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Characterization of the tumor-promoting activity of *m*-chloroperoxybenzoic acid in SENCAR mouse skin and its inhibition by gallotannin, oligomeric proanthocyanidin, and their monomeric units

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Abstract. *m*-Chloroperoxybenzoic acid (CPBA), which induces ornithine decarboxylase activity as much as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), was tested for its ability to induce DNA synthesis, hydroperoxide (HPx) production, and tumor promotion in mouse epidermis *in vivo*. After an early inhibition, CPBA stimulates DNA synthesis, a response which is maintained between 16 and 72 h and maximal after two treatments. CPBA at 0.6-5 mg stimulates DNA synthesis more than other organic peroxides, and nearly as much as TPA. The HPx-producing activity of the epidermis is maximally stimulated 48 h after two CPBA treatments at a 24-h interval. However, the HPx response to CPBA is much smaller than that to TPA. Aleppo gall tannic acid (AGTA) and loblolly pine bark condensed tannin (LPCT) inhibit both the DNA and HPx responses to CPBA. In contrast, their respective monomeric units, gallic acid (GA) and catechin (Cat) inhibit the DNA response to CPBA but fail to alter CPBA-stimulated HPx production. Although it is more potent than benzoyl peroxide, CPBA is a complete tumor promoter much weaker than TPA and even less effective than mezerein (MEZ). CPBA in stage 1 cannot enhance like TPA the tumor-promoting activity of MEZ in stage 2. And in contrast to that of MEZ, the very weak tumor-promoting activity of CPBA is not enhanced after stage 1 treatment with TPA. At equal mg doses, AGTA, GA, LPCT, and Cat pretreatments all remarkably inhibit complete skin tumor promotion by CPBA. In spite of their antioxidant activities, AGTA post-treatments have no or very little inhibitory effects on the development of skin tumors by CPBA during 2-stage or complete tumor promotion.

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Introduction

Multistage skin carcinogenesis is a sequence of tumor initiation, stage 1 (conversion) and stage 2 (propagation) promotion, and progression (1,2). Reactive oxygen species (ROS) may be involved at all steps of this process (3-5). Several organic peroxides partially mimic the ability of TPA to induce ornithine decarboxylase (ODC) activity, hyperplasia, dark basal keratinocytes, and tumor promotion (6-10). 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 (11,12). There is evidence to suggest that free radical (FR) generation may be involved in the mechanism of tumor promotion by organic peroxides. FRs can be trapped in different cell lines and in intact murine skin treated with organic peroxides (13-18). The tumor-promoting effects of organic peroxides can be inhibited by superoxide dismutase-mimicking agents (19), phenolic antioxidants (17), and inhibitors of the lipoxygenase pathway of arachidonic acid (AA) metabolism (20). Moreover, FR-derived active metabolites mediate tumor promotion by organic peroxides (21). The tumor-promoting activities of various peroxides may differ on the basis of percutanous absorption, metabolism, and rate of FR formation rather than chemical stability (6).

Recently, we found that CPBA was the most effective ODC inducer among various peroxides tested, multiple CPBA treatments increasing the activity of this enzyme as much as TPA (22). This ODC response to CPBA is dramatically inhibited by various hydrolyzable tannins (HTs), condensed tannins (CTs), and their monomeric units (22), which have already been shown to decrease the ODC-inducing and tumor-promoting activities of TPA (23-28).

Overexpression of ODC is required for epidermal tumor cell propagation *in vivo* (29-31), and since CPBA is much more effective than BPx at inducing this enzyme activity (22), it might also be a more potent tumor promoter than this compound. Therefore, the present study was undertaken to (i) determine if CPBA could mimic other biochemical events linked to skin tumor promtion by TPA, (ii) characterize the tumor-promoting activity of CPBA in the complete and 2-stage tumor promotion protocols, and (iii) assess the ability of polyphenolic antioxidants to inhibit the biochemical and biological effects of CPBA in mouse epidermis *in vivo*.

Materials and methods

Treatment of mice. Tumor promotion-sensitive female SENCAR mice (from Harlan Sprague Dawley Inc., Indianapolis, IN), 5-week old, were used throughout, and their dorsal skins were shaved before experimentation (32). The solutions of TPA and MEZ (both from LC Laboratories, Woburn, MA), CPBA, BPx, dicumyl peroxide (DPx), and 2-butanol peroxide (BUP) (all from Aldrich Inc., Milwaukee, WI) were delivered to the backs of individual mice in volume of 0.2 ml of acetone. AGTA (from Quercus infectoria), and commercial GA and Cat (both from Sigma, St. Louis, MO) were applied topically in 0.4 ml of acetone. LPCT (from *Pinus taeda*) was applied topically in 0.4 ml of H_2O:EtOH:acetone (18:18:64). Unless otherwise specified, the HT, CT and their monomeric units were applied 20 min before, and to the same area of skin as, each application of CPBA (23-28). Control mice were treated with vehicle only and in each experiment all mice received the same volume of solvent.

Determination of DNA synthesis. Except when otherwise specified, the rate of incorporation of [methyl-^{3}H]-thymidine (51 Ci/mmol; Amersham Corp., Arlington Heights, IL) into epidermal DNA was determined 16 h after single or multiple treatments with peroxides or TPA (33). The mice received an i.p. injection of 30 μCi of [^{3}H]-thymidine 40 min before the indicated times of sacrifice. Control mice treated only with acetone were sacrificed after the same 40-min period of pulse-labelling. Epidermal homogenates were prepared from two mice, the macromolecules were precipitated by acidification with $HClO_4$, and the acid-insoluble pellets were washed essentially as described previously (33). DNA was hydrolyzed from the precipitate with 2 ml of 0.5 N $HClO_4$ for 12 min at 90°C. The radioactivity incorporated in 0.2 ml aliquots of the above hydrolysates was estimated by liquid scintillation counting. The DNA contents of the samples were determined by the diphenylamine procedure (33).

