Oxygen Radical-dependent Epoxidation of (7S,8S)-Dihydroxy-7,8-dihydrobenzo[ajpyrene in Mouse Skin in Vivo

STIMULATION BY PHORBOL ESTERS AND INHIBITION BY ANTIINFLAMMATORY STEROIDS*

(Received for publication, May 4, 1992)

Chuan Ji and Lawrence J. Marnett‡
From the A. B. Hancock, Jr. Memorial Laboratory for Cancer Research, Department of Biochemistry, Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146

(7S,8S) - Dihydroxy - 7, 8 - dihydrobenzo[ajpyrene (+)-BP-7,8-diol) is epoxidized to (7S,8R)-dihydroxy-(9S,10R)-epoxy-7,8,9,10-tetrahydrobenzo[ajpyrene (+)-syn-BPDE) by cytochrome P-450 isoenzymes and to (7S,8R)-dihydroxy-(9R,10S)-epoxy-7,8,9,10-tetrahydrobenzo[ajpyrene (-)-anti-BPDE) by peroxyl free radicals. 32P postlabeling analysis of the diasteroemic BPDE-DNA adducts was used to investigate the pathways of (+)-BP-7,8-diol oxidation in mouse skin in vivo. The pattern of deoxynucleoside 3',5'-bisphosphate adducts in epidermal scrapings from female CD-1 mice indicated that cytochrome P-450 was the major oxidant. Similar results were obtained when the tumor-promoting phorbol ester tetradecanoylphorbolacetate (TPA) was coadministered with (+)-BP-7,8-diol. However, when animals were pretreated with TPA 24 h before coadministration of TPA and (+)-BP-7,8-diol, the pattern of BPDE-DNA adducts indicated that peroxyl radicals made a major contribution to (+)-BP-7,8-diol epoxidation. Peroxyl radical-dependent epoxidation was maximal when the time between the two TPA administrations was 24-72 h. No increase in (-)-anti-BPDE-DNA was observed when the non-tumor-promoting phorbol ester 4-O-methyl-TPA was substituted for TPA. The calcium ionophore A23187 stimulated peroxyl radical generation when substituted for the first, but not the second, TPA treatment. The antiinflammatory steroid fluocinolone acetonide inhibited (-)-anti-BPDE-DNA adduct formation when coadministered with the first but not the second TPA treatment. These findings demonstrate the existence of two independent pathways of metabolic activation of (+)-BP-7,8-diol in mouse epidermis, one dependent on cytochrome P-450 and the other dependent on peroxyl free radicals. The results also suggest that repetitive topical administration of tumor-promoting phorbol esters remodels epidermal metabolism leading to a significant increase in free radical generation.

Oxidation of foreign compounds is a key step in their conversion to toxic, mutagenic, and carcinogenic derivatives (1). Studies from many laboratories have provided compelling evidence for the involvement of cytochrome P-450 enzymes in the metabolic activation of numerous compounds, especially in the liver (2-4). However, in vitro experiments impli- cate several non-cytochrome P-450 pathways in foreign compound oxidation, and these pathways may be important in metabolic activation in extrahepatic tissues (5-7).

BP-7,8-diol\(^1\) is a proximate carcinogenic metabolite of benzo[a]pyrene (8). BP-7,8-diol is epoxidized at the 9,10-double bond to dihydrodiol epoxides that are highly mutagenic, tumor-initiating, and carcinogenic (9-11). Epoxidation is effected by the metal-oxo complex of cytochrome P-450, by peroxyl free radicals, or by the metal-oxo complexes of certain hemeproteins (12-15). BP-7,8-diol is also epoxidized by myeloperoxidase, but whether the oxidant is a protein-derived peroxyl radical or a metal-oxo complex is unknown (16). The stereosemichemistry of epoxidation of the (+)-enantiomer of BP-7,8-diol provides a basis for distinguishing oxidation by cytochrome P-450 and non-cytochrome P-450 pathways (Fig. 1). In contrast, non-cytochrome P-450 pathways oxidize (+)-BP-7,8-diol almost exclusively to (+)-syn-BPDE (12, 13). In contrast, non-cytochrome P-450 pathways oxidize (+)-BP-7,8-diol predominantly, but not exclusively, to (-)-anti-BPDE (17). The (+)-anti-/(+-)-syn-BPDE ratio usually varies from 2.5:1 to 10:1. The differential stereosemichemistry of epoxidation has been exploited to demonstrate the occurrence of non-cytochrome P-450 epoxidation of (+)-BP-7,8-diol by cultured C3H10T1/2 fibroblasts, by freshly isolated mouse keratinocytes, and by human neutrophils (16, 18, 19).

