Inhibition by 2(3)-tert-Butyl-4-hydroxyanisole and Other Antioxidants of Epidermal Ornithine Decarboxylase Activity Induced by 12-O-Tetradecanoylphorbol-13-acetate

Walter J. Kozumbo, John L. Seed, and Thomas W. Kense1

Departments of Environmental Health Sciences [W. J. K., T. W. K.] and Immunology and Infectious Diseases [J. L. S.], School of Hygiene and Public Health, Johns Hopkins University, Baltimore, Maryland 21205

ABSTRACT

The relationship between reactive oxygen and/or free radical species and tumor promotion was evaluated by investigating the inhibitory effects of 2(3)-tert-butyl-4-hydroxyanisole (BHA) and other antioxidants on the induction of ornithine decarboxylase (ODC) activity in mouse epidermis by a tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA). Mice maintained on a diet containing 0.75% BHA for 8 days showed a 50% reduction in maximal ODC induction following treatment with TPA when compared to mice fed a control diet. Topical application of BHA (55 μmol) 30 min prior to TPA treatment (17 nmol) elicited an 80% inhibition of promoter-induced ODC activity. BHA was ineffective as an inhibitor when administered either 18 hr before or 2 hr after the promoter. The inhibition by BHA was dose dependent with a dose producing a 50% inhibition of ODC induction of 6 μmol. A structure-activity study with BHA analogues (2-tert-butyl-4-hydroxyanisole, 3-tert-butyl-4-hydroxyanisole, 2-tert-butyl-1,4-dimethoxybenzene, tert-butylhydroquinone, 4-hydroxyanisole, p-hydroquinone, phenol, and 2-tert-butylphenol) showed that hydroxyl and tert-butyl substituents were important determinants of inhibitory activity. A spectrum of other antioxidants were also tested. Butylated hydroxytoluene was nearly equipotent to BHA; α-tocopherol, propyl gallate, and disulfiram were all less potent, and L-ascorbate was inactive. None of the antioxidants affected basal ODC activity in non-TPA-treated mice. Collectively, these results demonstrate an early and direct inhibition of TPA-induced ODC activity by lipophilic phenolic antioxidants and suggest a role for reactive oxygen and/or free radical species in tumor promotion.

INTRODUCTION

Phorbol diester tumor promoters act early and directly on the plasma membrane to trigger a host of biochemical and cellular responses that can be used to characterize and study the process of tumor promotion (1–3, 9). One of the earliest responses to TPA2 may be the generation of reactive oxygen species. Human polymorphonuclear leukocytes undergo a rapid burst of oxidative metabolism following exposure to TPA (13, 23). In these cells, the stimulation of both superoxide anion production and reactive oxygen-generated chemiluminescence by phorbol diester analogues correlates well with the tumor-promoting activities of these compounds (15). Antagonists of promotion, such as protease inhibitors, indomethacin, and retinoic acid, also inhibit reactive oxygen metabolism in polymorphonuclear leukocytes (12, 15). Free radical involvement in tumor promotion is more directly supported by studies showing that free radical generators, such as benzoyl peroxide and lauroyl peroxide, are also tumor promoters in mouse epidermis (26). In addition, TPA as well as nonphorbol tumor promoters produce a rapid and selective decrease in epidermal superoxide dismutase and catalase activity (27), the major detoxifying enzymes for oxygen radicals. This response may result in elevated levels of superoxide anion, hydrogen peroxide, and other higher energy radicals, such as hydroxy radical. Viewed collectively, such evidence implies a possible obligatory role for reactive oxygen and/or free radical species in the process of promotion.

