Role of Oxygen Radicals in Tumor Promotion

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Tumor promoters provoke the elaboration of oxygen radicals by direct chemical generation and through the indirect activation or alteration of cellular sources including membrane oxidases, peroxisomes, and electron transport chains in mitochondria and endoplasmic reticulum. Although direct measurement of amplified oxygen radical production in response to tumor promoters in target tissues remains problematic, studies with scavengers of reactive oxygen species demonstrate inhibition of biochemical and biological sequelae of tumor promoter exposure and provide strong presumptive evidence for oxygen radical involvement in this late stage of carcinogenesis. The critical macromolecular targets for these oxygen radicals remain undefined; however, they may include lipids, DNA, DNA repair systems, and other enzymes.

Key words: multistage carcinogenesis, tumor promotion, phorbol esters, reactive oxygen, free radicals, superoxide dismutase, antioxidants, DNA damage, lipid peroxidation

INTRODUCTION

Respiring cells produce free radicals from molecular oxygen through enzymatic and nonenzymatic reactions. The univalent pathway of oxygen reduction generates, in turn, the superoxide radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), the hydroxyl radical (·OH), and finally water. The intermediates of oxygen reduction to water are highly reactive and present a challenge to the morphological and functional integrity of cells. As either an oxidant or reductant O$_2^-$ can, directly or indirectly as a precursor for more potent radicals, modify a variety of biologically important molecules. For example, fluxes of O$_2^-$ generated enzymatically, photochemically, or radiochemically have been shown to peroxidize lipids, depolymerize polysaccharides, alter enzyme activity, cleave DNA, and kill cells [Bors et al, 1980; Fridovich, 1981]. While it has long been recognized that oxygen has a primary role in radiation toxicity and that radicals and peroxides derived from molecular oxygen participate in radiation damage...
to cells, it has only recently been appreciated that these reactive oxygen species are also important mediators of toxicity for a wide spectrum of chemical agents [Bus and Gibson, 1979; Holtzman, 1981; Trush et al, 1982; Hochstein, 1983]. The cumulative effect of oxygen radical-initiated reactions may be cell death, possibly resulting in tissue necrosis or fibrosis, or may be more subtle and delayed as evidenced by the development of neoplasms.

Epidemiological and experimental evidence has established that the induction of cancer by chemicals is represented by stages of cellular evolution from normal, through preneoplastic and premalignant cells, to highly malignant neoplasia. In the best-studied model system, mouse skin, three distinct stages can be defined: initiation, promotion, and progression. The initiation stage requires only a single application of a direct or an indirect carcinogen at a subthreshold dose and is essentially irreversible whereas the second stage, promotion, which follows initiation, requires repetitive treatments and, although initially reversible, later becomes irreversible. Progression entails a second round of exposure to initiating agents and allows for the selection of autonomous, malignant cells. The concept of multistage carcinogenesis has recently been extended to other species and tissues. For example, multistep carcinogenesis systems have been shown in many epithelial tissues including liver, lung, bladder, mammary gland, pancreas, esophagus, stomach, and colon, as well as cells in culture. Such findings provide strong presumptive evidence for the role of multistage processes in the etiology of human cancer.

Tumor promotion is a phenomenon of gene activation in which the latent altered genotype of the initiated cell becomes expressed through selection and clonal expansion. Thus, although tumor promoters are considered to be epigenetic in action, their effects are ultimately on the genome. Tumor promoters are not carcinogenic by themselves, but must work in sequence with initiators. The work of Rous and Kidd [1941], Berenblum [1941], and Mottram [1944] provided the early evidence for promotion. These studies were based primarily on studies on mouse skin using croton oil or its later identified active principal, 12-O-tetradecanoylphorbol-13-acetate (TPA), as the model promoter. Boutwell [1964] and later Slaga et al [1980a] have shown that promotion itself could be divided into two steps, conversion (I) and propagation (II). After initiation, the conversion step is accomplished by a limited number of TPA treatments, which with no further treatment produce only a few tumors. The propagation step is accomplished by repeated treatment with turpentine or mezereum, compounds that are ineffective as complete promoters. Selective inhibition of these two stages of promotion with various antipromoters suggests that stage I reflects a dedifferentiation to the embryonic state as manifested by the induction of dark basal keratinocytes while stage II reflects specific gene activation and cell proliferation [Slaga et al, 1980b, 1982].