Despite the extensive experimental evidence for non-cytochrome P-450-dependent (+)-BP-7,8-diol epoxidation in vivo, there is no compelling evidence for the operation of such pathways in an intact animal. Preliminary evidence from our laboratory suggested the formation of approximately equal amounts of BPDE-deoxynucleoside adducts in mouse skin in vivo via P-450 and non-P-450 pathways (20). However, a more extensive investigation of the levels of DNA adduction indicates that the P-450 pathway predominates by a ratio of 4:1-6:1 (21). Several years ago, Kessler et al. (22) reported that topical treatment of female CD-1 mice with TPA 24 h before topical coadministration of [\(^3\)H]-(-)-BP-7,8-diol and TPA increases the amount of [\(^3\)H] bound to epidermal DNA by 50% compared with controls performed without TPA pretreatment. The stereosemichemistry of BPDE-DNA adduction was not determined, but it was suggested that the dual TPA treatment stimulated the generation of non-P-450 oxidants. This suggestion is supported by the well known ability of TPA to recruit neutrophils to the dermis of mouse skin and

\(^1\) The abbreviations used are: (+)-BP-7,8-diol, (7S,8S)-dihydroxy-7,8-dihydrobenzo[a]pyrene; TPA, 12-O-tetradecanoylphorbol-13-acetate; 4-O-Me-TPA, 4-O-methyl-12-O-tetradecanoylphorbol-13-acetate; BPDE, 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; (+)-syn-BPDE, (7S,8R)-dihydroxy-(9S,10R)-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; (-)-anti-BPDE, (7S,8R)-dihydroxy-(9R,10S)-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; BHA, butylated hydroxyanisole; FA, fluocinolone acetonide.
to activate neutrophils to release copious amounts of superoxide anion radical (23, 24).

Determining the stereochmetry of BPDE-DNA adduct formation in mouse skin in vivo is complicated by the low level of BPDE-deoxyguanosine adducts that are isolated from epidermal DNA. This causes practical limitations related to expense, numbers of animals, analytical sensitivity, etc. We recently described a modification of \( ^{32} \text{P} \) postlabeling technology which enables separation and quantitation of individual diastereomeric BPDE-DNA adducts (25). We report herein the use of this methodology to explore the pathways of epoxidation of (+)-BP-7,8-diol in mouse skin following single or multiple applications of TPA and related compounds.

The results demonstrate that repetitive TPA administration dramatically enhances the non-P-450 pathway of epoxidation and that the extent of non-P-450-dependent epoxidation is roughly correlated to the tumor-promoting and proinflammatory activity of the compounds. The two TPA treatments appear to trigger different biochemical events as judged by their sensitivity to inhibition by antiinflammatory steroids and to stimulation by the calcium ionophore A23187. These results indicate that multiple pathways of oxidative activation of polycyclic hydrocarbons exist in mouse skin which can be modulated independently. The constitutive pathway is dependent on oxidation by a cytochrome P-450 isoenzyme, whereas the phorbol ester-inducible pathway utilizes oxidants produced as a result of the inflammatory response or by phenotypically remodeled keratinocytes.