Therefore, antioxidants, with their capacity to terminate free radical chain reactions, would be expected to modify the promotion process. Since BHA is a relatively nontoxic antioxidant and potent inhibitor of tumor promotion (25), we examined the effects of this compound as well as other antioxidants on the induction of ODC activity by TPA in mouse epidermis, an in vivo system that allows direct application of antioxidants to the target tissue. Because ODC induction is often viewed as a necessary event in tumor promotion and occurs within hr after TPA exposure (20), it represents a relatively reliable and yet early indicator of promoter activity. Thus, epidermal ODC induction should offer a useful first approach to the more direct and immediate in vivo effects of antioxidants on tumor promotion and, at the same time, provide further evidence for free radical involvement in promoter activity.

MATERIALS AND METHODS

Animals. Female CD-1 mice (7 to 9 weeks old) were obtained from Charles River Breeding Laboratories (Wilmington, Mass.) and were housed in an air-conditioned room with a light period from 6 a.m. to 6 p.m. Purina laboratory chow (Ralston-Purina Co., St. Louis, Mo.) and water were available ad libitum. Commercial BHA, which is a mixture of 95% 3-isomer and 5% 2-isomer, was incorporated into powdered chow by mixing thoroughly to a final concentration of 7.5 g/kg. Dorsal hair was shaved with surgical clippers 2 days before experimentation; only those mice not exhibiting hair growth were used. TPA (17 nmol) and antioxidants were dissolved in acetone and applied to the shaved area in a volume of 200 μl.

Chemicals. TPA, BHA, BHT, disulfiram, propyl gallate, L-ascorbate, α-tocopherol, and p-hydroquinone were purchased from Sigma Chemical Co. (St. Louis, Mo.); and phenol, 2-tert-butylphenol, and octanol were from Aldrich Chemical Co. (Milwaukee, Wis.). 3-BHA, 2-BHA, methyl-

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; BHA, 2(3)-tert-butyl-4-hydroxyanisole; ODC, ornithine decarboxylase; BHT, 3,5-tert-butyl-4-hydroxytoluene; 3-BHA, 3-tert-butyl-4-hydroxyanisole; 2-BHA, 2-tert-butyl-4-hydroxyanisole; methyl-BHA, 2-tert-butyl-1,4-dimethoxybenzene; t-BHQ, tert-butylhydroquinone; ID₅₀, dose producing a 50% inhibition of ornithine decarboxylase induction.

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BHA, t-BHQ, and 4-hydroxyanisole were prepared or purified by Hans J. Prochaska and were generous gifts of Dr. Paul Talalay. All antioxidants were recrystallized before experimental use. L-[1-14C]Ornithine hydrochloride (56 µCi/µmol) was obtained from Amersham/Searle Corp. (Arlington Heights, Ill.). Coefficients for the partitioning of phenolic antioxidants into octanol were determined as described by Leo et al. (17).

Assay of ODC. At appropriate times after treatment, mice were killed by cervical dislocation, and epidermis was isolated by brief heat treatment (19). The epidermal cells were disrupted by alternating freezing and thawing 3 times in 200 µl of 50 mM sodium phosphate (pH 7.2) containing 0.1 mM pyridoxal phosphate and 1 mM EDTA, followed by centrifugation at 12,000 × g for 3 min to obtain the soluble supernatant. ODC activity in the supernatant was determined by measuring the release of 14CO2 from L-[1-14C]ornithine hydrochloride using an Eppendorf microvessel assay system. Typically, a 10-µl aliquot of an assay mixture containing 50 mM sodium phosphate (pH 7.2), 1 mM EDTA, 0.2 mM pyridoxal phosphate, 4 mM dithiothreitol, 0.4 mM L-ornithine, and 0.125 µCi L-[1-14C]ornithine hydrochloride was placed in the apex of a 1500-µl Eppendorf vessel. The reaction was initiated by the addition of 20 µl of epidermal supernatant at the same time as a 5-µl droplet of 40% KOH was deposited on the underside of the vessel lid. The resultant assembly was closed rapidly and securely and then incubated at 37°C for 60 min, at 95°C for 2 min, and finally overnight at room temperature. At the end of these incubations, the lids were removed, and the radioactivity trapped in the alkali thereon was measured by scintillation spectrometry. Diffusion of released 14CO2 into the KOH droplet in the Eppendorf vessel assembly is quantitative (7). Assays were always performed in triplicate on supernatants prepared from individual mice. Protein content was determined as described by Bradford (4) using bovine serum albumin as standard.