The molecular mechanisms of action of tumor promoters are not fully understood and the bulk of the available information pertains to studies of the tumor promoting activity of phorbol esters in mouse skin. In general though, most tumor promoters stimulate cell proliferation. Several recent excellent reviews have dealt with this subject [Diamond et al, 1980; Blumberg, 1981; Hicks, 1983]. Phorbol esters act early and directly on cell membranes, presumably through their interaction with high-affinity, saturable receptors. This receptor appears to be a calcium and lipid binding protein kinase C that is activated upon binding by TPA. Phosphorylation of proteins by this kinase may in turn modulate many cell regulatory functions including
gene activation. Within minutes following exposure of cells to TPA increases in calcium ion flux, cyclic nucleotide and phospholipid metabolism, amino acid and glucose transport, and activation of the arachidonate cascade are observed. Sequential activation of RNA, protein, and DNA synthesis occurs later. The importance of any of these specific biochemical events in the tumor promotion process is not clearly defined.

Although reactive oxygen species appear to have a role in multiple steps in chemical carcinogenesis, a rapidly expanding literature presents a particularly compelling argument for the involvement of oxy radicals in tumor promotion and is the subject of this review. The reader is also referred to other commentaries on free radicals in carcinogenesis [Ts'o et al. 1977; Floyd, 1982; Pryor, 1982; McBrien and Slater, 1982; Ames, 1983; Copeland, 1983; Kensler and Trush, 1984]. Figure 1 provides a general scheme for the elaboration of oxy radicals by tumor promoters and the subsequent cascade of aggressive oxygen species that can interact with biomolecules and contribute to the development of a neoplastic state. As is the case with mechanistic studies on tumor promotion in general, most experimental observations linking oxygen-free radicals to the tumor promotion process are derived from studies on the action of agents active in the skin, particularly the phorbol esters. However, additional presumptive evidence suggests that these oxygen species might also be involved in tumor promotion induced at nonepidermal organ sites. Table I summarizes the classes of tumor promoters that may evoke, by direct or indirect mechanisms, the elaboration of oxy radicals and hence a cellular pro-oxidant state.

**STUDIES WITH EPIDERMAL TUMOR PROMOTERS**

**Tumor Promotion by Free Radical Generating Compounds**

A number of free-radical generating peroxides used in the chemical and pharmaceutical industries are skin irritants and tumor promoters. Benzoyl peroxide, lauryl

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**Diagram Description**

**Fig. 1.** General scheme for the elaboration of reactive oxygen by tumor promoters and the accompanying enzymatic and non-enzymatic pathways leading to more aggressive oxygen species that in turn may modify critical molecular targets and participate in the development of a neoplastic state.
TABLE I. Classes of Tumor Promoters That May Act Through an Oxidant Mechanism

UV radiation
Reactive oxygen-generating compounds: Peroxides, anthralin, gossypol, complete carcinogens that autooxidize
Membrane active agents: phorbol esters, indole alkaloids
Peroxisome proliferators: hypolipidemic drugs, phthalate esters
Mixed function oxidase inducers: TCDD, DDT, phenobarbital, polychlorinated biphenyls, α-hexachlorocyclohexane

Hyperoxia

peroxide, decanoyl peroxide, cumene hydroperoxide, perbenzoic acid, m-chloroperbenzoic acid, and p-nitroperoxybenzoic acid are very active in the mouse epidermis system, while tert-butyl hydroperoxide and methylethylketone peroxide are weak promoters and \( \text{H}_2\text{O}_2 \) is only marginally active [Bock et al., 1975; Slaga et al., 1981, 1983; Klein-Szanto and Slaga, 1982]. Interestingly, however, when \( \text{H}_2\text{O}_2 \) is examined in the multistage promotion scheme, it is found to be an effective stage I promoter when followed by phorbol ester during stage II promotion. Both benzoyl peroxide and lauroyl peroxide induce epidermal hyperplasia and the production of dark basal keratinocytes, a phenotypic marker of stage I promotion, in approximately 10% of the basal cell population during the first week after single topical application. \( \text{H}_2\text{O}_2 \)-induced epidermal hyperplasias also exhibited numerous dark cells, but their presence was less sustained [Klein-Szanto and Slaga, 1982]. Although active as tumor promoters, these peroxides are largely inactive as either initiators or complete carcinogens [Kotin and Falk, 1963; Van Duuren et al., 1963; Slaga et al., 1981].