### MATERIALS AND METHODS

**Chemicals**—Micrococcal nuclease, potato appraxe, ribonuclease T1, (grade IV: *Asperillus oryzae*), ribonuclease A (bovine pancreas, type 1), proteinase K (*Trichatrum album*, type XI fungal), dithiothreitol, calcium ionophore A23187, TPA, 4-0-Me-TPA, and fluocinolone diacetate were purchased from Sigma. Mezerein was purchased from LC Services (Woburn, MA). T4 polynucleotide kinase was obtained from U. S. Biochemical Corp. \( ^{32} \text{P} \)ATP was synthesized from \( ^{32} \text{P} \)phosphate with Gamma Prep according to the Promega Technical Bulletin. To determine the specific activity of \( ^{32} \text{P} \)ATP, a known amount of deoxyadenosine 3'-monophosphate was \( ^{32} \text{P} \) labeled in triplicate, purified by TLC, and counted. The procedure was similar to that described by Reddy and Banderier (27). The specific activity was approximately 4,000 Ci/mmol and did not vary significantly among different batches of \( ^{32} \text{P} \)phosphate.

**Enzymatic DNA Digestion and \(^{32}P\) Postlabeling Analysis of BPDE-DNA Adducts**—Digestion and \(^{32}P\) labeling conditions were similar to those described by Gupta (28). Briefly, DNA deoxyguanosine 3'-monophosphates were incubated with 10 µg of DNA with 10 µg each of micrococcal endonuclease and calf spleen phosphodiesterase in 30 µl of 10 mM sodium succinate, 5 mM CaCl₂ (pH 6.0) at 38 °C for 4 h. The digest was then diluted to 40 µl with distilled water (0.25 µg DNA/µl). To enrich the adducts (by removing normal nucleotides from 10 µg of the above digest), 40 µl of the mixtures were applied to TLC plates with 6.5 µl each of 100 mM ammonium formate (pH 3.5) and 10 mM tetrabutylammonium chloride. After adding 47.4 µl of distilled water, the mixture was extracted twice with 100 µl of water-saturated 1-butanol. The organic phase was then neutralized by adding 3 µl of 200 mM Tris-HCl (pH 9.5) and evaporated to dryness. The residue was dissolved in 16 µl of water. To this solution was added a 15-µl aliquot of a radioactive mixture containing 5 µl of T-buffer (300 mM Trizma base, 100 mM MgCl₂, 100 mM dithiothreitol, and 10 mM spermidine (pH 9.5)), 7.8 µl of carrier-free \( ^{32} \text{P} \)ATP (155 Ci/mmol), 0.4 µl of poly(dA-dT) (170 units), and 1.8 µl of H₂O. The solution was incubated at 38 °C for 40 min. The adducted deoxyribonucleoside 3',5'-biphosphates were separated using four directional TLC on polyethyleneimine-cellulose plates as follows: D1, 1.0 mM sodium phosphate (pH 6.3); D2, 4.0 mM lithium formate, 7.0 mM urea (pH 3.5); D3, 0.5 mM Tris-HCl, 0.5 mM HBO₂, 0.5 mM MgCl₂, and 5 mM urea (pH 8.0); D5, 1.7 mM sodium phosphate, pH 6.0 (25). D2 was omitted. Adducts were located by screen-enhanced autoradiography and counted.

**\(^{32}P\) Labeling of Total Nucleotides**—To measure the total amount of nucleotides, an aliquot containing 5 ng of DNA digest in 5 µl of water was mixed with 7.5 µl of the radioactive mixture that was used for adduct labeling. The reaction was carried out at 38 °C for 40 min and terminated by adding 2.5 µl of potato appraxe (2 µg/µl). After an additional 30 min, the labeled digest was then diluted to 250 µl with 10 mM Tris-HCl, 5 mM EDTA (pH 8.2). Five µl of the diluted digest (0.02 ng of DNA/µl) was applied to a polyethyleneimine plate (8 cm long) and developed with 10 mM ammonium formate. The spots were visualized by autoradiography and quantitated by Cerenkov counting.

**Measurement of Myeloperoxidase Activity in Mouse Skin**—TPA or mezerein (10 nmol) was topically applied to mouse skin in 100 µl of acetone with or without inhibitors. The animals were killed at the specified times, and the skin was excised. A small piece of normal side down on an ice-cold Petri dish, and subdermal materials were separated by scraping. The skins were minced with scissors and homogenized in 5 ml of 0.5% hexadecyltrimethyl ammonium bromide, 50 mM potassium phosphate buffer (pH 6.0) using a Poltron homogenizer. The homogenates were then boiled at 10 °C at 4 °C for 5 min and centrifuged at 10,500 rpm for 20 min at 4 °C and the supernatants pipetted off. Myeloperoxidase activity was assayed by a procedure described by Metcalfl et
RESULTS