Determination of HPx production. Unless otherwise specified, the mice were sacrificed 48 h after the last peroxide or TPA treatments. The epidermal preparations from two mice were pooled in 12.5 ml of 50 mM potassium phosphate buffer, pH 7.4, containing 118 mM NaCl, 5.36 mM KCl, 1 mM $CaCl_2$, 0.84 mM $MgSO_4$ and 5 mM dextrose, homogenized, filtered through 3 layers of surgical gauze, and centrifuged at 30,000 x g for 30 min. Aliquots of these final supernatants were incubated in the presence or absence of 5 mM NaN_3 for 3 h at 37°C and the HPx contents of the epidermal samples were assayed spectrophotometrically by a modification of the ferrithiocyanate method essentially as reported previously (25,32,34-36). The absorption of the red ferrithiocyanate complex formed in the presence of peroxide was measured against a reagent blank at 480 nm, and the levels of HPx were quantitated with reference to calibration curves prepared under similar conditions with standards of H_2O_2 ranging from 10-200 μM. The background levels of HPx in control epidermal samples incubated without NaN_3 were subtracted from each value. Data of all biochemical experiments were analyzed using Student's t-test with the level of significance set at $P<0.05$.

Tumor promotion experiments. Skin tumors were initiated in all SENCAR mice by a single topical application of a subcarcinogenic dose of 25 nmol of 7,12-dimethylbenz(a)-anthracene (DMBA). Two weeks later, groups of mice were promoted twice a week (on days 1 and 4) for the rest of experiment, using either the complete or the 2-stage tumor promotion protocols (1-3). For complete tumor promotion, mice were treated for 30 weeks with either 3.24 nmol of TPA, 4 mg of BPx, or 0.64 mg, 1.6 mg and 4 mg of CPBA. AGTA, GA, LPCT, and Cat were applied at a dose of 12 mg 20 min before each promotion treatment with CPBA. In addition, 12 mg of AGTA were tested 1 h before or 1 h after each promotion treatment with CPBA. In the 2-stage promotion protocol, mice were treated 4 times in two weeks with 3.24 nmol of TPA to achieve stage 1, and then 36 times in 18 weeks with 3.05 nmol of MEZ to achieve stage 2. Four applications of TPA in stage 1 are insufficient to produce any tumor (1,2,37). However, MEZ was applied alone in stage 2 to assess the very weak complete tumor-promoting activity of this agent in the absence of stage 1 promotion with TPA (1,2,37). The dose of 4 mg of CPBA was tested for its ability to induce either stage 1 when applied only 4 times before the stage 2 promotion treatment with MEZ or stage 2 when applied 36 times after the stage 1 promotion with TPA. AGTA was applied at a dose of 12 mg 2 h after each treatment with CPBA in either stage 1 or stage 2 promotion. Initially, there were 30 mice in each treatment group. The incidence and yield of skin tumor were respectively recorded weekly and once every two weeks. Statistics for the differences between the means of papillomas (PAs)/mouse were performed using Student's t-test, whereas differences between PA incidences were compared using the χ^2 test. The level of significance was set in both cases at $P<0.05$.

Results

After a single treatment with 5 mg of CPBA, epidermal DNA synthesis is slightly decreased at 8 h, nearly maximally stimulated at 16 h, and reaches 381% of the control level at 58 h (Fig. 1A). Interestingly, CPBA-stimulated DNA synthesis remains 300% above control at least up to 3 days (Fig. 1A). The ability of a single dose of CPBA to stimulate DNA synthesis at 16 h starts above 0.31 mg and is maximal at about 2.5-5.0 mg (Fig. 1B). However, the DNA-stimulating activity of CPBA is sharply reduced at 10 mg, suggesting that doses greater than 5.0 mg may be cytotoxic (Fig. 1B).

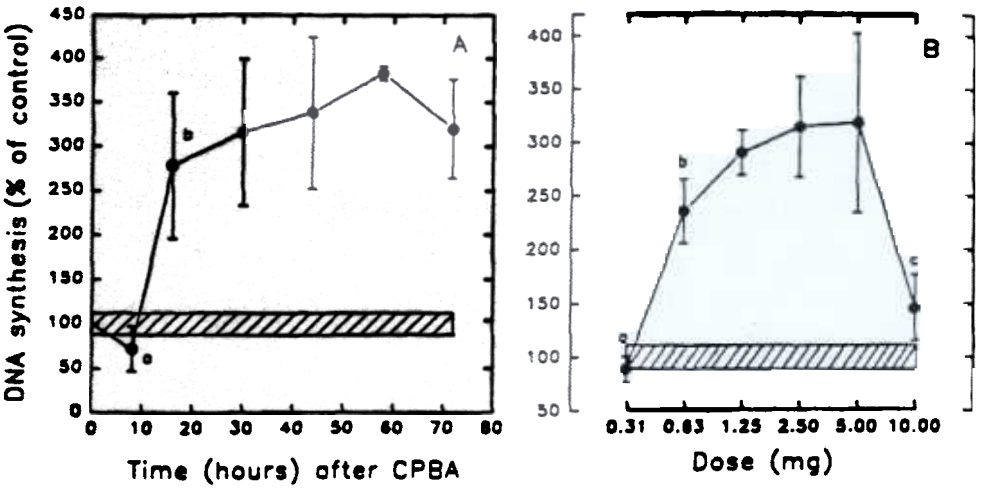

Figure 1. A. Time curve showing the effects of CPBA on DNA synthesis in mouse epidermis *in vivo*. The rate of ^{3}H-thymidine incorporation into DNA was determined at the indicated times after a single application of 5 mg of CPBA at time 0. All mice were sacrificed after a 40-min period of pulse-labelling with 30 µCi of ^{3}H-thymidine. Bars: means ± SD (n=4). The mean value of basal DNA synthesis determined at each time point in acetone-treated control mice was 51.1±12.3 cpm/µg DNA (100±24%). [a]Not significantly different from control; [b]P<0.005, greater than control, not significantly different from 30, 44, and 72 h, P<0.025, smaller than 58 h. B. Dose-dependent stimulation of DNA synthesis observed 16 h after a single application of CPBA in mouse epidermis *in vivo*. Doses are plotted on a logarithmic scale. Basal DNA synthesis in control mice was 52.7±13.2 cpm/µg DNA (100±25%). [a]Not significantly different from control; [b]P<0.005, greater than 10 mg, but not significantly different from 1.25, 2.5, and 5.0 mg; [c]P<0.05, greater than control.