The antipsoriatic agent anthralin is another mouse skin tumor promoter [Bock and Burns, 1663] that can directly produce free radicals. Anthralin may be readily oxidized by air and light to form intermediate hydroperoxyl and anthralin radicals [Ashton et al., 1983]. The hydroperoxyl radical can then react with basic groups to form \( \text{O}_2^\cdot \). The anthralin free radical undergoes further oxidation to anthraquinone and anthralin dimer. Neither of the end products of anthralin oxidation, anthraquinone nor dimer, have tumor promoting activity [Segal et al., 1971]. Interestingly, anthralin can also inhibit tumor promotion when applied before application of TPA [Boutwell et al., 1981].

Gossypol, the toxic principle of cottonseed oil, is a tumor promoter in the skin of mice initiated with 7,12-dimethylbenz[a]anthracene [Haroz and Thomasson, 1980]. Gossypol occurs in three tautomeric forms, hemiacetal, phenol quinoid, and aldehyde, which are reactive with oxygen [Berardi and Goldblatt, 1969]. This compound is presently being evaluated for antifertility activity [Zatuchni and Osborn, 1981] and Troll and coworkers [Coburn et al., 1980] have suggested that the spermicidal action of gossypol results from the production of oxygen metabolites inasmuch as SOD and catalase inhibit this activity. De Peyster et al. [1984] have observed that gossypol enhances the formation of oxygen radicals when incubated with rat liver microsomes and human sperm. Thus, it is possible that the promoting activity of gossypol relates to its oxygen radical generating action.

Several polycyclic aromatic hydrocarbons including benzo(a)pyrene are carcinogenic to the skin [Slaga et al., 1978]. Benzo(e)pyrene is a skin promoter [Slaga et al,
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1979. A major pathway of activation of benzo(a)pyrene entails sequential metabolism to 7,8-di-9,10-epoxy benzo(a)pyrene, the putative ultimate metabolite. However, this metabolite lacks complete carcinogen activity in mouse skin although it is an initiator in the multistage model [Slaga and Fischer, 1983]. Other metabolic pathways also generate reactive benzo(a)pyrene intermediates and these may contribute to the “promoter” action of benzo(a)pyrene. One electron oxidation at the C-6 position results in the formation of a radical cation which can undergo nucleophilic attack by water to yield the labile 6-hydroxy benzo(a)pyrene derivative, which in turn rearranges to the 6-oxo radical [Ts'o et al., 1977]. Interaction of molecular oxygen with this radical yields 1,6-, 3,6-, and 6,12-quinone metabolites of benzo(a)pyrene [Lesko et al., 1975; Lorentzen et al., 1975]. In the presence of molecular oxygen the quinones undergo oxidation-reduction cycling that is accompanied initially by the generation of O$_2^-$ and subsequently by H$_2$O$_2$ and 'OH [Lesko et al., 1975; Lorentzen and Ts'o, 1977]. Accordingly, Lorentzen et al. [1979] have shown that the toxicity of benzo(a)pyrene quinones in Syrian hamster embryo fibroblasts is oxygen dependent. In further support of this concept, we find that a biomimetic superoxide dismutase (SOD) inhibits the oxidative metabolism of 7,8-diol-benzo(a)pyrene [Trush et al., 1984]. Biomimetic SOD also inhibits both the initiating [Solanki et al., 1984] and complete carcinogenic action [Egner PA and Kensler TW, unpublished observations] of 7,12-dimethylbenz[a]anthracene in mouse skin. Other carcinogens with diverse target specificities, such as benzene, anthracyclines, diethylstilbestrol, bleomycin, and aromatic amines, can also produce oxy radicals that may be important to their respective “promoter” activities [Greenlee et al., 1981; Doroshow, 1983; Metzler, 1981; Borek and Troll, 1983; Nakayama et al., 1983; Stier et al., 1980; Kensler and Trush, 1984].

