Skin Tumor–Promoting Activity of Benzoyl Peroxide, a Widely Used Free Radical–Generating Compound

Abstract. *Benzoyl peroxide, a widely used free radical–generating compound, promoted both papillomas and carcinomas when it was topically applied to mice after 7,12-dimethylbenz*[a]*anthracene initiation. Benzoyl peroxide was inactive on the skin as a complete carcinogen or as a tumor initiator. A single topical application of benzoyl peroxide produced a marked epidermal hyperplasia and induced a large number of dark basal keratinocytes, effects similar to those produced by the potent tumor promoter 12-O-tetradecanoyl phorbol-13-acetate. Benzoyl peroxide, like other known tumor promoters, also inhibited metabolic cooperation (intercellular communication) in Chinese hamster cells. In view of these results caution should be recommended in the use of this and other free radical–generating compounds.*

Skin tumors can be induced on mice by a wide variety of chemical carcinogens or by ultraviolet light (*1*). Most chemical carcinogens have to be given repetitively to induce a large number of tumors (*1, 2*). Skin tumors can also be induced by application of a subthreshold dose of a carcinogen (initiation phase) and subsequent repetitive treatment with a weak or noncarcinogenic promoter (promotion phase) (*2*). The initiation phase requires only a single application of either a direct or indirect carcinogen (metabolic activation is necessary) and is essentially irreversible. The promotion phase is reversible at first but later becomes irreversible (*2*). Every known skin carcinogen, when appropriately tested, has been shown to have initiating activity. However, urethane and several polycyclic aromatic hydrocarbons and derivatives (*3*) appear to act only as initiators, and not as complete carcinogens, on mouse skin.

The promotion phase of two-stage skin carcinogenesis in mice can be accomplished by repetitive treatment with croton oil, certain phorbol esters found in croton oil, some synthethic phorbol esters, certain euphorbia latices, Anthralin, a number of phenolic compounds, certain fatty acid methyl esters, certain long-chain alkanes, surface-active agents such as sodium lauryl sulfate and Tween 60, citrus oil, iodoacetic acid, and extracts of unburned tobacco and tobacco smoke condensate (*2, 4*). Benzo[*e*]pyrene (*5*), 1-fluoro-2, 4-dinitrobenzene (*6*), and dihydroteleocidin B (*7*) reportedly have skin tumor–promoting activity.

The phorbol ester tumor promoters, as well as many of the other promoters, cause morphological and biochemical changes in the skin in addition to inflammation and epidermal hyperplasia (*2, 8*). Of the observed tumor promoter–related effects on the skin, the induction of epidermal cell proliferation, ornithine decarboxylase, and dark basal keratinocytes show the best correlation with promoting activity (*9*). Of the various short-term tests used to detect possible tumor-promoting agents, the two-stage transformation system in mouse embryo $10T^{1}/_{2}$ cells (*10*) and the inhibition of metabolic cooperation (intercellular communication) in Chinese hamster V79 cells (*11*) show the best correlation with tumor-promoting activity of a wide variety of compounds.

Benzoyl peroxide is a widely used free radical–generating compound with an estimated production of 7 million pounds per year (*12*). It is primarily used in the polymer industry as a polymerization initiator, as a curing agent, and as a cross-linking agent. It is also used as an additive in cosmetics and pharmaceuticals, especially those related to the treatment of acne. When benzoyl peroxide was shown to cause irritation of human skin (*13*), it came under scrutiny as a potential carcinogen. In earlier skin-painting studies, benzoyl peroxide had shown negative results as a carcinogen (*14*). Because the hyperplastic and morphological effects of benzoyl peroxide on mouse skin (*15*) are similar to those of the strong promoter 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), and because free radicals may be important in tumor promotion (*16*), we tested benzoyl peroxide as a skin tumor promoter. Our results show that, although benzoyl peroxide is not a complete skin carcinogen or a skin tumor initiator, it is an effective promoter of both papillomas and squamous cell carcinomas (Table 1).