Previous experiments have demonstrated that topical administration of 200 nmol (+)-BP-7,8-diol to female CD-1 mice leads to the formation of two DNA adducts detectable by 32P postlabeling analysis (21). The major adduct chromatographs with (+)-syn-BPDE-N²-dG and the minor adduct chromatographs with (-)-anti-BPDE-N²-dG. The levels of both adducts increase with time and dose of (+)-BP-7,8-diol (21). The relative abundance of DNA adducts detected by postlabeling analysis suggests that cytochrome P-450 is the major oxidant of (+)-BP-7,8-diol in mouse skin from untreated animals. Co-administration of (+)-BP-7,8-diol with 10 nmol of TPA did not change the pattern of adducts produced 6 or 24 h after treatment (Fig. 2, left). However, if the animals were treated topically with TPA 48 h before coadministration of (+)-BP-7,8-diol and TPA, the adduct spot arising from (-)-anti-BPDE was enhanced relative to the spot arising from (+)-syn-BPDE (Fig. 2, center). In addition, a faint spot was seen which cochromatographed with a standard of (-)-anti-BPDE-N²-dA (30). The increase in (-)-anti-BPDE adducts required both TPA treatments. If the animals were treated once with TPA 24 h before (+)-BP-7,8-diol, the adduct profile was identical to that of Fig. 2, left.

The increase in the level of the (-)-anti-BPDE-N²-dG and (-)-anti-BPDE-N²-dA adducts in Fig. 2, center, suggested that sequential TPA administration increased the production of non-cytochrome P-450 oxidants (possibly oxygen radicals) in mouse skin. Oxygen radicals can be generated in the processing of tissue so an experiment was performed to test if oxidants were produced after killing the animals. Mice were pretreated with TPA 24 h before coadministration of TPA and (+)-BP-7,8-diol. One group of mice was killed immediately thereafter, whereas a second group was maintained for 1 h before being killed. The levels of DNA adducts were analyzed by postlabeling. Although the levels of adducts were much greater in mice that were kept for 1 h before being killed, relatively strong adduct spots were detected in the "zero time" DNA samples (Fig. 3). Radioactive spots were detected that corresponded to both P-450-dependent and P-450-independent adducts. This suggested that considerable (+)-BP-7,8-diol oxidation occurred postmortem during tissue isolation and processing. To prevent postmortem oxidant generation, animals were treated with a large amount of BHA (5 mg in 100 µl of acetone) immediately prior to being killed. BHA is both an antioxidant and a substrate for cytochrome P-450 so it prevents (+)-BP-7,8-diol oxidation by either pathway (31, 32). When animals were treated with BHA and killed immediately after coadministration of (+)-BP-7,8-diol and TPA, postlabeling analysis of the epidermal DNA revealed no adduct spots (Fig. 3c). Therefore, topical administration of BHA just before killing was incorporated into the standard protocol to prevent postmortem oxidation of (+)-BP-7,8-diol. This limited the period during which oxidation of (+)-BP-7,8-diol could occur to the time between its application and the application of BHA, i.e. while the animals were alive.

Experiments were then performed in which the time between the two TPA treatments was varied from 3 to 72 h. After the second TPA treatment the mice were kept for 6 h before BHA application and death. The levels of the (+)-syn-BPDE adduct and the (-)-anti-BPDE adducts/µg of DNA are plotted in Fig. 4a. The data demonstrate that no change in adduct profile occurred up to 12 h, but between 12 and 24 h a significant stimulation (~10-20-fold) of adduction by (-)-anti-BPDE occurred. An apparent decrease in the level of the (+)-syn-BPDE adduct was evident in the same time frame. This decrease is an artifact of the method used to express adduct levels. A single administration of TPA stimulates DNA synthesis in mouse epidermis with a time course that is similar to the time course for adduction exhibited in Fig. 4a. The increase in the level of epidermal DNA decreased the level of adducts when they were expressed per µg of DNA. To correct for this, total adduct levels were calculated by multiplying the level of adduct/µg of DNA by the total amount of DNA (Fig. 4b). When expressed in this fashion, the level of the (+)-syn-BPDE adduct did not change with time, whereas the levels of the (-)-anti-BPDE adducts increased sharply from 12 to 24 h. Furthermore, the levels of the (-)-anti-BPDE adducts were nearly the same when the time between the two TPA treatments was 24–72 h.