RESULTS

Time Courses of TPA-induced Epidermal ODC Activity in Mice Fed BHA-supplemented or Control Diets. Mice fed control diets and treated with TPA (17 nmol) exhibited a rapid and transient induction of epidermal ODC activity (Chart 1), which increased to a peak of 18 nmol/hr/mg protein at 7 hr after phorbol ester treatment and subsided to approximately 30% of this activity by 11 hr. Mice fed previously a diet supplemented with BHA (0.75%) for 8 days displayed a similar profile of ODC activities when treated with TPA, but the levels were significantly reduced (approximately 50%) from those found in the TPA-treated group receiving acetone. Neither the mice fed antioxidant nor those on control diets responded to acetone treatment, indicating that the diets alone did not elicit an increase in ODC activity.

The possibility that BHA directly inhibited the decarboxylation of ODC was investigated by adding the test compound to the assay tube containing the supernatant from TPA-treated epidermis and then measuring ODC activity as described in “Materials and Methods” (data not shown). No inhibitory effects were observed, eliminating a direct intermolecular ODC:BHA interaction as the cause of inhibition.

Dose Response of the Inhibition of TPA-induced ODC Activity by Topically Applied BHA. To attenuate possible indirect effects of BHA on the inhibition of phorbol ester-induced ODC activity and to focus on the direct antioxidant properties of BHA, graded doses (0.055 to 55.5 µmol) were topically administered 30 min prior to phorbol ester application. As shown in Chart 2, direct topical treatment of BHA evoked a dose-related inhibitory response with an ID50 value of 6 µmol. If the ID50 value of BHA is compared to those values of 3 other inhibitors of mouse epidermal ODC induction by TPA, putrescine, indomethacin, and retinoic acid, then BHA is approximately 2, 2 × 10⁻², and 2 × 10⁻⁵ times as potent, respectively (32, 33, 36).

Effect of Time of Addition of Topically Applied BHA on TPA-induced ODC Activity. The degree of inhibition of ODC was dependent on the time of application of BHA relative to TPA treatment (Chart 3). Maximum inhibition occurred when the antioxidant was applied up to 4 hr before the promoter, and application 16 hr prior to TPA produced no inhibitory effect. Application of the BHA simultaneously with promoter, or up to 2 hr after, resulted in a reduction of the inhibition, such that the antioxidant was completely ineffective 2 hr after TPA.

Inhibitory Effects (ID50) of BHA, BHA Analogues, and Other Antioxidants on TPA-induced ODC Activity. To establish
whether the ODC-inhibitory activity is due to an antioxidant (radical-scavenging) effect or to a specific structural characteristic of BHA, antioxidants structurally unlike BHA as well as BHA analogues were tested for their inhibitory potencies. The ID50 values for each of these compounds were compared and used as an indication of their relative inhibitory potencies. The compounds listed in Chart 4 represent a group of classical but structurally dissimilar antioxidants. BHA was the most potent antioxidant examined. BHT was nearly equipotent to BHA; however, α-tocopherol, propyl gallate, and disulfiram were decreasingly less potent, and L-ascorbate was inactive. These results indicate that antioxidant character alone is insufficient to produce an inhibitory effect. A structure-activity study of BHA analogues is shown in Chart 5. In this instance, t-BHQ was the most potent analogue tested, whereas p-hydroquinone and phenol were only marginally active. The increasing potencies of methyl-BHA, 2-

tert-butylphenol, 2- or 3-BHA, and t-BHQ, respectively, show the importance of the hydroxyl substituent(s), while the relative ineffectiveness of p-hydroquinone and phenol underscores the significant contribution of the tert-butyl group. Addition of a tert-butyl moiety to p-hydroquinone and phenol results in a substantial increase in their lipophilicity, as adjudged by the octanol:H2O partition coefficients (Chart 5), and in a corresponding enhancement of their inhibitory activities. Thus, the structure-activity study implies that the hydroxyl and tert-butyl substituents of BHA together play a significant role in antioxidant-mediated antagonism of the induction of ODC by TPA.