Even at a dose of 40 mg given twice weekly for 1 year, benzoyl peroxide was not effective as a complete carcinogen. Benzoyl peroxide did not show any tumor-initiating activity when it was applied once at various dose levels, with subsequent repetitive applications of the

Table 1. Tumor-initiating and tumor-promoting activities and skin carcinogenicity of benzoyl peroxide in Sencar mice. Female Sencar mice, originally obtained from R. K. Boutwell, Madison, Wisconsin, are now being raised at Oak Ridge, Tennessee. Mice, 7 to 9 weeks old, were shaved with surgical clippers 2 days before the treatment, and only those in the resting phase of the hair cycle were used. Thirty animals were used for each treatment group. Groups 1 to 5 received a single topical application of 10 nmole of DMBA in 0.2 ml of acetone, or acetone only (group 5). After 1 week, groups 1 to 4 received topical applications of various dose levels of benzoyl peroxide twice weekly for 52 weeks. Groups 6 to 10 received one topical application of various dose levels of benzoyl peroxide in 0.2 ml of acetone, or acetone only (group 10), and after 1 week were given applications of 2 μg of TPA twice weekly for 52 weeks. Groups 11 to 15 received topical applications of various dose levels of benzoyl peroxide in 0.2 ml of acetone, or acetone only (group 15), twice weekly for 52 weeks. The number and incidence of papillomas and carcinomas were recorded weekly; they were removed at random for histological verification. Details of the procedures for the skin tumor induction studies have been described (*5*).

Group	Amount of benzoyl peroxide per application (mg)	Mice alive at 30 weeks (No.)	Papillomas per mouse at 30 weeks (No.)	Mice with papillomas at 30 weeks (%)	Mice with carcinomas at 52 weeks (%)
	Tumor-promoting activity (after DMBA initiation)				
1	1	29	0.8	32	4
2	10	28	3.8	72	22
3	20	27	5.2	79	43
4	40	24	5.4	85	40
5	Acetone*	28	0.03	3	0
	Tumor-initiating activity (with TPA promotion)				
6	1	29	0.1	10	0
7	10	28	0.1	10	0
8	20	28	0.1	10	0
9	40	27	0.2	15	0
10	Acetone*	29	0.2	15	0
	Complete carcinogenic activity				
11	1	28	0	0	0
12	10	29	0.03	3	0
13	20	27	0.03	3	0
14	40	25	0.03	3	0
15	Acetone*	29	0	0	0

*Acetone only, 0.2 ml.

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known promoter TPA. However, when benzoyl peroxide was applied topically twice weekly after initiation of tumors with 7,12-dimethylbenz[*a*]anthracene (DMBA) it proved to be an effective promoter with a reasonable dose-response relationship. As little as 1 mg given twice weekly produced a significant number of papillomas and carcinomas. The tumor response appeared to plateau at a dose level of 20 mg, with 79 percent of the mice developing papillomas (average of 5.2 papillomas per mouse at 30 weeks). At the same dose level, 40 percent of the mice developed squamous cell carcinomas at 52 weeks. Benzoyl peroxide also increased the tumor response in a dose-dependent manner when it was given simultaneously with TPA (data not shown). Separate experiments on skin carcinogenicity and tumor-initiating and -promoting activities of benzoyl peroxide gave results similar to those above.

When TPA and other tumor promoters are applied topically to the backs of mice, they cause inflammation and epidermal hyperplasia. However, not all agents that cause inflammation and hyperplasia are tumor promoters (*8*). Furthermore, tumor promoters induce the appearance of dark cells, electron-dense ribosome-rich basal keratinocytes, which are intensely stained with toluidine blue in semithin Epon sections. We have found a good correlation between the dark keratinocyte–inducing capacities of several promoters and their respective promoting efficiencies (*9*). When a promoting dose of benzoyl peroxide is applied topically to the backs of mice, it induces epidermal hyperplasia and morphological changes similar to those caused by TPA (Fig. 1). At 48 and 72 hours after benzoyl peroxide treatment, hyperplasia was marked and there were a large number of dark keratinocytes (approximately 15 percent of the total basal cell population). In normal adult mouse skin, the dark keratinocytes represent 2 to 3 percent of the total basal cell population. The hyperplastic epidermis was about 100 μm thick (normal epidermis is 25 μm thick) and was characterized by increased surface keratinization as well as an increase in the number of spinous and granular layers (Fig. 1A). The dermis showed moderate leukocyte infiltration, and the hair follicles and sebaceous glands appeared normal.

