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Ability of *m*-Chloroperoxybenzoic Acid to Induce the Ornithine Decarboxylase Marker of Skin Tumor Promotion and Inhibition of this Response by Gallotannins, Oligomeric Proanthocyanidins, and their Monomeric Units in Mouse Epidermis *in Vivo*

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Abstract. *m-Chloroperoxybenzoic acid (CPBA) was tested for its ability to induce the ornithine decarboxylase (ODC) marker of skin tumor promotion. In contrast to benzoyl peroxide, dicumyl peroxide, and 2-butanol peroxide, 5 mg of CPBA applied twice at a 72-h interval induce ODC activity at least as much as 3 μg of 12-O-tetradecanoylphorbol-13-acetate (TPA). ODC induction peaks 36 h after a single CPBA treatment but is maximal 5 h after two applications of CPBA at a 48-h interval. The ODC-inducing activity of CPBA is dose dependent and sustained after chronic treatment. In contrast to TPA, two CPBA treatments at 12-24 h intervals produce no refractory state against ODC induction. The mechanism of ODC induction by CPBA is iron dependent. Various hydrolyzable tannins, condensed tannins (CTs) and their monomeric units remarkably inhibit the ODC response to multiple CPBA treatments. At 12 mg, gallic acid, Aleppo gall tannic acid (TA), catechin, and loblolly pine bark CT inhibit the most CPBA-induced ODC activity. Aleppo gall TA is even effective when applied several hours before CPBA. The tumor-promoting activity of CPBA and its inhibition by plant tannins remain to be evaluated.*

Because the mutagenic events of tumor initiation are irreversible, one important approach to the prevention of cancers is to characterize and inhibit the common biochemical markers of tumor promotion by different agents. Recent studies suggest that the different tumor-promoting activities of various 12-*O*-tetradecanoylphorbol-13-acetate (TPA)- and non-TPA-type agents may be related to their abilities to trigger different combinations of events stimulating or limiting tumor promotion, such as ornithine decarboxylase (ODC) induction, DNA synthesis, hydroperoxide (HPx) production, and intracellular Ca^{2+} mobilization (1,2). Reactive O_2 species (ROS) generated directly or indirectly by carcinogens and tumor promoters are implicated at all stages of skin tumorigenesis (3). ROS may play a role in tumor promotion and progression by damaging membranes, causing oxidation of DNA bases and DNA strand breaks, and inducing chromosomal aberrations. Organic peroxides and free radical (FR)-generating systems exhibit tumor-promoting activities and mimic or enhance some of the molecular events linked to tumor promotion (4-12). Benzoyl peroxide (BPx), the most studied compound, is a weak tumor promoter and a potent tumor progressor in mouse skin, but neither a tumor initiator nor a complete carcinogen (5, 13, 14). Several organic peroxides have different ODC-inducing activities and may possess additional properties that limit their tumor-promoting stimuli (11). Since overexpression of ODC is required for clonal expansion of epidermal tumor cells *in vivo* (15), a major objective is to identify the organic peroxide treatments that induce the most this enzyme and have the best chance of promoting skin tumor development. Another goal is to determine if naturally occurring polyphenolic antioxidants, which inhibit the ODC-inducing and tumor-promoting activities of TPA (16,17), can also decrease the effects of organic peroxides. Therefore, the present study was under-taken to characterize the potent ODC-inducing activity of m-chloroperoxybenzoic acid (CPBA) and

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Key Words: *m*-Chloroperoxybenzoic acid, mouse skin, ornithine decarboxylase, tumor promotion, hydrolyzable tannins, condensed tannins.