Phorbol Ester-Mediated Oxy Radical Generation

Specific interaction of either soluble or particulate stimuli with membranes of inflammatory cells such as leukocytes and macrophages causes these cells to consume and metabolize oxygen to reactive intermediates. Concentrations of TPA as low as 5 ng/ml provoke rapid and marked changes in oxidative metabolism of polymorphonuclear leukocytes (PMNs) including increased oxygen consumption [Repine et al., 1974], increased oxidation of glucose via the hexose monophosphate shunt pathway [Briggs et al., 1975]; increased generation of O$_2^-$ and H$_2$O$_2$ and chemiluminescence [Goldstein et al., 1975; DeChatelet et al., 1976; Kensler and Trush, 1981]. These processes appear to be mediated through the interaction of the phorbol ester and a specific membrane receptor [Goodwin and Weinberg, 1982] and the subsequent activation of a pyridine nucleotide-dependent oxidase system localized to the plasma membrane [Briggs et al., 1975]. Structure-activity studies comparing phorbol ester analogs of varying tumor promoter activity with their ability to act as stimulators of active oxygen metabolism, as monitored by O$_2^-$ production and chemiluminescence, have shown strong concordance between the two processes [Goldstein et al., 1981; Kensler and Trush, 1981]. Phorbol and phorbol triacetate, which are inactive as tumor promoters are also inactive as stimulators of active oxygen production. Phorbol diacetate, phorbolol, phorbol dibutyrate, phorbol dibenzoate, and phorbol didacartate, which range from weak to potent tumor promoters, show a comparable range of activities in the PMN system. TPA is the most active phorbol ester examined. Several nonphorbol-promoting agents, iodoacetic acid, Tween 60, and anthralin, and
the inflammatory but nonpromoting agent, ethylphenylpropionate, were without stimulatory activity. Chemiluminescence is also stimulated by the calcium ionophore A23187 [Wilson et al., 1978], a stage-I promoter [Slaga et al., 1983]. Indole alkaloids such as dihydroteleocidin B and teleocidin, which are chemically distinct from the phorbol esters, represent another class of epidermal tumor promoters that activate oxygen metabolism in PMNs [Troll et al. 1982]. Mezerein, a diterpene related to the phorbol esters, is only a weak tumor promoter [Mufson et al., 1979], but a potent stimulator of the generation of oxygen production and chemiluminescence [Kensler and Trush, 1981; Troll et al., 1982]. Mezerein is however a potent stage-II promoter [Slaga et al., 1980a], suggesting that elaboration of oxygen radicals may be particularly important in the propagation stage of promotion.

Utilizing lymphocytes from bovine lymph nodes, Mueller and coworkers [Mueller et al., 1980; Wertz and Mueller, 1980; Mueller and Wertz, 1982; Wrighton and Mueller, 1982] have presented evidence that TPA activates certain preexisting enzymatic processes and induces gene expression for other enzymes through an oxygen-mediated mechanism. Early membrane events that are oxygen independent involve the facilitation of glucose transport and the capping reaction observed using fluorescein-labeled concanavalin A. The oxygen-dependent group of lymphocyte responses to TPA include the stimulation of choline phospholipid synthesis, amino acid transport, cytidyl transferase activity, and the alkylation of protein by arachidonic acid, all of which occur after a 20–30 min lag period. The possibility that the requirement for molecular oxygen in these later events reflects the involvement of reactive oxygen species remains conjectural at present. However, in contrast to the oxygen-independent events, the oxygen-dependent events are sensitive to inhibition by antipromoting retinoids and arachidonate cascade inhibitors that also impede oxygen radical elaboration in phorbol ester-activated PMNs.

Other than the inferential nature of inhibition of tumor promotion by free-radical scavengers discussed in a subsequent section, direct evidence for the elaboration of oxygen radicals in response to phorbol esters in target tissue, i.e., mouse epidermis, is very limited. This limitation reflects in large part the difficulty in measuring these species in intact biological systems. However, Fischer and Adams [1984] have observed a dose-dependent generation of chemiluminescence in mouse epidermal cells following TPA treatment. Additionally, Goldstein et al. [1983] have demonstrated the TPA-mediated production of H$_2$O$_2$ in mouse skin in vivo by measuring the ability of 3-amino-1,2,4-triazole to inhibit catalase, a process dependent on H$_2$O$_2$. Treatment with increasing concentrations of 3-amino-1,2,4-triazole produces a dose-dependent decrease in catalase activity in mouse skin. This inhibitory effect is markedly enhanced when 2.5 µg TPA is also applied, although this dose of TPA alone has no effect on catalase activity. The cellular source of the H$_2$O$_2$ remains undefined, although it is hypothesized by these investigators that it is produced by infiltrating inflammatory cells activated by the phorbol ester. The findings of Fischer and Adams [1984] suggest that cells are not necessarily be so, since epidermal cells themselves can be shown to produce radicals. Discussions of the overall role of inflammation in carcinogenesis can be found in several recent reviews [Demopoulos et al., 1980, 1983; Roman-Franco, 1982].