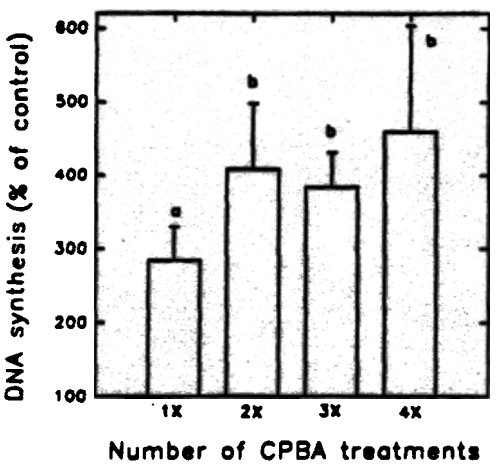

Figure 2. Effects of repeated applications of CPBA on DNA synthesis in mouse epidermis *in vivo*. DNA synthesis was measured 16 h after a single or the indicated number of applications of 2.5 mg of CPBA at 72-h intervals. Bars: means ± SD (n=4). Basal DNA synthesis in control mice receiving acetone only was 58.9±15.0 cpm/µg DNA (100±25%). [a]P<0.001, greater than control; [b]P<0.025, greater than 1X, but not significantly different from each other.

Two applications of 2.5 mg of CPBA at a 72-h interval stimulate DNA synthesis at 16 h to a greater degree than a single treatment (Fig. 2). Additional applications do not further increase the magnitude of this DNA response to CPBA (Fig. 2). On an equal dose basis, two applications of 2.5 mg CPBA at a 72-h interval stimulate DNA synthesis at 16 h to a greater degree than BUP or BPx and almost as much as 5 nmol of TPA, whereas DPx has no effect (Fig. 3A). When applied 20 min before each CPBA treatment, 12 mg of LPCT, Cat, AGTA, and GA all equally inhibit the stimulation of DNA synthesis observed 16 h after two applications of 2.5 mg of CPBA at a 72-h interval (Fig. 3B). However, LPCT, Cat, AGTA, and GA post-treatments are ineffective against CPBA-stimulated DNA synthesis (data not shown).

Two applications of 5 mg of CPBA at a 24-h interval stimulate HPx production at 48 h to a much greater degree than a single treatment (Fig. 4A). Additional administrations of CPBA do not further increase the HPx response to this compound (Fig. 4A). The magnitude of HPx production at 48 h is greater than when two doses of 5 mg of CPBA are applied at a 12- or 24-h interval than at a 48- or 72-h interval (Fig. 4B). The HPx-producing activity of the epidermis is increased 24 h after two applications of 5 mg of CPBA at a 24-h interval and remains maximally stimulated between 48 and, at least, 72 h (Fig. 5). On an equal dose basis, two applications of 5 mg of CPBA at a 24-h interval stimulate HPx production at 48 h as much as BPx but to a much lesser degree than 5 nmol of TPA, whereas BUP and DPx have no effects (Fig. 6A). When applied 20 min before each CPBA treatment, 12 mg of LPCT or AGTA inhibit the stimulation of HPx production observed 48 h after two applications of 5 mg of CPBA at a 24-h interval (Fig. 6B). In contrast to their ability to inhibit the DNA response to CPBA (Fig. 3B), Cat or GA fail to alter CPBA-stimulated HPx production (Fig. 6B). Interestingly, LPCT and AGTA administered 1-2 h after each CPBA treatment totally block the HPx response to this compound (data not shown).

As compared to TPA, all peroxide treatments tested were very weak complete tumor promoters and we were obliged to use different scales to fit the TPA tumor data in the same graphs (Fig. 7). With 4 mg of CPBA the first PAs appear at week 14 instead of week 6 for TPA and, at plateau, there are about 39% of mice with tumors instead of 97% for TPA and less than 1 tumor/mouse instead of 14 for TPA (Fig. 7).

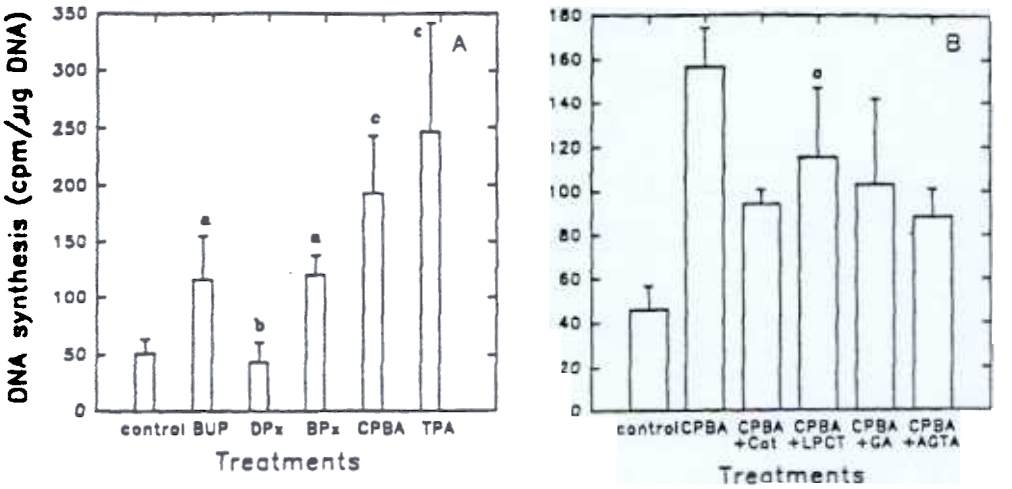

Figure 3. A. Comparison of the DNA-stimulatory effects of TPA and organic peroxides in mouse epidermis *in vivo*. DNA synthesis was determined 16 h after two applications of 5 nmol of TPA and 2.5 mg of either CPBA, BPx, DPx, or BUP at a 72-h interval. Bars: means ± SD (n=4). Basal DNA synthesis in control mice receiving acetone only was 49.9±12.1 cpm/µg DNA (100±24%). [a]P<0.01, greater than control; [b]Not significantly different from control; [c]P<0.05, greater than BUP and BPx, but not significantly different from each other. B. Comparison of the inhibitory effects of HT, CT, and their monomeric units on the DNA response to CPBA in mouse epidermis *in vivo*. DNA synthesis was determined 16 h after two applications of 2.5 mg of CPBA at a 72-h interval. Treatments with 12 mg of LPCT, Cat, AGTA and GA were administered 20 min before each CPBA treatment. Basal DNA synthesis in control mice was 46.1±10.7 cpm/µg DNA (100±23%). [a]P<0.05, smaller than CPBA, but not significantly different from CPBA+Cat, CPBA+GA and CPBA+AGTA.