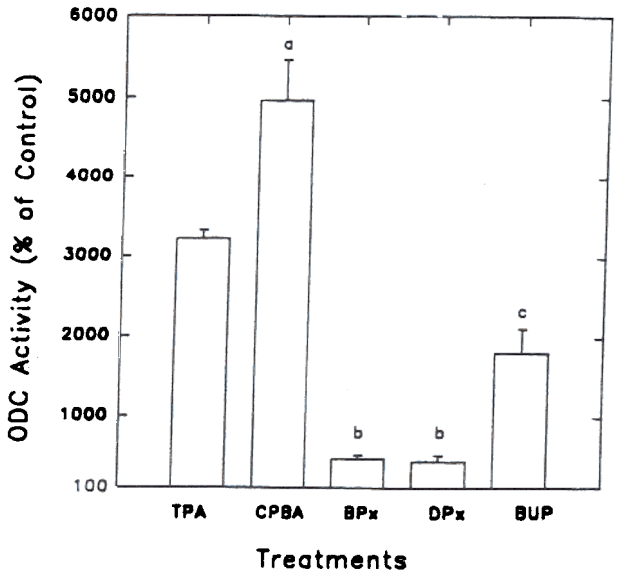

Figure 1. *Comparison of the ODC-inducing activities of TPA and organic peroxides in mouse epidermis in vivo. ODC activity was measured 5 h after the last application of two TPA (5 nmol), CPBA (5 mg), BPx (5 mg), DPx (5 mg) and BUP (5 mg) treatments at a 72-h interval. Bars: means±SD (n=4). Basal ODC activity in control mice receiving acetone only was 0. 17±0.03 nmol CO_2/h/mg protein (100±18 %). [a]P<0.01, significantly greater than TPA; [b]P<0.001, significantly greater than control; [c]P<0.005, significantly greater than BPx and DPx.*

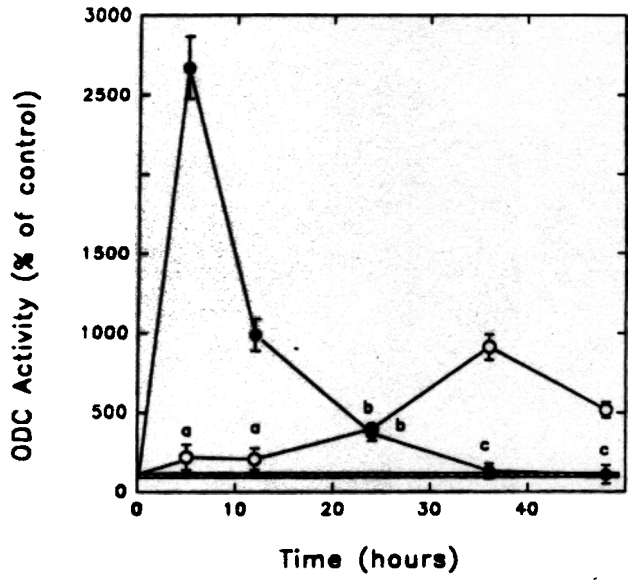

Figure 2. *Comparison of the time courses for ODC induction after a single (O) or Two applications of 2 mg of CPBA at a 72-h interval (●). Bars: means±SD (n=4). The mean value of the basal ODC activities determined at each time point in acetone-treated mice was 0.10±0.01 nmol CO_2/h/mg protein (100±10%). [a]Not significantly different from each other; [b]P< 0.01, significantly greater than 5 h and 12 h after a single CPBA treatment; [c]Not significantly different from control.*

its inhibition by hydrolyzable tannins (HTs), condensed tannins (CTs), and their monomeric units.

Materials and Methods

Treatment of mice. Female CF-1 mice (from Sasco Inc., Omaha, NE), 7 weeks old, were housed and maintained, and their dorsal skins were shaved with surgical clippers one day before experimentation. The solutions of TPA (from LC Services Corp., Woburn, MA), CPBA, BPx, dicumyl peroxide (DPx), and 2-butanol peroxide (BUP) (all from Aldrich Inc., Milwaukee, WI) were prepared in acetone and delivered to the backs of individual mice in a volume of 0.2 ml. Desferal mesylate (DSF), a gift from CIBA-GEIGY Corp. (Suffern, NY and Basel, Switzerland), was dissolved and applied topically to the skin in 0.4 ml of H_2O:EtOH:acetone (18:18:64). Aleppo gall tannic acid (TA) (from *Quercus infectoria*), sumach leaf TA (from *Rhus coriaria*), tara pod TA (from *Casealpinia spinosa*), commercial TA, gallic acid, and catechin (all from Sigma, St. Louis, MO) were dissolved and applied topically in 0.4 ml of acetone. Oligomeric CTs from guamuchil bark (*Pithecellobium dulce*), southern red oak inner bark (*Quercus falcata*), pecan nut pith (*Carva illinoiensis*), and loblolly pine bark (*Pinus taeda*) were dissolved and applied topically in 0.4 ml of H_2O:EtOH:acetone (18:18:64). Unless otherwise specified, these HTs and CTs and their monomeric units were applied 20 min before, and to the same area of skin as, each application of CPBA (16, 17). Control animals were treated with vehicle only and in each experiment all mice received the same volume of solvent.