The efficiency with which tumor promoters eliminate metabolic cooperation in Chinese hamster cells is correlated with tumor-promoting activity (*11*). Wild-type Chinese hamster V79 cells (6-thioguanine–sensitive) reduce the recovery of 6-thioguanine–resistant cells—when they are cultured together at high densities—through a form of intercellular communication (*11*). The phorbol ester tumor promoters effectively inhibit this metabolic cooperation, and benzoyl peroxide also inhibits it in a dose-dependent manner (Table 2). The mechanism by which benzoyl peroxide promotes skin tumors may thus result from membrane changes caused by free radicals that are generated by benzoyl peroxide.

Table 2. Effects of benzoyl peroxide on the recovery of 6-thioguanine–resistant Chinese hamster V79 cells. The procedure has been described (*11*); S.E., standard error.

Dose (μg/ml)	Recovery (% ± S.E.)
Benzoyl peroxide	
0	18.6 ± 1.81
0.1	28.5 ± 1.12
0.5	24.5 ± 2.08
1.0	36.5 ± 1.66
1.5	46.6 ± 2.63
TPA	
0.01	109.6 ± 6.07

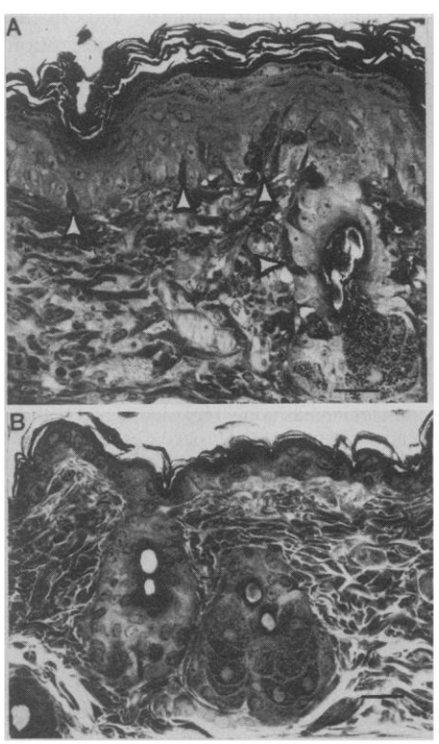

Fig. 1. Histological sections of skin from Sencar mice (Epon–toluidine blue, ×250) 48 hours after treatment with one single topical application of 40 mg of (A) benzoyl peroxide or (B) acetone. The benzoyl peroxide–treated skins (A) show a marked epidermal hyperplasia and hyperorthokeratinization; numerous dark basal keratinocytes (arrowheads) are seen in the interfollicular and infundibular epidermis. In the acetone-treated (control) skin (B), the epidermal layers are thin and lack dark basal keratinocytes. The histological procedures have been described (*9*).

The phorbol ester tumor promoters cause many other membrane changes and interact with specific membrane receptors (*17*). A rapid decrease in large external transformation-sensitive glycoprotein, an increase in 2-deoxyglucose transport, alterations in membrane fluidity, an increase in phospholipid synthesis, changes in ion movement, and interaction of TPA with epidermal growth-factor receptors have been reported (*17*). These membrane changes are believed to be instrumental in the altered phenotype induced by TPA.

Free radicals may play an important part in the carcinogenic effects produced by radiation and chemicals (*18*). Although the role of free radicals in radiation-induced carcinogenesis is widely accepted, their role in chemical carcinogenesis has been questioned because of the importance of electrophiles interacting with critical nucleophiles in macromolecules. However, if chemical carcinogens act by a mechanism of initiation and promotion, the initiation phase could involve a critical interaction of electrophilic forms of carcinogens with some cellular nucleophile (*19*), and the generation of free radicals during the promotion phase could lead directly or indirectly to membrane peroxidation. Other evidence also suggests that free radicals are important in tumor promotion. (i) Phorbol ester tumor promoters stimulate superoxide anion radical production by human polymorphonuclear leukocytes (*16*). (ii) The tumor promoter–induced free radicals are inhibited by antipromoters such as protease inhibitors, retinoids, and anti-inflammatory steroids (*20*); we found that the antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are potent inhibitors of TPA promotion (*21*) and of chemical carcinogenesis in general (*22*). (iii) Other free radical–generating compounds, such as chloroperbenzoic acid, lauroyl peroxide, and 2,2'-azobis-2-methylpropionitrile, are effective skin tumor promoters (*23*). In view of these results, caution should be recommended in the use of benzoyl peroxide and other free radical–generating compounds.