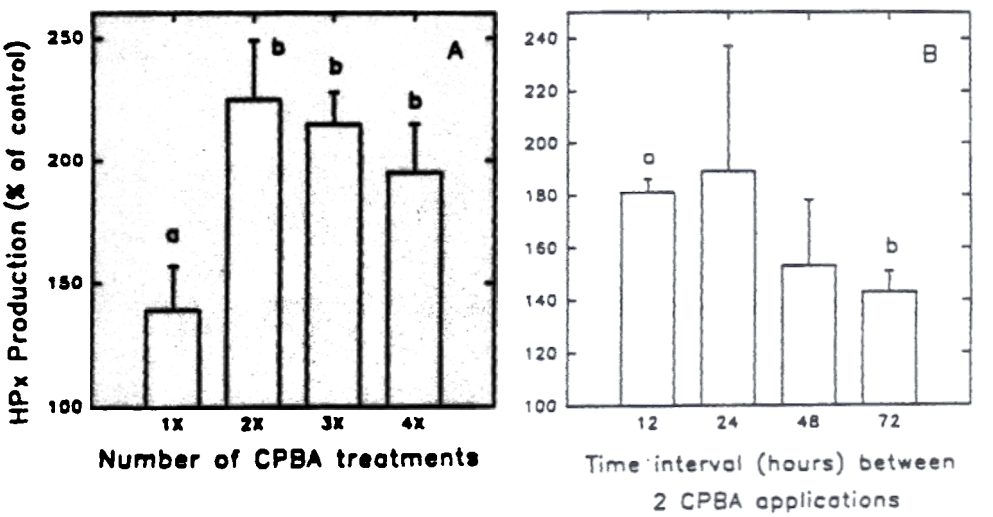

Figure 4. A. Comparison of the stimulatory effects of single or multiple CPBA treatments on HPx production in mouse epidermis *in vivo*. HPx production was determined 48 h after a single or the indicated number of applications of 5 mg of CPBA at 24-h intervals. Bars: means ± SD (n=4). Basal HPx production in acetone-treated control mice was 9.4±0.9 nmol H_2O_2/3 h/ mg protein (100±10%). [a]P<0.005, greater than control; [b]P<0.005, greater than 1X, but not significantly different from each other. B. Effect of the time interval between two applications of CPBA on HPx production in mouse epidermis *in vivo*. HPx production was determined 48 h after two applications of 5 mg of CPBA at the indicated time intervals. Basal HPx level in control mice was 7.9±1.1 nmol H_2O_2/3 h/mg protein (100±14%). [a]Not significantly different from a 24-h interval, but P<0.05, greater than a 48-h interval; [b]P<0.001, greater than control.

In the group promoted with 4 mg of CPBA, the average tumor weight/survivor is only 12% of that observed in TPA-promoted mice (Table I). However, when equal 4 mg doses are tested, CPBA is a complete tumor promoter about 4 times more potent than BPx, although tumors appear at the same time in both cases (Fig. 7). In contrast, smaller doses of 1.6 and 0.64 mg of CPBA have larger latency periods and negligible tumor-promoting activities (Fig. 7).

AGTA, GA, LPCT, and Cat applied 20 min before each promotion treatment with 4 mg of CPBA dramatically delay and inhibit the produce of skin tumors by this peroxide (Figs. 8 and 9). The latency period for PA development by CPBA is delayed by 7, 13, and 16 weeks in the presence of LPCT, GA, and AGTA, respectively. In the presence of Cat, CPBA is totally unable to promote any tumors for at least 30 weeks. The incidence, yield and weight of skin tumors

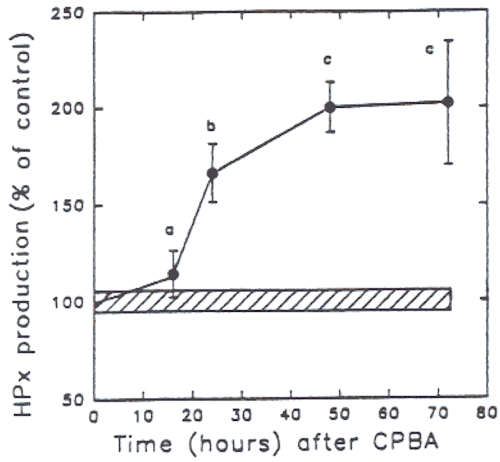

Figure 5. Time course for the stimulation of HPx production observed after two applications of 5 mg of CPBA at a 24-h interval in mouse epidermis *in vivo*. Bars: means ± SD (n=4). Basal HPx level in control mice receiving acetone only was 8.1±1.0 nmol H_2O_2/3 h/mg protein (100±12%). [a]Not significantly different from control; [b]P<0.001, greater than 16 h; [c]P<0.01, greater than 24 h, but not significantly different from each other.

promoted by CPBA are respectively inhibited by 75-92%, 82-97%, and 98-100% in the presence of LPCT, GA, and AGTA (Figs. 8 and 9; Table I). The antitumor-promoting activity of AGTA is decreased when this HT is applied 1 h before each promotion treatment with CPBA and totally lost when AGTA is applied 1 h after each peroxide treatment (Fig. 8). Such AGTA post-treatment, however, appears to shorten the latency period for tumor promotion by CPBA (Fig. 8).