Determination of ODC activity. The epidermis was separated from the dermis by the cold-scraping (about 4°C) method (10, 18). The epidermal preparations from two mice were pooled in 3 ml of 25 mM Tris-HCl buffer, pH 7.6, containing 4 mM dithiothreitol, 1 mM EDTA and 0.2 mM pyridoxal 5-phosphate, homogenized, centrifuged, and ODC activity was determined in 0.1 ml aliquots of the clear soluble supernatants by measuring the release of $^{14}CO_2$ from L [1-^{14}C] ornithine-HCl (55 mCi/mmol; American Radiolabelled Chemicals, St. Louis, MO) essentially as described previously (1, 2). The protein concentration of the epidermal samples was assayed with Bio-Rad dye reagent (Bio-Rad Laboratories, Richmond, CA).

Results and Discussion

The epidermis must be scrapped off the dermis in ice-cold conditions because the tumor promoter-induced ODC enzyme is susceptible to heat inactivation and it is often desirable to avoid the heat treatment (55°C for 30 sec) method for epidermal isolation when assaying the small ODC responses to weak non-TPA-type tumor promoters, such as BPx (10, 18-20). On an equal dose basis, CPBA is by far the most effective ODC inducer among the various organic peroxides tested (Figure 1). An interesting finding is that two applications of 5 mg of CPBA at a 72-h interval induce ODC activity at 5 h to a greater degree than similar treatment with 3 μg of TPA (Figure 1). Similar effects can be observed when these large ODC responses to TPA and CPBA are assayed in epidermal samples prepared after a brief heat treatment (data not shown). However, the weak ODC response to BPx in samples prepared by the cold scraping method is at least 2-fold higher than in epidermis isolated by heat treatment (10). In contrast, the ODC-inducing activities of BUP, and especially, BPx and

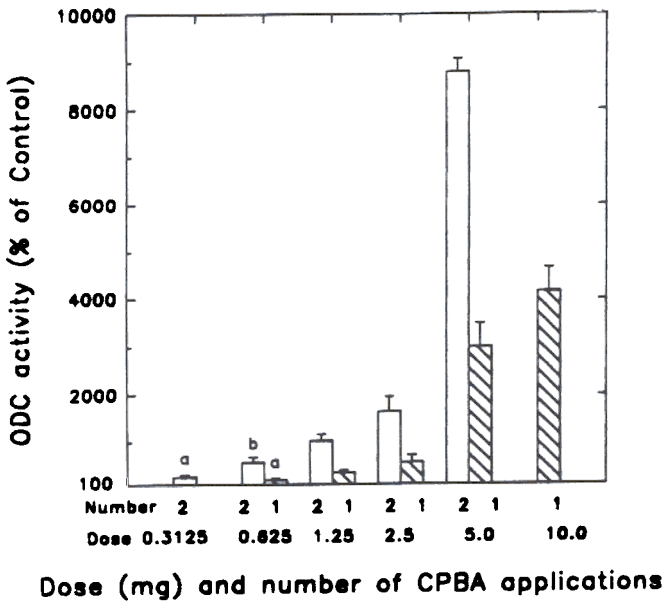

Figure 3. *Dose-dependent induction of ODC activity observed 5h after two applications of CPBA at a 72-h interval (open bar) or 36 h after a single CPBA treatment (striped bar). Bars: mean±SD (n=4). Basal ODC activity in control mice receiving acetone only was 0.13±0.02 nmol CO_2/h/mg protein (100±15%).* [a]*P< 0.001, significantly greater than a single application of 0.625 mg of CPBA;* [b]*P<0.005, significantly greater than control.*