An extensive structure-activity series exists for tumor-promoting activity by phorbol ester analogs (33). Mezerein and 4-O-Me-TPA are phorbol ester derivatives that exhibit weak and no tumor promoting activity, respectively. Experiments were conducted in which equivalent doses of TPA, mezerein, and 4-O-Me-TPA were administered in two doses each at varying time intervals between the applications. (+)-BP-7,8-diol was coadministered with the second dose. Six h later, BHA was applied, and the animals were killed. Adducts from (+)-syn-BPDE and (-)-anti-BPDE were determined by postlabeling. To normalize the response between different agents,
data were expressed as the ratio of anti to syn adducts. Since the levels of the (+)-syn-BPDE adducts do not change with time (Fig. 4), increases in the ratio of anti to syn adducts primarily reflect increases in the level of (-)-anti-BPDE adducts. Fig. 5 compares the dependence of adduct ratios on the time interval between the two treatments with each compound. Mezerein stimulated an increase in the anti/syn ratio comparable to TPA but delayed by 24 h. This paralleled the time course for the increase in DNA synthesis following treatment with mezerein (data not shown). In contrast, no increase in the anti/syn ratio or DNA synthesis was seen following two treatments with 4-O-Me-TPA.

The requirement for two TPA treatments to generate radical-derived adducts may reflect independent biochemical events. Operationally, we term the events triggered by the first TPA treatment priming and those resulting from the second treatment activation. Experiments were conducted to investigate the specificity of priming and activation. TPA was administered at time zero to prime the skin, and 72 h later (+)-BP-7,8-diol was coadministered with TPA, mezerein, or 4-O-Me-TPA. After an additional period of time, the animals were killed and the adduct levels determined. Fig. 6 displays the chromatographic patterns of the deoxynucleoside bisphosphate adducts detected by postlabeling. Increases in the level of (-)-anti-adducts were observed when TPA and mezerein were used to activate the primed animals. The extent of stimulation of radical adduct formation appeared to be greater with TPA than with mezerein. 4-O-Me-TPA induced no increase in the level of (-)-anti-adducts above that observed with TPA, but delayed by 24 h. This paralleled the time course for the increase in DNA synthesis following treatment with mezerein (data not shown). In contrast, no increase in the anti/syn ratio or DNA synthesis was seen following two treatments with 4-O-Me-TPA.
Tumor-promoting phorbol esters and A23187 are potent proinflammatory agents when administered topically to mice. Neutrophil infiltration, which is a component of inflammation, may be a key event in priming skin for oxidant generation by subsequent phorbol ester treatment (37). We measured the increase in myeloperoxidase activity in skin following phorbol ester treatment as an indicator of neutrophil infiltration. The assays developed by others for myeloperoxidase activity were modified to provide maximum sensitivity (29, 38). Fig. 7 displays the time course for the increase in myeloperoxidase activity following topical application of a single dose of 10 nmol of TPA. The level of myeloperoxidase activity was undetectable in untreated skin but rose dramatically within 6 h after TPA administration. The increase was maximal by 18 h, and activity declined slowly thereafter. The level of activity decreased by approximately 60% by 80 h (data not shown). Mezerein triggered a comparable increase in myeloperoxidase activity but with a slower time course (Fig. 7). The increase in activity at 6 h was approximately half that seen with TPA, and the maximal activity was detected at 24 h rather than 18 h. The decrease in activity from the maximum value was faster with mezerein than with TPA.