As already demonstrated in SENCAR mice (1,2,37), MEZ alone is a very weak complete tumor promoter producing about 30% of mice with PAs and on average of

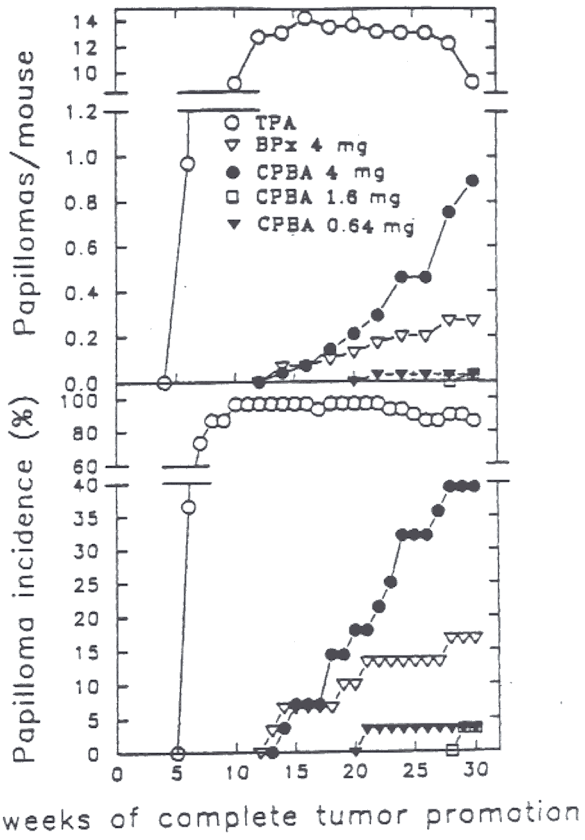

Figure 7. Comparison of the potencies of CPBA, BPx and TPA in the complete tumor promotion protocol. The conditions of the tumor experiments are detailed in Materials and methods. SENCAR mice initiated with 25 nmol of DMBA were promoted twice weekly for 30 weeks with 3.24 nmol of TPA (○), 4 mg of BPx (▽), and 4 mg (●), 1.6 mg (□), 0.64 mg (▼) of CPBA.

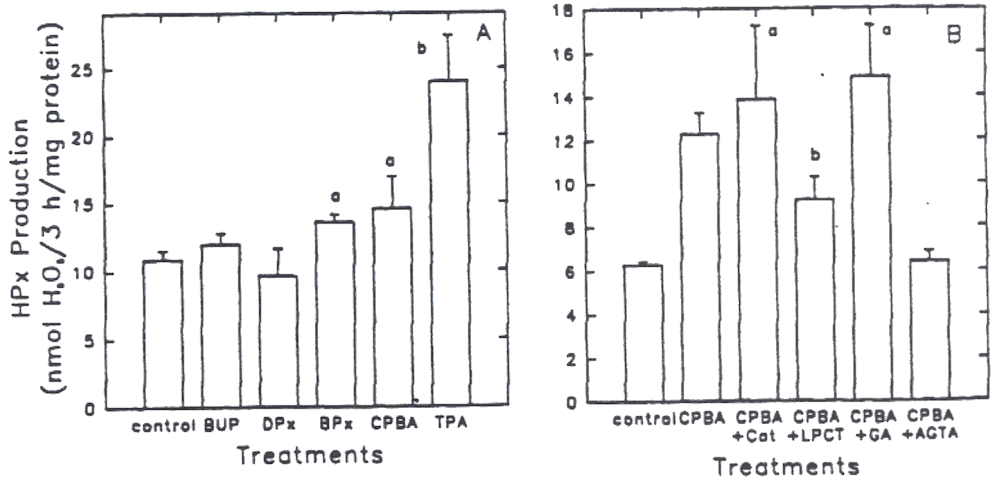

Figure 6. A. Comparison of the HPx responses to TPA and organic peroxides in mouse epidermis *in vivo*. HPx production was determined 48 h after two applications of 5 nmol of TPA and either 5 mg of CPBA, BPx, DPx or BUP at a 24-h interval. Bars: means ± SD (n=4). Basal HPx level in control mice receiving acetone only was 10.9±0.7 nmol H_2O_2/3 h/mg protein (100±6%). [a]P<0.025, greater than control, but not significantly from each other; [b]P<0.05, greater than CPBA. B. Comparison of the inhibitory effects of HT, CT and their monomeric units on CPBA-stimulated HPx production in mouse epidermis *in vivo*. HPx production was determined 48 h after two applications of 5 mg of CPBA at a 24-h interval. Treatments with 12 mg of Cat, LPCT, GA and AGTA were administered 20 min before each CPBA treatment. Basal HPx production in control mice was 6.3±0.2 nmol H_2O_2/3 h/mg protein (100±2%). [a]Not significantly different from CPBA; [b]P<0.05, smaller than CPBA, and P<0.05, greater than CPBA+AGTA.

Table I. Complete tumor-promoting activity of CPBA and its inhibition by HT, CT and their monomeric units.

Treatment[a] dose/application (time in relation to CPBA)	wk of 1st PA	Observations at 30 wks				
		wt/mouse (g)	% of survival	Tumor weight/mouse		
				mg	% of TPA	% of CPBA 4 mg
TPA 3.24 nmol	6	37.5	97	1218.90	100.00	
BPx 4 mg	13	40.1	100	39.77	3.26	
CPBA 0.64 mg	21	40.0	97	0.38	0.03	
CPBA 1.6 mg	29	41.0	100	1.07	0.09	
CPBA 4 mg	14	39.2	90	146.04	11.98	100.00
+GA (-20 min)	27	39.1	100	2.52		1.73
+AGTA (-20 min)	30	40.0	100	0.47		0.32
+AGTA (-1 h)	15	40.5	87	70.30		48.14
+AGTA (+1 h)	7	39.6	97	93.23		63.84
+Cat (-20 min)	-	41.2	100	0.00		0.00
+LPCT (-20 min)	21	38.9	100	1.74		1.19

[a]Experimental condition were as described in Figures 7-9.