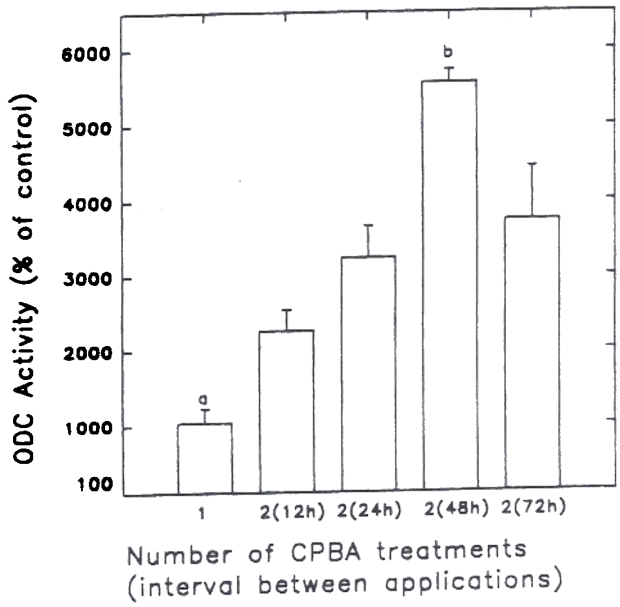

Figure 4. *Effect of the time interval between two applications of CPBA on ODC induction in mouse epidermis in vivo. ODC activity was determined 5h after a.single or two CPBA treatments (3.5 mg) applied at the indicated time intervals. Bars: mean±SD (n=4). Basal ODC activity in control mice receiving acetone only was 0.34±0.06 nmol CO_2/h/mg protein (100±18%).* [a]*P<0.001, significantly greater than control;* [b]*P<0.005, significantly greater than two CPBA treatments at a 72-h interval.*

DPx are much smaller than that of TPA (Figure 1). Single or multiple applications of 0.5-2 mg of CPBA were also more effective than several other organic peroxides at stimulating short-term markers of skin tumor promotion, such as ODC activity, epidermal thickness, and dark basal keratinocytes (11), Since ODC induction and polyamine biosynthesis are so critical for tumor promotion, especially at later stages (21), the potent ODC-inducting activity of CPBA in Fig. 1 suggests that this compound may be a more effective tumor promoter than the other organic peroxides tested before (5-7, 9, 12, 14). Therefore, the ODC-inducing activity of CPBA was further characterized to identify the best CPBA treatments to be tested in future long-term tumor experiments.

The ODC response to a single TPA treatment peaks at 5 h and returns to control levels within 12-24 h (20). In contrast, a single application of 2 mg of CPBA produces a 9-fold and longer-lasting ODC response, which peaks at 36 h (Figure 2). However, after two CPBA treatments at a 72-h interval, the peak of ODC induction shifts back at 5 h and is three times higher than that observed 36 h after a single CPBA treatment (Figure 2). Although this ODC response to multiple CPBA treatments resembles that to TPA, the discrepancy between the time courses for the effects of single CPBA and TPA treatments on ODC activity suggests that the initial mechanisms of ODC induction by these agents may be different. Since the broad peaks of ODC induction occurring after sin-

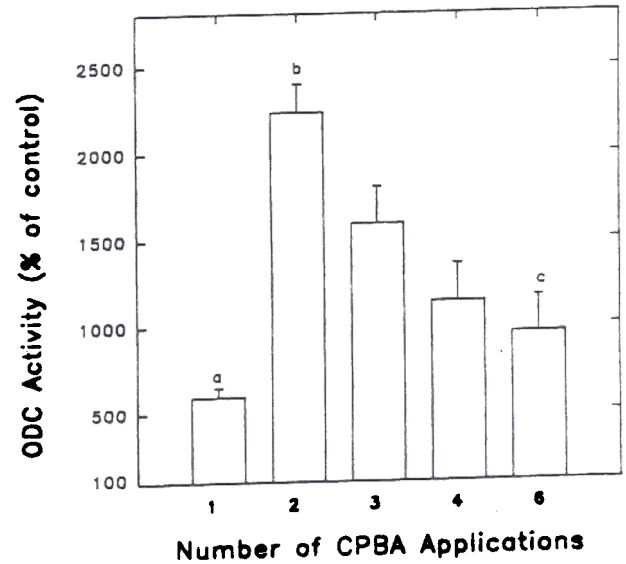

Figure 5. *Comparison of the ODC responses observed 5h after a single or multiple applications of 2 mg of CPBA at 48-h intervals in mouse epidermis in vivo. Bars: mean±SD (n=4). Basal ODC activity in control mice receiving acetone only was 0.32±0.04 nmol CO_2/h/mg protein (100±14%).* [a]*P<0.001, significantly greater than control;* [b]*P<0.005, significantly greater than three applications of CPBA;* [c]*P<0.01, significantly greater than a single CPBA application.*