**DISCUSSION**

Phenolic antioxidants, such as BHA, have long been recognized as effective inhibitors of carcinogenesis against numerous carcinogens in a variety of animal species and tissues (30, 34, 35) when administered before or during carcinogen exposure. Current evidence suggests that these antioxidants can modulate the initiation process by altering the metabolism of carcinogens, particularly by increasing the activities of several enzymes involved in the detoxification of electrophilic compounds (28). The alternative possibility that antioxidants serve as noncritical nucleophiles which bind electrophilic carcinogens, thus protecting critical macromolecules such as DNA, remains largely unsupported. Chan and Black (6) have suggested that feeding mice a 2% antioxidant diet containing BHT, α-tocopherol, L-ascorbate, and glutathione inhibits both the initiation and promotion stages of tumorigenesis in mouse epidermis. More recently, Slaga et al. (25) have shown that topically applied BHA acts as an effective...
antipromoter. However, the direct or indirect action of phenolic antioxidants as antipromoters has not to this point been evaluated.

The results illustrated in Chart 1 show that 8-day dietary administration of BHA (0.75%) clearly inhibited mouse epidermal ODC induction by TPA, although the mode of inhibition is unclear. These results stand in contrast to those of Peterson et al. (21) who found that 0.5% BHT in the diet was without notable effect on epidermal ODC induction by TPA. Since antioxidant-associated properties of BHA are considered relatively quick and direct acting, their evaluation was impossible with the prolonged feeding regimen. Antioxidant inhibition in the feeding study may have resulted from a BHA-mediated elevation in the endogenous antioxidant, glutathione, and not from BHA directly. To obviate such indirect effects on TPA-stimulated ODC induction, BHA was administered topically to the epidermis shortly (0.5 hr) before phorbol ester application. The resulting dose-dependent inhibition (Chart 2) suggests a direct antioxidant mode of action. Inhibition, which is maximal when BHA is applied 30 min before TPA, is markedly reduced when BHA treatment occurs 1 or 2 hr after TPA (Chart 3), underscoring the importance of BHA application antecedent to phorbol ester and suggesting that subcellular prelocalization of BHA is necessary to attenuate an early TPA-initiated event linked to ODC induction. The absence of an antagonistic effect by BHA when applied 16 hr prior to TPA treatment mitigates against such indirect effects that may be related to antioxidant metabolism or enzyme induction.