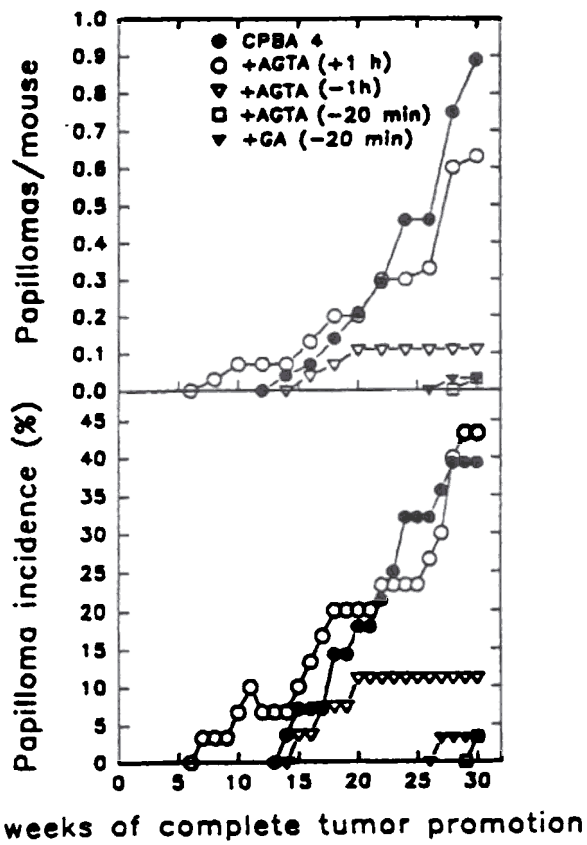

Figure 8. Inhibitory effects of HT and its monomer on complete tumor promotion by CPBA. SENCAR mice initiated with 25 nmol of DMBA were promoted twice weekly for 30 weeks with 4 mg of CPBA (●). GA (12 mg) was applied 20 min before each promotion treatment with CPBA (▽) and AGTA (12 mg) was applied either 1 h before (▼), 20 min before (□) or 1 h after (○) each CPBA treatment.

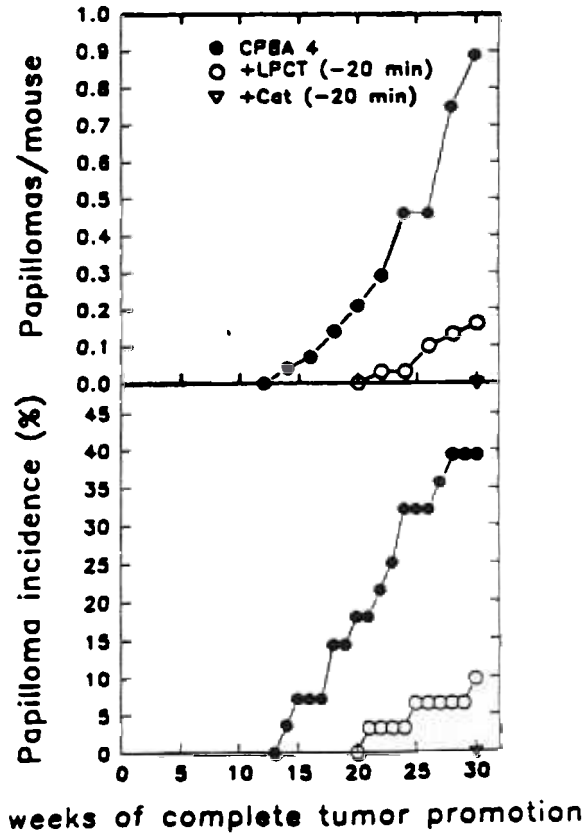

Figure 9. Inhibitory effects of CT and its monomer on complete tumor promotion by CPBA. SENCAR mice initiated with 25 nmol of DMBA were promoted twice weekly with 4 mg of CPBA for 30 weeks (●). Doses of 12 mg of LPCT (○) or Cat (▽) were applied 20 min before each promotion treatment with CPBA.

0.8 PA/mouse (Fig. 10). But CPBA alone is a complete tumor promoter even weaker than MEZ since the tumors appear 4 weeks later and their incidence and yield are 4-9 times lower after CPBA than after MEZ (Fig. 10). As shown before (1,2,37), TPA in stage 1 clearly enhances the tumor-promoting activity of MEZ (Fig. 10), and that is why it is concluded that MEZ is an ineffective complete tumor promoter but a good second stage tumor promoter. When

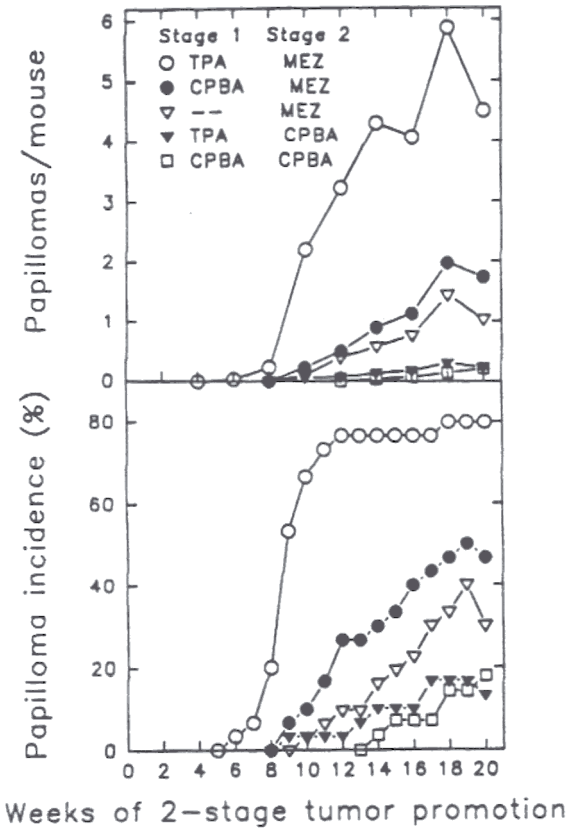

Figure 10. Effects of CPBA in the 2-stage tumor promotion protocol. SENCAR mice initiated with 25 nmol of DMBA were promoted twice a week for two weeks with 3.24 nmol of TPA to achieve stage 1 and then twice a week for 18 weeks with 3.05 nmol of MEZ to achieve stage 2 (○). Control mice received only 36 applications of MEZ in stage 2 (▽). Doses of 4 mg of CPBA applied twice a week were tested either during stage 1 before MEZ (●) or during stage 2 after TPA (▼). The complete tumor-promoting activity of 4 mg of CPBA applied twice a week for 20 weeks is indicated for the sake of comparison (□).