Corticosteroids are potent antiinflammatory agents and inhibitors of multiple stage mouse skin tumorigenesis (33–41). Therefore, flucinolone acetonide (FA) was tested for its effect on (+)-BP-7,8-diol epoxidation. Experiments were conducted in which FA was coadministered with either the first or second dose of mezerein to assess its effect on priming or activation. (+)-BP-7,8-diol was applied with the second dose of mezerein as in previous experiments. Fig. 8 presents the effect of increasing doses of FA applied in the priming phase on the pattern of BPDE-deoxynucleoside bisphosphate adducts. A dramatic decrease in the level of the (-)-anti-BPDE-dG adduct was observed from 0.1 to 10 μg of FA. A concommitant increase in the level of the (+)-syn-BPDE-dG adduct was observed at the same doses. When parallel experiments were conducted in which 1–50 μg of FA was coadministered with the second mezerein dose, no inhibition of (-)-anti-BPDE-dG formation was observed (data not shown). An increase in the level of (+)-syn-BPDE-dG was observed at 1 and 10 μg (2- and 3-fold, respectively). The increase at 50 μg was 1.5-fold over the no-FA control.

The effect of FA on neutrophil infiltration in response to a single dose of mezerein was determined by monitoring the level of myeloperoxidase in skin 24 h after administration. Significant inhibition of myeloperoxidase activity was observed at 0.01–10 μg. No inhibition was evident at 0.001 μg of FA (not shown).

**DISCUSSION**

The present results provide strong evidence for the existence of two pathways of metabolic activation of BP-7,8-diol in mouse skin in vivo. The pathway of epoxidation in naive skin utilizes cytochrome P-450 as the oxidant. When animals are treated with inflammatory agents such as phorbol esters or a calcium ionophore, a second pathway of epoxidation is induced which does not appear to involve cytochrome P-450 as oxidant. The independence of the basal and induced pathways is demonstrated by their differential stereochemistry, differential response to proinflammatory agents, and differential response to antiinflammatory agents.

Two applications of phorbol esters are required to trigger oxidant production in vivo. The available data suggest that each application triggers a separate biochemical event (Fig. 9). Tumor-promoting phorbol esters trigger both events, whereas nonpromoting phorbol esters do not. A23187 is able to effect the first change (priming) but not the second (activation). Likewise, FA inhibits priming but not activation. The molecular events responsible for priming and activation are uncertain. Possibilities for priming include recruitment of inflammatory cells or remodeling of keratinocyte metabolism. The ability of TPA and A23187 to recruit neutrophils is well established and was confirmed in the present investigation by monitoring the increase in myeloperoxidase activity following TPA treatment (23, 34, 37, 42). Furthermore, TPA is capable of activating a respiratory burst in neutrophils that results in O_2 generation (43). Thus, ample precedent exists in support of the hypothesis of Kensler et al. (22) that neutrophils recruited by the chemotactic activity of TPA produce oxidants in response to the second treatment. Also consistent with this hypothesis is the close correlation of the dose dependence of inhibition of priming and neutrophil infiltration by FA.

However, some of the present results appear to be inconsistent with a mechanism in which neutrophils are the source of the oxidant. First, A23187 does not trigger oxidant production in either TPA- or A23187-primed skin although it activates O_2 generation and myeloperoxidase secretion by neutrophils ex vivo (35, 36). Second, a time lag of ~8–12 h exists between the time course of neutrophil infiltration and the time course for priming. Third, neutrophil infiltration is ex-
clusively into the dermis, whereas the levels of BPDE adducts are highest in the epidermis.

Although these observations seem inconsistent with neutrophil involvement, they do not absolutely preclude it. For example, the time lag of 12 h between neutrophil infiltration and (+)-BP-7,8-diol oxidation could be explained by depletion of endogenous antioxidants by neutrophil-derived oxidants. The antioxidant enzymes catalase and superoxide dismutase decrease in mouse epidermis following application of TPA (44-46). However, there is no decrease in epidermal glutathione levels following TPA treatment (47). As the antioxidant levels decrease, (+)-BP-7,8-diol epoxidation could increase. Likewise, the preponderance of adducts in the epidermis rather than the dermis may indicate that a diffusible oxidant triggers (+)-BP-7,8-diol oxidation. Neutrophils release copious amounts of hydrogen peroxide when stimulated, and keratinocytes contain a low molecular weight iron complex that reacts with peroxides to generate radical oxidants (48).