The fact that inhibitors of ODC induction, such as t-BHQ and BHA, possess hydroxyl substituents, which facilitate the transfer of electrons and augment antioxidant potential, suggests that an antioxidant component is necessary to the inhibition of ODC induction by TPA. However, at the same time, the relative inactivities of phenol, L-ascorbate, propyl gallate, p-hydroquinone, and 4-hydroxyanisole imply that antioxidant potential per se is an insufficient determinant of inhibition. The distinct inhibitory activity of methyl-BHA, which lacks antioxidant potential, may reflect its O-demethylation by epidermal cytochrome P-450 to form BHA. Furthermore, the ineffectiveness of p-hydroquinone and phenol relative to their tert-butyl derivatives highlights the important role of the tert-butyl group in the activity of the phenolic antioxidants. Since the antagonism of TPA-induced ODC is an in vivo response, the facility to translocate to the target cells and then to partition to the appropriate subcellular loci may be as important as the antioxidant properties of these phenolic inhibitors. For example, a tert-butyl substituent enhances lipophilicity (Chart 5) and conceivably promotes the delivery and/or partitioning of the phenolic compounds to sites of reactive oxygen and/or free radical generation and propagation. Probable sites could be membrane:cytoplasmic interfaces, particularly the lipid:protein complexes that are associated with superoxide generation. Plasma membrane, endoplasmic reticulum, and inner mitochondrial membrane with their associated NAD(P)H oxidase, mixed-function oxidase, and cytochrome electron transport system, respectively, are such sites of superoxide generation and free radical propagation (5, 10, 11, 24, 29). An appropriately lipophilic phenolic antioxidant, such as t-BHQ, could easily penetrate to and concentrate at these membrane:cytoplasmic interfaces where the scavenging of free radicals from superoxide-generating sites would be most efficient. In fact, studies using pulse radiolysis techniques in aqueous solution (pH 7.0) have shown that phenolic compounds in general demonstrate appreciable reactivity to superoxide-generated free radicals as well as superoxide itself (22). Concordantly, the inactivity of p-hydroquinone, phenol, and L-ascorbate could be explained by their high degrees of aqueous solubility and their corresponding lack of partitioning to these sites.

An additional consideration relating to the importance of the tert-butyl group to t-BHQ, for example, arises from the potential of alkyl substituents in general and tert-butyl in particular to donate electrons to the aromatic ring. This enhances the electron density of the ring, the degree of which depends upon the substitution pattern of the aromatic nucleus and the substituents themselves. Due to the high electron negativity of the oxygen atom of the hydroxyl substituent, the delocalized electrons of the aromatic ring or electrons from other sources within the cell (i.e., free radicals) may be attracted to the hydroxyl group. This action tends to facilitate a transfer of electrons via the hydroxyl substituent, thus serving as the focal point of flux of electrons, thereby enhancing the free radical-scavenging potential of the compound. In light of this rationale, BHT displayed a somewhat anomalous inhibitory activity, for in spite of 2 tert-butyl substituents per aromatic ring, this compound was a less effective inhibitor of ODC induction by TPA than were t-BHQ and BHA. This relative lack of potency may be due to the vicinal relationship of the dialkyl substituents to the hydroxyl group in BHT, such that the donation of electrons may be sterically hindered as is suggested by other studies (31) in which rate constants and oxidation-reduction potentials have been used to characterize the transfer process. The resultant diminution of antioxidant properties could account for a corresponding decrease in inhibitory potency. In essence, the optimal placement, number, and size of alkyl substituents relative to the hydroxyl group on the aromatic ring nucleus of phenolic compounds determine the optimal lipophilic and electron transfer properties that together are necessary for maximal inhibition of ODC induction by TPA in mouse epidermis. However, it is important to recognize that, while these properties appear to influence the inhibitory activity of phenolic compounds, they may not be the sole chemical determinants to inhibition of TPA-induced ODC activity. For example, the possibility exists that the purported inhibition of cyclooxygenase activity by phenolic antioxidants (8, 18) is related to their inhibition of ODC induction by TPA. Furthermore, the relative inactivities of propyl gallate and α-tocopherol are unexplained in spite of their lipophilic and phenolic antioxidant-like properties.

That there exists a cause-and-effect relationship between free radical scavenging by phenolic antioxidants and inhibition of ODC induction by TPA is further supported by other results from our laboratory. In one study, BHA was a potent inhibitor of TPA-stimulated chemiluminescence and cytochrome c reduction by superoxide generated by human polymorphonuclear leukocytes,4 indicating a potential for BHA to scavenge superoxide and/or other oxygen radicals. In another study, a biomimetic superoxide dismutase with antitumor promoter activity was more potent than was t-BHQ in inhibiting TPA-induced ODC activity in mouse epidermis (14). Taken together with the present results, the suggestion is made that reactive oxygen and/or free radical species could play important roles in the action of tumor promoters.

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