**Modulation of Oxidant Defenses by Tumor Promoters**

Multiple defense mechanisms are present in cells for coping with oxidant stress. Protection of cell constituents from damage by oxygen and its metabolites can be
accomplished through enzymatic and nonenzymatic means. SOD, catalase, and perox- idases (particularly glutathione peroxidase) catalyze reactions to remove \( \cdot \text{O}_2^- \) or \( \text{H}_2\text{O}_2 \). Enzymes that catalyze the removal of \( \cdot \text{OH} \) or \( \cdot \text{O}_2 \) have not been described so that these species must be removed by quenching molecules such as glutathione and vitamins A, C, and E. Levels or activities of these defenses are known to be modulated in response to oxidant stress.

Solanki et al. [1981] have investigated the effects of tumor promoters on epidermal SOD and catalase activities. Treatment of mouse skin with TPA results in a rapid and sustained decrease in SOD and catalase activities which is diminished 75% and 60%, respectively. The decline in SOD activity occurs within 3 h of TPA application and is maximal at 16 h. The alterations in both enzymes occur against a background of enhanced protein synthesis suggesting that the effect of phorbol ester is selective for SOD and catalase. Nonphorbol tumor promoters such as anthralin and mezerein (a potent stage-II promoter) are also active. In a separate study [Logani et al., 1982], it was observed that these tumor promoters have no effect on glutathione peroxidase activity. The underlying mechanism for the reduction in SOD and catalase activities is unclear. Although \( \cdot \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) inhibit catalase and SOD, respectively [Bray et al., 1974; Kono and Fridovich, 1982], the requirement for high oxidant concentrations and the lack of protective effect of either 2(3)-tert-butyl-4-hydroxyanisole (BHA) or 3,5-tert-butyl-4-hydroxyanisole (BHT) may mitigate against this possibility. Kinsella et al. [1983] report that TPA provokes a lowering of SOD activity in both human lymphocytes and fibroblasts, although maximal reduction is not observed until 48 h. The role of lowered SOD levels and cancer has been recently reviewed [Oberley, 1982].

**Inhibition of Tumor Promotion by Free Radical Scavengers**

Antioxidants have been shown to protect against tumor induction by a spectrum of chemical carcinogens in several rodent tissues [Wattenberg and Lam, 1981]. Current evidence suggests that the protective effects may arise from enhanced carcinogen inactivation through selective induction of enzymatic detoxification pathways [DeLong et al. 1983]. Alternatively, antioxidants may directly or indirectly through elevation of free thiol pools serve as scavengers of electrophilic reactants, thus protecting critical biomolecules from attack. Such a mode of action may be more pertinent to the antipromoting activity of phenolic antioxidants such as BHA and BHT. Wattenberg and Lam [1983] have observed inhibition of carcinogenesis of the large bowel when 3-BHA (the major isomer in commercial preparations) was given subsequent to dimethylhydrazine. Slaga et al. [1983] have reported that BHA and BHT inhibit skin tumor promotion after 7,12-dimethylbenz[a]anthracene initiation by both benzoxy peroxide and TPA. In addition to the phenolic antioxidants, other antioxidants, namely, \( \alpha \)-tocopherol and disulfiram, are also effective inhibitors. Many inhibitors of tumor promotion, such as retinoids, protease inhibitors, antioxidants, and arachidonate cascade inhibitors, block oxy radical production by activated PMNs [Goldstein et al., 1979; Witz et al., 1980; Kensler and Trush, 1981, 1983; Yavelow et al. 1982; Hoffman and Autor, 1982] and epidermal cells [Fischer and Adams, 1984].