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Calcium-Dependent Prolonged Effects on Melanophores of [4-Norleucine, 7-D-Phenylalanine]-α-Melanotropin

Abstract. *A single injection of the melanotropin analog [4-norleucine, 7-D-phenylalanine]-α-melanotropin into frogs* (Rana pipiens) *caused near maximum darkening of the skins of the frogs for at least 6 weeks. Injections of the natural hormone α-melanotropin or of the analog [Nle*^{4}*]-α-melanotropin also caused darkening, but this effect lasted only a few days. Morphological examination of the skins of frogs injected with [Nle*^{4}*, D-Phe*^{7}*]-α-melanotropin revealed that both dermal and epidermal melanophores were dispersed during the entire 6-week period. In vitro [Nle*^{4}*, D-Phe*^{7}*]-α-melanotropin also causes prolonged darkening of the skin of the lizard* Anolis carolinensis. *In the absence of the melanotropin, skins previously darkened with the analog could be lightened by removal of calcium from the incubation medium but could then be redarkened by adding calcium. The cycle could be repeated indefinitely without addition of melanotropin. These results demonstrate the role of calcium in receptor signal transduction and the prolonged biological effects of [Nle*^{4}*, D-Phe*^{7}*]-α-melanotropin long after its removal from the assay medium.*

α-Melanotropin (Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2, α-MSH) is a tridecapeptide hormone that is synthesized in the pars intermedia of the vertebrate pituitary (*1*). It reversibly darkens amphibian skins by stimulating melanosome movement (dispersion) within melanophores. This melanotropin also affects mammalian melanocytes, both normal and transformed (melanoma) cells, by stimulating adenylate cyclase activity, tyrosinase activity, and melanin production. In addition, recent studies suggest that this hormone may have important functions in fetal development and in neural mechanisms related to learning and memory (*2*).

The amino acid residues that are important to the biological activity of α-melanotropin have been elucidated by systematic structure-function studies of α-MSH, α-MSH fragments, and related analogs on amphibian melanophores (*3, 4*) and, more recently, on mammalian melanoma cells (*5–8*). Treatment of melanotropins with heat and alkali leads to partial racemization of some amino acid residues within these peptides, and early investigators showed that these peptides darkened the skins of hypophysectomized frogs for at least 6 hours (*9*). However, the exact stereostructural changes responsible for these prolonged activities were not determined. Recently, we have investigated quantitatively the extent of racemization at individual amino acid residues of several melanotropins as a result of heat-alkali treatment (*8, 10*). On the basis of our results we synthesized [4-norleucine, 7-D-phenylalanine]-α-melanotropin ([Nle^4, D-Phe^7]-α-MSH) and demonstrated its prolonged (> 48 hours) melanosome-dispersing effect on frog (*Rana pipiens*) melanophores in vitro, high biological potency in stimulating mouse melanoma adenylate cyclase and tyrosinase activities, and resistance to degradation by serum enzymes that inactivate α-MSH (*8*).

The extraordinary potency and prolonged biological activity of this molecule in vitro led us to examine the biological effects of the peptide in vivo, using the frog (*Rana pipiens*) and the lizard (*Anolis carolinensis*). We report here that a single injection of [Nle^4, D-Phe^7]-α-MSH into a frog will darken its skin for periods up to 6 weeks, and that a similar injection into a lizard will darken the skin of this animal for several days. In addition, we have used the analog to investigate the mechanism of melanotropin receptor–mediated signal transduction on lizard melanophores in vitro and the role of calcium in the biological action of this peptide.

Frogs of both sexes were placed in white plastic containers with a small amount of water under overhead illumination. Under these conditions, the animals became light green in color, presumably because they were not releasing any endogenous MSH. Forty-eight hours later, light reflectance from the dorsal surface of the animals was measured with a Photovolt reflectometer (*11*). At this time, the frogs were injected subcutaneously with Ringer solution (controls) or Ringer containing α-MSH, [Nle^4]-α-MSH, or [Nle^4, D-Phe^7]-α-MSH (100 μl of a 10^{-4} *M* solution per 10 g of body weight) to provide a final body concentration of approximately 18 μg/10 g. Subsequent reflectance values were taken at 2- to 3-day intervals for 6 weeks (Fig. 1).

Maximum darkening of the frogs was obtained with [Nle^4, D-Phe^7]-α-MSH; at

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