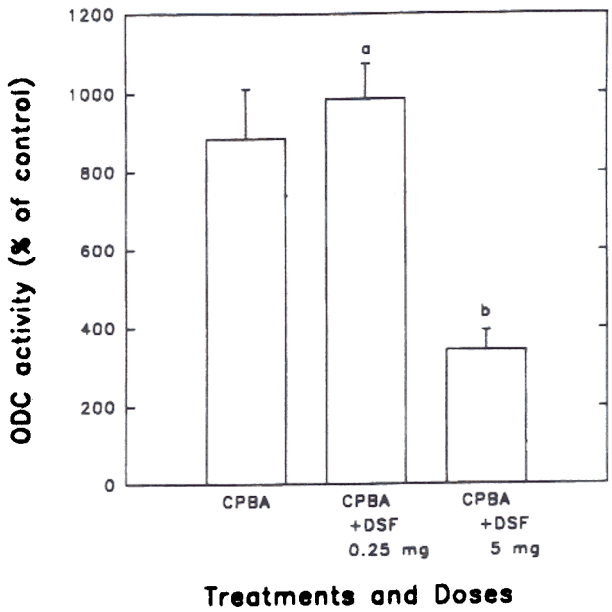

Figure 6. *Effect of DSF on CPBA-induced ODC activity in mouse epidermis in vivo. Epidermis ODC activity was determined 5 h after two applications of 5 mg of CPBA at a 48-h interval. The indicated doses of DSF were applied 20 min before each CPBA treatment. Basal ODC activity in acetone-treated control mice (1.13±0.10 nmol CO_2/h/ mg protein) was subtracted from the results. Bars: mean±SD (n=4).* [a]*Not significantly different from CPBA;* [b]*P< 0.001, significantly smaller than CPBA.*

Table I. *Comparison of the effectiveness of HTs, CTs, and their monomeric units as inhibitors of CPBA-induced ODC activity in mouse epidermis in vivo.*

Treatment[a] (Dose/application)	ODC activity at 5h[b] nmol CO_2/h/ mg protem mean±SD (n=4)	% of control	% of CPBA
Control	0.27±0.04	100	
CPBA (5 mg)	9.76±1.68	3615	100
+Catechin (12 mg)	0.55±0.16	204	
+Loblolly pine bark CT (12 mg)	2.83±0.62	1048	27
+Guamuchil bark CT (12 mg)	4.07±0.82	1507	40
+Southern red oak bark CT (12 mg)	3.12±0.41	1156	30
+Pecan nut pith CT (12 mg)	9.67±0.73[c]	3581	99
+Gallic acid (12 mg)	1.88±0.26	696	17
+Commercial TA (12 mg)	3.40±0.27	1259	33
+Sumac leaf TA (12 mg)	2.26±0.42	837	21
+Aleppo gall TA (12 mg)	1.79±0.26	663	16
+Tara pod TA (12mg)	4.07±0.97	1507	40

[a]HTs, CTs and their monomeric units were applied 20min before each CPBA treatment;
[b]After two CPBA treatments at a 72-h interval;
[c]Not significantly different from CPBA.

gle BPx, chrysarobin, and thapsigargin can also be shifted to earlier times after multiple applications of these compounds (2, 10, 22), it is speculated that CPBA may induce ODC activity like these weak non-TPA-type tumor promoters.

A single application of 0.3125 mg of CPBA does not induce epidermal ODC activity. When measured 36 h after a single application of 0.625-10 mg, the ODC response to CPBA is clearly dose dependent (Figure 3). In accord with the different magnitudes and time courses for ODC induction by one or two CPBA treatments (Figure 2), the dose dependent induction of ODC activity observed 5 h after two applications of 0.3125-5 mg of CPBA at a 72-h interval is about three times higher than that measured 36 h after a single of these treatments (Figure 3). Above 5 mg, multiple applications of CPBA induce increasing levels of ulceration, coagulative necrosis, and toxicity and cannot be tested (11). The toxicity of CPBA, therefore, might explain why no plateau can be reached in Figure 3.