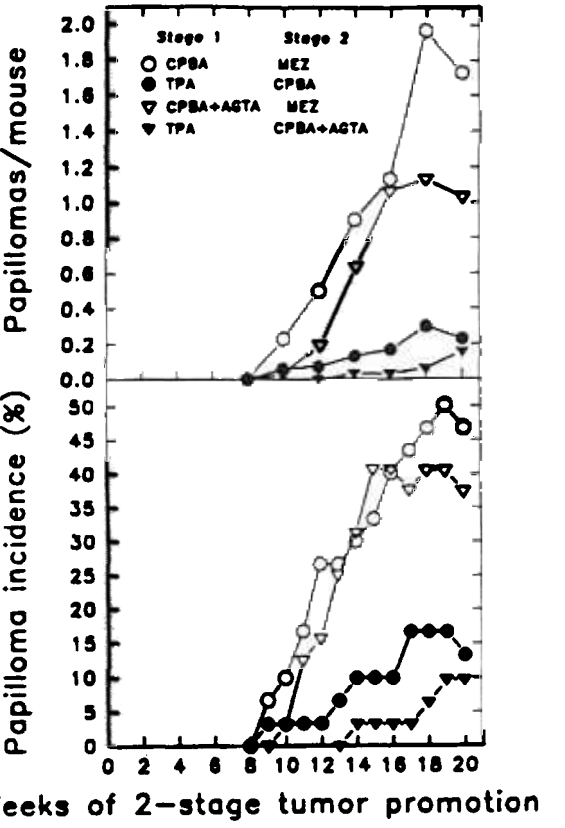

Figure 11. Ability of AGTA post-treatment to alter the effects of CPBA in the 2-stage tumor promotion protocol. SENCAR mice initiated with 25 nmol of DMBA were promoted with the following treatments: 4 mg of CPBA twice weekly for two weeks followed by 3.05 nmol of MEZ twice weekly for 18 weeks (○); 3.24 nmol of TPA twice weekly for two weeks followed by 4 mg of CPBA twice weekly for 18 weeks (●). AGTA was applied at a dose of 12 mg 2 h after either each CPBA treatment in stage 1 (▽) or each CPBA treatment in stage 2 (▼).

tested during stage 1, 4 applications CPBA do not enhance like TPA the subsequent tumor-promoting activity of MEZ in stage 2 (Fig. 10). Moreover, TPA in stage 1 does not further increase like it does for MEZ the subsequent tumor-promoting activity of CPBA in stage 2 (Fig. 10). AGTA post-treatments do not alter the tumor-promoting activity of 4 applications of CPBA during stage 1 but delay the appearance and slightly decrease the incidence and yield of PAs promoted by 36 CPBA treatments during stage 2 (Fig. 11).

Discussion

The major finding of this study is that CPBA is a skin tumor promoter about 4 times more potent than BPx. In cooperation with other genetic defects, sustained high levels of ODC and polyamines may be required for neoplastic transformation, clonal expansion of tumor cells, maximal PA formation, and progression to malignancy (29-31). Since CPBA and BPx have similar weak HPx-producing activities in our study, the greater tumor-promoting activity of CPBA may be linked to its ability to ODC activity (22) and DNA synthesis (Fig. 3A) to a greater degree than BPx. However, the very weak complete tumor-promoting activity of CPBA as compared to other agents so far tested is inconsistent with the fact that this organic peroxide is capable of inducing ODC activity (22) and DNA synthesis (Fig. 3A) as much as the most potent skin tumor promoter TPA. Either CPBA lacks critical stimuli for PA development, triggers events that prevent the full expression of its promoting activity, of both.

The very different tumor-promoting activities of CPBA and TPA in SENCAR mice are not due to different sensitivities of this strain to organic peroxides and phorbol esters since a common genetic pathway may control susceptibility to mouse skin tumor promotion by diverse classes of promoting agents (38). Several studies suggest that ODC induction, HPx production, and DNA synthesis may stimulate complementary effects required to fully maintain the prolonged hyperplastic response involved in tumor promotion (4,32,35-37). In our study, CPBA is much weaker than TPA at stimulating the HPx-producing activity of the epidermis. Interestingly, this HPx marker of skin tumor promotion is not linked to ODC induction or protein kinase C activation and down-regulation but rather to the lipoxygenase pathway of AA metabolisms, inflammation and sustained hyperplasia, the best parameters of tumor promotability (2,4,32,35,36,39). In spite of its ability to mimic the ODC

and DNA responses to TPA, therefore, CPBA may be a very weak complete tumor promoter because it fails to stimulate the HPx-producing activity of the epidermis as much as TPA.

Full stimulation of HPx production by TPA- and non-TPA-type tumor promoters requires protein synthesis and xanthine oxidase (XO), phospholipase A_2, and lipoxygenase activities (36,40). Moreover, antihistamines, anti-inflammatory steroids, and NADPH oxidase inhibitors decrease the HPx-producing activity of the epidermis treated with tumor promoters *in vivo*, suggesting that increased vascular permeability and the recruitment and activation of ROS-generating inflammatory cells play a role in this process (39,41). Although at a lower level, the time course for HPx production by CPBA (Fig. 5) resembles that by TPA (25), suggesting that both compounds may use the same mechanism to trigger this response. Further studies should determine whether the weak HPx-stimulating activity of CPBA is caused by its inability to stimulate AA metabolism and the synthesis of enzymic sources of ROS. Moreover, CPBA-treated epidermis may generate and accumulate less HPx than after TPA treatment because organic peroxides do not inhibit the antioxidant protective system as much as TPA. Indeed, the marginal decrease in glutathione peroxidase activity caused by H_2O_2 and BPx *in vivo* is substantially smaller than that observed after TPA treatment (42,43). After 18 h, the water contents of SENCAR mouse skins treated with a single application of CPBA or TPA are about 125 and 200% of the control, respectively (data not shown). Apparently, the extent of skin edema observed after CPBA is similar to that reported in the same system after a single BPx treatment (38). Unlike TPA, none of the organic peroxides previously tested produced more than slight inflammatory alterations in the dermis (6). Thus, the very weak HPx-producing and tumor-promoting activities of organic peroxides might be related to their inability to produce significant inflammatory and vascular changes in mouse skin. Even though organic peroxides generate free radicals directly, it is possible that inflammation, HPx products of the lipoxygenase pathway of AA metabolism, and enzymic sources of ROS such as xanthine oxidase, NADPH oxidase, and myeloperoxidase represent necessary steps in tumor promotion that are not or only partially stimulated by CPBA and BPx. Indeed, in contrast to TPA, a promoting dose of BPx was reported to cause no edema and only a marginal elevation of epidermal XO activity (44).