On the other hand, there is also precedent for TPA stimulation of oxidant generation in skin by a neutrophil-independent pathway. Fischer and co-workers (49, 50) demonstrated that TPA treatment of freshly isolated mouse keratinocytes triggers a burst of oxidant production detectable by luminol-dependent chemiluminescence. Chemiluminescence was detectable 5 min after TPA treatment, reached a maximum at 15 min, then declined. This suggests that epidermal cells contain an oxidation system that can be activated by TPA treatment ex vivo. TPA also induces changes in oxidative capacity in epidermis. Fürstenberger et al. (51) have established that TPA enhances the activity of an arachidonic acid 5-lipoxygenase within 24 h after administration. Lipooxygenases oxidize arachidonic acid to hydroperoxy fatty acids that react with metals and epoxidize BP-7,8-diol (52, 53). Induction of this lipooxygenase may represent the priming event, and TPA-enhanced release of arachidonic acid may represent activation.

The control experiment summarized in Fig. 3 indicates that considerable oxidant generation occurs during postmortem tissue processing of TPA-treated skin. Topical administration of the phenolic antioxidant BHA immediately prior to killing effectively prevents this artificial oxidant generation. BHA is an effective radical scavenger and inhibitor of lipid peroxidation. Lipid peroxidation is often associated with cell death or lysis which can be induced by mechanical disruption. In fact, Reiners et al. (47) have recently reported that the procedures used to prepare "intact" keratinocytes or skin homogenates result in rapid depletion of 90% of the cellular glutathione which should dramatically enhance the cells' susceptibility to oxidative stress. Examination of the autoradiogram in Fig. 3 indicates that BHA also inhibits (+)-syn-BPDE adduct formation, which is primarily a cytochrome P-450-dependent process. BHA is itself a P-450 substrate and most likely inhibits by competing with BP-7,8-diol (43).

These findings cast doubt on the conclusions regarding oxidant generation reached from experiments that do not employ procedures to limit postmortem radical generation. This criticism applies to in vitro experiments or in vivo experiments employing freshly isolated cells. For example, previous work from our laboratory demonstrated that (+)-BP-7,8-diol is oxidized primarily to (-)-anti-BPDE by freshly isolated epidermal cells from untreated mice, thereby suggesting the importance of radical-dependent epoxidation in vivo (20). However, by inspection of the autoradiogram in Fig. 2, it is clear that radicals make only a minor contribution to BP-7,8-diol epoxidation in animals that are not treated with TPA. The differences in experimental results between the in vitro and in vivo experiments are reconcilable by the likely induction of oxidative stress during the isolation of epidermal cells. Similar considerations are likely to apply to a variety of reports of oxidant generation in vivo or ex vivo (19, 22, 54, 55).

It seems likely that peroxyl radicals are the oxidants that epoxidize BP-7,8-diol in mouse skin. Hydrogen peroxide, hydroxyl radical, singlet oxygen, superoxide anion, and alkoxyl radicals do not epoxidize BP-7,8-diol nor do mixtures of hydrogen peroxide and iron complexes (56-58). Myeloperoxidase epoxidizes BP-7,8-diol but in TPA-treated skin one anticipates that formation of BPDE adducts would be highest in dermis if myeloperoxidase was the oxidant (16). This was not the case in the present experiments. BP-7,8-diol reacts rapidly with peroxyl radicals to form BPDE, and peroxyl radicals have relatively long half-lives compared with other oxidants (14, 59). Furthermore, the hydrophobicity of BP-7,8-diol would localize it in membrane-bound areas of the cell, whereas the oxidant released from the neutrophil diffuses to the epidermis and reacts with iron complexes in the vicinity of DNA to generate an oxidant.