The effects of various time intervals between two successive CPBA treatments on the induction of epidermal ODC activity have been compared in Figure 4. Again, the ODC response 5 h after two applications of 3.5 mg of CPBA at a 72-h interval is about four times higher than 5 h after a single of these treatments because of the different time courses illustrated in Figure 2. CPBA-induced ODC activity is maximal 5 h after two treatments at a 48-h interval (Figure 4), suggesting that CPBA might be a more effective tumor promoter if it is applied every 2 days rather than every 3 days or 2x/week, the usual frequency of tumor promotion treatments. Theoretically, 12 and 24 h after TPA there is a potent refractory state against ODC induction so that a 2nd application of TPA during this period is totally unable to induce ODC activity (23). But this is not the case for CPBA. Two applications of CPBA at 12- or 24-h intervals induce ODC activity to greater degrees than after a single CPBA treatment (Figure 4). One explanation is that the level of ODC induction 12-24 h after the 1st CPBA treatment (Figure 2) is still very low at the time when the 2nd applications of CPBA are administered in Figure 4. Therefore, the weak ODC-inducing activities caused by CPBA (Figure 2) and thapsigargin (2, 20) at early times after a single treatment might explain why, in contrast to the potent ODC inducer TPA that causes a prolonged down-regulation of protein kinase C (PKC), applications of non-TPA-type tumor promoters repeated at 12- to 24-h intervals do not induce refractory states against ODC induction by the last of these treatments (Figures 2 and 4).

The development of skin tumors requires repeated applications of TPA or BPx to initiated skin, and the magnitudes of the biochemical and biological effects of TPA- and non-TPA-type agents are generally maximal after about 3-6 treatments (1, 10, 24, 25). Therefore, the magnitudes of CPBA-induced ODC activity have been compared after 1-6 applications of this compound (Figure 5). At the best frequency indicated in Figure 4, the effects of multiple applications of CPBA are all more pronounced than that of a single of these treatments (Figure 5). Maximal ODC induction is achieved 5 h after 2

Table II. *Effect of the time of Aleppo gall TA post treatment on CPBA-induced ODC activity in mouse epidermis in vivo.*

Time of TA application after CPBA treatment	Treatments[a]	ODC activity: 5 h after 2CPBA treatments % of control ±SD (n=4)	% of CPBA	5 h after 1CPBA treatment % of control ±SD (n=4)	% of CPBA	36 h after 1CPBA treatment % of control ±SD (n=4)	% of CPBA
	control[b]	100±10		100±10		100±10	
	CPBA	3052±336	100	781±64	100	2714±408	100
+20 min	+TA	3265±422[d]	107	344±35	44	2877±441[c]	106
+1 h	+TA					3067±137[c]	113
+2h	+TA					2985±277[c]	110

[a] Mice were treated either with a single dose of 10 mg of CPBA or two doses of 5 mg CPBA applied at a 72-hr interval; 12 mg of Aleppo gall TA was applied at the indicated times after each CPBA treatment.
[b] Basal ODC activity in acetone-treated controls was 0.23±0.02 nmol CO_2/h/mg protein.
[c] Not significantly different from CPBA.
[d] Not significantly different from CPBA.

applications of CPBA at a 48-h interval but additional CPBA treatments are increasingly less effective at producing such response (Figure 5), perhaps because the cumulative negative or toxic effects of chronic treatments increasingly limit their tumor-promoting stimuli. Since the potency of a tumor promoter may be linked to its ability to induce sustained biochemical responses triggering tumor development (reviewed in ref. 26), the declining ODC-inducing activity of chronic CPBA treatments probably reflects more accurately the potential of this agent in long-term tumor experiments.

The iron-specific chelator DSF has been shown *in vitro* to block ODC induction by butylated hydroxytoluene hydroperoxide, a mouse skin tumor promoter (27). Therefore, the ability of CPBA to induce epidermal ODC activity *in vivo* was assessed in the presence of DSF to determine whether this mechanism is iron dependent. As shown in Figure 6, pretreatment with 5 mg of DSF inhibits the ODC-inducing activity of CPBA by 69%, whereas 0.25 mg of DSF is ineffective. A dose of 2.5 mg of DSF also inhibits CPBA-induced ODC activity by about 70% (data not shown). This inhibitory effect of DSF *in vivo* suggests that free iron may play a role in the molecular mechanism by which CPBA induces ODC activity. Iron may catalyze the generation of free radicals by peroxides and/or the activation of peroxides to reactive intermediates involved in the induction of ODC activity (27).