Incidentally, two CPBA treatments at a 48-h interval produce an ODC response that is better than that after two CPBA treatments at a 72-h interval (22). Moreover, two CPBA treatments at a 12- to 24-h interval produce an HPx response that is better than that after two CPBA treatments at a 72-h interval (Fig. 4B). Taken together, these observations suggest that CPBA might be a more effective tumor promoter if it is applied every 24-48 h rather than 2X/week, the usual frequency of TPA promotion treatment.

The molecular mechanisms of 2-stage tumor promotion are not clearly defined (1-4). Nevertheless, undisturbed DNA synthesis is required for stage 1 tumor promotion by TPA since nontoxic hydroxyurea treatment inhibits those two events (45). The occurrence of dark basal keratinocytes has also been correlated to the stage 1 tumor-promoting activity of various agents (1). Since dark basal keratinocytes develop after organic peroxide treatments (8) and CPBA triggers a DNA response, which is almost as high (Fig. 3A) and even longer (Fig. 1A) than that to TPA (4,33), it is surprising that CPBA fails to mimic the stage 1 tumor-promoting activity of TPA. Moreover, CPBA is a potent inducer of ODC activities (22), and excellent biochemical marker of stage 2 tumor promotion (1), but its effectiveness in stage 2 following stage 1 treatment with TPA is not better than when CPBA is tested alone in complete tumor promotion. Then effects of CPBA, or lack thereof, substatiate the hypothesis that, in contrast to TPA, various weak or incomplete tumor promoters may trigger different combinations of stimulatory and inhibitory events that limit their effectiveness as promoters of skin tumors in initiated mice (2,37). To resolve this question, future studies should identify the missing or limiting factors that prevent CPBA from expressing its full potential in stage 1, stage 2, and complete tumor promotion. Obviously, TPA in stage 1 does not provide the missing events required to enhance the tumor-promoting activity of CPBA in stage 2 or is not sufficient to overcome the negative/toxic effects limiting the tumor promoting activity of this peroxide.

Since the DNA response to CPBA is almost abolished at 10 mg (Fig. 1B) and chronic applications of CPBA above 5 mg cannot be tested because of their lethality, the tumor-promoting activity of CPBA is likely to be severely limited by extensive oxidative stress, macromolecule damage, genetic lesions, cytotoxic events, and signals of terminal differentiation that prevent the survival or the proliferation of initiated cells.

BPx, lauryl peroxide, decanoyl peroxide, DPx, and H_2O_2 have no initiating or complete carcinogenic activities of their own in mouse skin (5,6), suggesting that free radical generation alone is probably unable to directly cause initiating mutations in epidermal cells *in vivo*. To initiate skin carcinogenesis, an agent should be a good point mutagen (2,4). Therefore, it is possible that free radical generators applied topically to mouse skin fail to initiate tumors because they are unable to cause point mutations or DNA-damaging events resulting in point mutations. An alternate explanation is that FR-generating agents do cause initiating mutations but are still inactive as skin tumor initiators because they are too toxic to permit the survival of a population of initiated cells large enough to be promoted (4,8). The genotoxic effects of the FR generator BPx may not be sufficient to produce specific initiating lesions but may contribute to other DNA damage or chromosomal aberrations accelerating the progression of PAs toward a higher degree of aneuploidy and malignancy (46). FR generation by organic peroxides, therefore, may be more important to the progression of PAs to carcinomas than in the initial promotion of PAs.

Any type of promoting regimen can select the mutation-bearing initiated cells and induce their transformation and clonal expression into skin tumors (47). The ability of HT, CT, and their monomeric units to inhibit the tumor-promoting effects of TPA, MEZ, TG, and CPBA suggests that these polyphenolic phytochemicals universally inhibit the mechanism(s) of tumor promotion by chemically unrelated TPA- and non-TPA-type agents (23-28,48). AGTA inhibits both ODC induction (22) and tumor

promotion (Fig. 8) to greater degrees when it is applied 20 min rather than 1 h before each CPBA treatment. Since AGTA post-treatment can also totally block the HPx responses to tumor promoters, its lack of antitumor-promoting activity may be due to its inability to inhibit the ODC (22) and DNA responses to chronic CPBA treatment. On an equal mol dose basis, HTs and CTs have more antitumor-promoting effects than GA and Cat (23-25,27) but, when equal mg doses are compared, these polymerized or monomeric molecules have similar inhibitory activities on ODC induction, DNA synthesis, and tumor promotion by CPBA. This finding suggests that identical amounts of galloyl or flavan-3-ol monomeric units have similar antitumor-promoting activities, whether or not these molecules are depsidically linked in the polygalloyl chains of gallotannins or bound together by interflavanoid linkages in oligomeric or polymeric proanthocyanidins. It should be noted that GA and Cat, which respectively inhibit ODC induction, DNA synthesis and tumor promotion by CPBA at least as much as AGTA and LPCT, do not mimic the inhibitory effects of the HT and CT on HPx production by CPBA (Fig. 6B) and TPA (25,28), suggesting that the antitumor-promoting activity of polyphenols is not solely related to their antioxidant activity, which requires polymerization. Because a few PAs developed earlier with the AGTA post-treatment (Fig. 8), we thought that the antioxidant and FR-scavenging activities of such post-treartments might protect or rescue the epidermis from some of the negative/toxic effects limiting the tumor-promoting activity of CPBA. But this is not the case since AGTA post-treatments do not enhance the incidence and yield of PAs promoted by CPBA in the complete or 2-stage promotion protocols and are probably applied too late to elicit potent inhibitory effects on tumor formation. AGTA post-treatments appear a little more effective against stage 2 than stage 1 promotion but this might simply be due to the fact that 9 times more applications are given during stage 2 rather than stage 1.

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