Numerous studies demonstrate the radical-dependent epoxidation of BP-7,8-diol in isolated cells, cellular homogenates, or subcellular fractions (16, 18, 19, 60, 61). The present results establish that radical-dependent epoxidation also occurs in vivo. Since epoxidation of BP-7,8-diol represents the terminal activation step in the metabolic activation of benzo[a]pyrene, our findings suggest that non-cytochrome P-450 processes can provide an alternate pathway for oxidative activation of benzo[a]pyrene and its derivatives (62). In the case of benzo[a]pyrene, its proximate carcinogenic form is (+)-BP-7,8-diol (10, 63). (+)-BP-7,8-diol is not a useful diagnostic probe for the type of metabolic experiments described here because it is metabolized to the same product (+)-anti-BPDE by cytochrome P-450 and peroxyl radicals (17). Nevertheless, it would be epoxidized by the oxidant responsible for (+)-BP-7,8-diol oxidation. In some multiple stage skin carcinogenesis experiments, TPA has been administered before the administration of the tumor initiator to enhance the initiation process (64). It has been presumed that enhancement is a result of the TPA stimulation of DNA synthesis, but the probability of mutation by replication of adducted DNA. However, it is also possible that TPA remodels epidermal metabolism thereby increasing the extent of formation of reactive electrophiles. BP-7,8-diol requires two TPA treatments to increase metabolism, but other compounds may require only one.

Although BP-7,8-diol is a tumor initiator, the metabolic experiments described above do not provide information about the mechanism of TPA promotion. The doses of TPA used in the present experiments are in the tumor promoting range (1-10 nmol) (65). In fact, the formation of radical adducts appears maximal at 5 nmol TPA.2 The non-tumor-promoting phorbol ester 4-O-Me-TPA does not stimulate radical formation, but the weak promoter mezerein does (albeit with a delayed time course). Finally, the fact that a single TPA treatment primes the skin for at least 72 h is relevant to the

C. Ji, unpublished result.

2 (+) -BP-7,8-diol Epoxidation

17847
The present experiments reveal that multi- 
itatively equal to or greater than P-450 pathways. The mo-
le treatments with tumor-promoting phorbol esters remodel 
form of cytochrome P-450 is primarily responsible for acti-
determination of total DNA binding would not have revealed 
form of cytochrome P-450 is primarily responsible for acti-
heterogeneity of the metabolic activation is apparent not 
doing of (+)-syn-BPDE adduct formation. Simple 
the case of BP-7,8-diol, an uncharacterized 
first TPA administration, the skins are primed for radical 
FA inhibition experiments. FA simultaneously inhibits prim-
Dipple, A., Moschel, R. C., and Bigger, C. A. H. 
Academia, New York. 
briefed on our findings, after the 
The utility of the stereochimical approach for characteriza-
proliferation and stimulates oxidation of (+)-BP-7,8-diol by cytochrome P-450. The two effects are 
metabolism so that radical pathways of oxidation are quan-
the pathway of metabolic activation is apparent not only 
TMS, New York. 
Sota, T., J. Fischer, S. M., Weeks, C. E., Klein-Szanto, A. J. P., and 
trials with tumor-promoting phorbol esters remodel the 
-anti-BPDE adducts and increase of (+)-syn-BPDE adducts. 
the metabolic steps leading to activation of chemical 
the case of BP-7,8-diol, an uncharacterized form of cytochrome P-450 is primarily responsible for acti-
films reveal that multi- 
the TPA induction experiments but also from the 
heterogeneity of the metabolic activation is apparent not only from the TPA induction experiments but also from 
redetermination of total DNA binding would not have revealed an effect of steroids because of the 
the case of BP-7,8-diol, an uncharacterized form of cytochrome P-450 is primarily responsible for activation to BPDE. The present experiments reveal that 
mechanism so that radical pathways of oxidation are quanti-
titatively equal to or greater than P-450 pathways. The mole-
cular events responsible for the alteration of skin metabolism and BP-7,8-diol epoxidation should be amenable to 
Investigation using the stereochimical approach outlined herein. 
Experiments in mouse skin have provided many insights into the metabolic steps leading to activation of chemical carcinogens. In the case of BP-7,8-diol, an uncharacterized form of cytochrome P-450 is primarily responsible for activation to BPDE. The present experiments reveal that multiple treatments with tumor-promoting phorbol esters remodel metabolism so that radical pathways of oxidation are quantitatively equal to or greater than P-450 pathways. The molecular events responsible for the alteration of skin metabolism and BP-7,8-diol epoxidation should be amenable to investigation using the stereochimical approach outlined herein. 

Acknowledgement—We are grateful to Dr. H. Wei for helpful discussions on assaying myeloperoxidase activity. 

REFERENCES 