HTs and CTs, which have been shown to inhibit ODC induction and skin tumor promotion by TPA (16, 17, 28, 29), were tested for their ability to inhibit the ODC-inducing activity of two CPBA treatments *in vivo* (Table I). Gallotannins have a sugar core with pendant esterified gallic acid substituents and possess a variable number of depsidically linked galloyl units in a polygalloyl chain (17). Proanthocyanidins or polyflavanoids derive from the condensation of flavan-3,4-diol. Oligomeric CTs contain variable numbers of similar flavan-3-ol monomeric units/molecule, such as catechin, epicatechin, or their pyrogallol B-ring analogues (16). Except pecan nut pith CT, all the other CT samples tested inhibit the ODC response to CPBA (Table I). The discrepancy between the effects of pecan nut pith CT and loblolly pine bark CT was

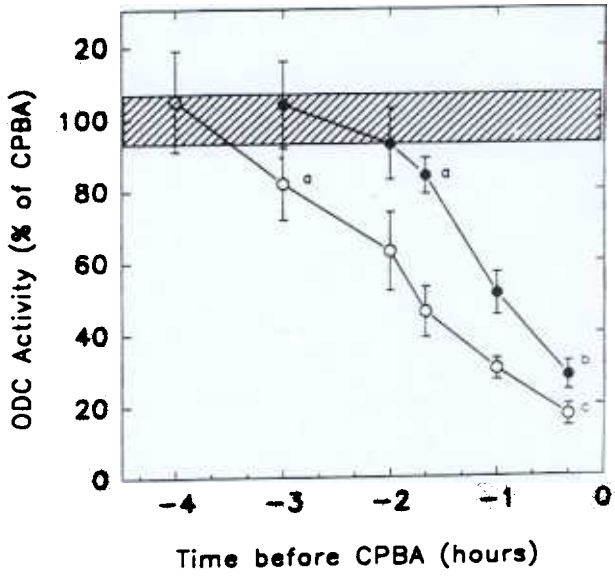

Figure 7. *Effect of the time of Aleppo gall TA or Loblolly pine bark CT pretreatment on CPBA-induced ODC activity in mouse epidermis in vivo. CPBA (5mg) was applied twice at a 72-hr interval. ODC activity was determined 5 h after the last CPBA treatment (time 0): 9.27±0.99 nmol CO_2/h/mg protein (100±11%). Treatments with 12 mg of Aleppo gall TA (O) or Loblolly pine bark CT (●) were administered at the indicated times before each application of CPBA. Basal ODC activity in acetone-treated control mice (0.27±0.04 nmol CO_2/h/ mg protein) was subtracted from the results. Bars: mean±SD (n=4). [a]Significantly different from CPBA treatment only (time 0).*

also observed on the ODC response to TPA (16). However, catechin inhibits CPBA-induced ODC activity to a greater degree than loblolly pine bark CT (Table I) even though this monomeric flavanoid is consistently less effective than this oligomeric CT against ODC induction by TPA (16). TA samples extracted from various sources all inhibit about equally the ODC responses to CPBA (Table I) and TPA (17). Interestingly, 12 mg of gallic acid and gallotannins inhibit to the same degree the ODC response to CPBA (Table I), suggesting that the different amounts of galloyl groups contained in equal μmol doses of gallic acid and gallotannin might have been the reason why commercial TA appeared more effective than its monomer at inhibiting TPA-induced ODC activity and skin tumor promotion (28, 29).

