxygenase activity [20, 21], facilitation of lipid peroxidation [22, 23], cooxygenation of certain substrates [24–30], and attack of the radicals on the heme moiety with formation of a distinctive class of damage products and loss of activity of the hemeprotein [31–38]. These phenomena are established for hemoglobin, peroxidases, and prostaglandin synthetase, as well as cytochrome P-450. The adrenocortical mitochondrial cytochrome P-450$_{11\beta}$ appears to be subject to a similar type of destruction [39–41]. In the interaction of hemeproteins with peroxides, various substances that are able to serve as hydrogen donors for peroxidase react with radicals formed, compete with the heme moiety as target for attack by the radicals, and thus protect the hemeprotein from degradation [16, 34, 37, 42]. Phenols are good hydrogen donors for peroxidase [43, 44] and are observed to protect hemeproteins in this way. The protective action of phenols on prostaglandin synthetase has been well-studied [45–47]. Phenols that protect hemeprotein enzymes apparently must have the properties of a hydrogen donor and also have access to the active site of the enzyme (the site of generation of the radical resulting from peroxidase activity [16, 42]). Thus, such compounds may show biphasic effects, protecting the enzyme at low concentrations and inhibiting at higher concentrations [46, 48].

In the present experiments, when a variety of phenols were compared with BHA for activity in preventing loss of 11β-hydroxylase activity, phenol itself was about equipotent with BHA. Addition of methyl, methoxy and benzyl groups to phenol did not diminish its activity, but addition of one and particularly two *tert*-butyl groups greatly diminished activity. Thus, although BHA was active, BHT was inactive. The essential feature of the protective phenols is the phenol structure without the steric hindrance provided by two *tert*-butyl groups. Polyhydric phenols were not more effective than phenol itself. Apparently, addition of hydroxy groups to phenol generally lowers its activity. Thus, low hydrophilicity may be necessary for activity.

Phenol is not a good primary antioxidant [49–51]. Although phenol reacts with peroxy radicals, the resultant phenoxy radical is not stabilized and reacts readily with other hydrogen donors [52, 53]. Phenoxyl radical stabilization and the production of a good antioxidant require the addition of electron-withdrawing groups on the benzene ring [50, 54–57]. Thus, phenols do not appear to act as antioxidants in protecting 11β-hydroxylase.

It is useful to have an internal control that phenols are capable of acting as antioxidants in this cell system. Such an internal control is provided by experiments previously reported on substances capable of preventing toxicity of aminooxyacetate in bovine adrenocortical cells. The phenomenon of aminooxyacetate toxicity is discussed in detail elsewhere [58]; it is hypothesized that the autotoxidizable cellular constituent that is protected by the antioxidants is ubiquinone. BHA effectively prevents toxicity of aminooxyacetate, whereas phenol is inactive here [58]. For prevention of aminooxyacetate toxicity, the order of effectiveness of phenolic compounds is apparently the same as that for chemical antioxidant potency [58]. In the present experiments, we investigated the activity for protection of 11β-hydroxylase of a group of phenols that had been tested previously for activity in prevention of toxicity of aminooxyacetate, and the relative effectiveness of these substances in the two sets of experiments is tabulated in Table 3. The order of effectiveness of the phenols in the two systems is entirely different. The data of Table 3 were obtained using the same cell type, and the location of the putative target molecules (the inner mitochondrial membrane) is the same. This provides a control that inactive compounds were not ineffective simply because they did not have access to the target molecules. Among the simple phenolic substances, prevention of aminooxyacetate toxicity is made more effective by the presence of ortho *tert*-butyl groups and a para methyl hydroxymethyl or methoxy group. In the case of 11β-

Table 3. Comparison of phenols in protection of 11β-hydroxylase versus prevention of toxicity of aminooxyacetate*

	11β-Hydroxylase	Aminooxyacetate
Phenol	+++	−
2-*tert*-Butyl-4-methoxyphenol	+++	+++
2-(4-Hydroxybenzyl)phenol	+++	++
2-*tert*-Butylphenol	++	+
2,6-di-*tert*-Butylphenol	−	+
2,6-di-*tert*-Butyl-4-methylphenol	−	++
2,6-di-*tert*-Butyl-4-hydroxymethylphenol	−	+++
Trolox C	−	+
α-Tocopherol	−	++++

* Comparison of effectiveness of phenols in prevention of loss of 11β-hydroxylase activity in the presence of pseudosubstrates and in prevention of toxicity of aminooxyacetate, both in cultured bovine adrenocortical cells. 11β-Hydroxylase data are from the present experiments. Aminooxyacetate data are from Ref. 58. Key: (−) indicates not effective; (+) to (++++) indicate effective, with increasing potency.

hydroxylase, the para substituents did not decrease effectiveness. One ortho *tert*-butyl somewhat decreased effectiveness and two *tert*-butyl groups completely blocked it. Since ortho *tert*-butyl groups in antioxidants act to stabilize the phenoxyl radical and thus lower chain propagation [50, 51, 54], it is likely that stability of this radical is not important in 11β-hydroxylase protection. However, two ortho *tert*-butyl groups apparently provide steric hindrance that prevents interaction of the compounds with the radical involved. This suggests that the radical is not freely accessible to these substances and, so, may be bound. Although protective compounds do not appear to act by scavenging free peroxy radicals, i.e. do not appear to act as antioxidants, an oxygen radical nevertheless appears to be involved because of the synergism of the effective compounds with a lowered concentration of oxygen.

Apparently the hydrogen-donating capacity of phenol is required, since benzene, fluorobenzene, and aniline were ineffective, but a stabilized phenoxyl radical is not. It is possible that the phenoxyl radical resulting from reaction of phenol with the enzyme-generated radical reacts with other cellular components (e.g. thiols [3]) with regeneration of phenol. This may account for the absence of a detected product of phenol or of aniline in the presence of pseudosubstrates.

Previously we demonstrated that 11β-hydroxylase inhibitors, particularly metyrapone, protect very effectively against loss of 11β-hydroxylase and corticosterone methyl oxidase activities in the presence of pseudosubstrates. Additionally, we demonstrated that BHA and DMSO do not significantly inhibit 11β-hydroxylase, under the conditions previously employed, at concentrations that offer protection against loss of activity. In contrast, the inhibitor, metyrapone, at a concentration that does offer protection against loss of 11β-hydroxylase activity (5 μM) inhibited activity almost completely. Inhibitors may protect cytochrome P-450 against degradation by preventing peroxidase activity and formation of destructive radicals [59]. Thus, certain compounds may exert a protective action both by scavenging of radicals and by inhibition of peroxidase activity.

The significance of these observations on protection of a cytochrome P-450 activity by phenols may lie in comparison of this phenomenon with observations on a destructive radical formed by the peroxidase activity of the prostaglandin synthetase enzyme [45, 47, 60–65]. This radical has been studied by ESR and is produced apparently by arachidonic acid hydroperoxides [66]. Unless scavenged it causes destruction of the heme of the prostaglandin synthetase. Phenol is an effective scavenger and protects the enzymes [45, 46]. The radical appears to be an oxy radical bound to the enzyme. By analogy, the radical causing loss of 11β-hydroxylase may be similar. Effective phenols would need to have access to the active enzyme site and would be restricted from access by steric hindrance. This may account for their activity both as protectors of the enzymes and as enzyme inhibitors. It is probable that alteration of the activity of the prostaglandin synthetase system by peroxides is important in normal cell regulation [45–47, 60–66]. We have reviewed elsewhere the possible role of regulation of cytochromes P-450 by peroxides in the aging and zonation of the adrenal cortex [2].

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