The HT and CT samples that inhibit the most CPBA-induced ODC activity in Table I were selected to determine the times at which they must be applied before each CPBA treatment (time 0) in order to alter the most the induction of ODC activity caused by this organic peroxide at 5 h (Figure 7). Aleppo gall TA and loblolly pine bark CT both inhibit maximally CPBA-induced ODC activity when they are applied 20 min before 5 mg of CPBA but their effectiveness declines at treatment times further from the time of application of CPBA. On an equal dose basis, the HT is more effective than the CT at all time points studied (Figure 7). As a result, the gallotannin is able to inhibit the ODC response to CPBA when it is administered over a long period of time extending from 3 h to 20 min before the application of the peroxide, whereas the oligomeric proanthocyanidin becomes ineffective when applied more than 100 min before CPBA (Figure 7).

A single dose of commercial TA has been shown to inhibit the peak of TPA-induced ODC activity at 5 h when applied from 3 h before to 1 h after the time of tumor promoter treatment (29). Similarly, Aleppo gall TA administered 20 min after a single dose of CPBA can inhibit the early ODC-inducing activity of this peroxide measured at 5 h (Table II). The ability of tannin post-treatments to inhibit the inductions of ODC activity 5 h after single TPA (29) or CPBA treatments (Table II) suggests that the pretreatments of skin with HTs and CTs do not simply produce a barrier that inhibits the penetration of tumor promoters and prevent their interaction with target epidermal cells. But gallotannin post-treatments administered 20 min-2 h after a single dose of CPBA all fail to decrease the peak of ODC induction observed 36 h after the organic peroxide (Table II). This result suggests that gallotannin post-treatments have short-lasting protective effects which can only inhibit the early and weak ODC responses to single CPBA treatments. The inability of Aleppo gall TA post-treatment to inhibit the peak of ODC induction occurring 36 h after the first application of CPBA might explain why this gallotannin subsequently fails to inhibit the greater ODC response observed 5 h after the second CPBA treatment (Table II).

The constitutive activation and overexpression of epidermal ODC activity are critical for neoplastic transformation, clonal expansion of tumor cells, and maximal papilloma formation (15, 30). But ODC induction is not sufficient for tumor promotion and there are some indications in our study that CPBA, especially chronic treatments, may trigger additional toxic or antagonizing effects that limit its potential in complete or stage two tumor promotion. Because the molecular events involved in the induction of ODC activity by peroxides are largely unknown (10, 11, 27), it is difficult to speculate on the precise mechanism by which plant polyphenols inhibit such response. The dissociation previously observed between tumor promoter-induced ODC activity and HPx production *in vivo* (1, 2, 20) suggests that the ability of organic peroxide treatments to produce ROS and oxidative stress in the skin may not play a major role in their ODC-inducing activities.

Much of the biochemical significance of tannins may be linked to macromolecule complexation, mineral chelation, and antioxidation (31, 32). In general, tanning ability appears at the trimeric level and increases in parallel with the molecular weight of HTs and CTs (31). Since gallotannins and oligomeric proanthocyanidins do not inhibit CPBA-induced ODC activity more than their respective monomeric units (Table I), their inhibitory effects are unlikely to be explained solely on the basis of their tanning activity or degree of polymerization. Moreover, polyphenolic antioxidants do not inhibit the HPx response to TPA because of their potency against ODC induction, and vice versa (16, 17, 25, 29). Therefore, it is unclear whether tannins inhibit CPBA-induced ODC activity because of their FR-scavenging activity and increased antioxidant protection during peroxide treatment. For instance, catechin is totally unable to mimic the inhibition of TPA-stimulated HPx production by loblolly pine bark CT (16) but is more effective than this compound at inhibiting the ODC response to CPBA in the present study (Table I).

As previously observed, the inhibition of CPBA-induced ODC activity by HTs, CTs, and their respective monomeric units is reversible and can not be explained on the basis of cytotoxicity, pH fluctuation, or traces of polyphenols directly interacting with components of the enzyme assay, suggesting that both HTs and CTs interfere with the action of CPBA or TPA and/or the molecular pathways regulating enzyme activities (16, 17, 29). The inhibitory effects of tannins *in vivo* are not simply due to nonspecific protein complexation and enzyme inactivation since these compounds can concomitantly enhance the activities of epidermal enzymes involved in xenobiotic detoxification and antioxidation such as glutathione S-transferases (reviewed in ref. 25). Finally, the ability of tannins to inhibit the biochemical effects of both TPA- and non-TPA-type agents suggests that plant polyphenols do not solely decrease binding to the phorbol ester receptor or PKC activation and downregulation (33).

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