O'Brien et al. [1975] have reported an excellent correlation between the tumor promoting activity of various compounds and their ability to induce ornithine decarboxylase (ODC), the rate limiting step in polyamine biosyntheses. Kozumbo et al. [1983] have utilized epidermal induction of ODC by TPA to examine the immediate
effects of antioxidants as inhibitors of tumor promoter action. Structure-activity relationships among BHA analogs show that both free-radical scavenging potential and lipophilicity are essential properties. Phenolic antioxidants are particularly reactive with oxygen-centered radicals such as peroxy and hydroxyl radicals, but show little reactivity with $O_2^-$ [Simic and Hunter, 1983]. However, recent observations by Kensler et al [1983] demonstrate the probable role of $O_2^-$ as well in tumor promotion. Here a low molecular weight, lipophilic copper coordination complex with SOD-mimetic activity inhibits phorbol ester-mediated tumor promotion in the skin of CD-1 mice. This complex, copper(II)(3,5-diisopropylsalicylate)$_2$ (CuDIPS), catalyzes the disproportionation of $O_2^-$ and displays a rate constant comparable to native Cu-Zn SOD [Lengfelder and Weser, 1981]. Compounds devoid of SOD-mimetic activity such as the ligand alone (DIPS) and the corresponding zinc complex (ZnDIPS) are without antipromoter activity. CuDIPS also blocks the induction of ODC by TPA, anthralin, and mezerein. Friedman and Cerutti [1983] also find that antioxidants with specificity for $O_2^-$, $H_2O_2$, and $OH^-$, namely, SOD, catalase, and mannitol, partially inhibit the induction of ODC by TPA in mouse mammary tumor cells.

A number of systems have been proposed as models of in vitro promotion of which the mouse embryo fibroblast C3H10T1/2 cells are the most thoroughly studied. Exposure of these cells at low density to carcinogenic agents such as polycyclic hydrocarbons, ultraviolet, or X-ray irradiation followed by sustained exposure to phorbol esters results in augmented formation of transformed foci [Mondal and Heidelberger, 1976; Mondal et al, 1976; Kennedy et al, 1980]. In general, promoting agents produce similar effects for in vivo carcinogenesis and in vitro transformation. Utilizing the C3H10T1/2 system, Little et al [1983] have observed that the enhancement of X-ray-induced transformation by TPA can be suppressed by concomitant incubation with either SOD or catalase. A similar finding has been made by Borek and Troll [1983] who demonstrated that SOD and to a lesser extent catalase inhibit the TPA enhancement of radiogenic transformation of freshly explanted hamster embryo cells. Zimmerman and Cerutti [1984] have recently reported that reactive oxygen acts directly as a promoter of transformation in C3H10T1/2 cells. Cells initiated with either X-rays or benzo(a)pyrene-diol-epoxide I at doses that produce minimal transformation show a strong enhancement in formation of malignant foci following daily treatment for three weeks with xanthine oxidase/xanthine. Addition of this extracellular $O_2^-$ generating system produces an effect comparable to that engendered by TPA addition, and like the TPA response, is inhibited by simultaneous addition of SOD. Phenolic antioxidants, SOD, and CuDIPS also inhibit promotion of neoplastic transformation by TPA in JB-6 mouse epidermal cells [Nakamura et al, 1984], implying that oxygen radicals may be elaborated directly without the involvement of inflammatory cells.

**STUDIES WITH NONEPIDERMAL TUMOR PROMOTERS**

Prolonged inhalation of asbestos produces pulmonary fibrosis and two forms of cancer, mesothelioma and bronchogenic carcinoma [Nettesheim et al, 1981; Mossman et al, 1983]. The action of asbestos in bronchogenic carcinoma appears to be primarily cocarcinogenic and promoting. Topping and Nettesheim [1980] have shown that asbestos has a promoting effect in rodent tracheal grafts previously exposed to subcancerogenic amounts of 7,12-dimethylbenz(a)anthracene. Phenotypic markers of
promotion, such as induction of ODC, increased DNA synthesis, hyperplasia, and metaplasia are induced by asbestos in tracheal organ cultures and tracheal epithelial cells [Mossman et al, 1983]. Exposure and subsequent phagocytosis of asbestos by tracheal epithelial cells in culture produces cell damage that can be greatly diminished by SOD [Mossman and Landesman, 1983], suggesting that O$_2^-$ may be an important mediator of asbestos toxicity. Catalase or singlet oxygen scavengers are ineffective. Prolonged exposure (3–4 days) to asbestos produces marked increases in endogenous SOD activities, perhaps reflecting an adaptive response. Concordantly, Gulumian et al [1983] observed a dose-dependent increase in lipid peroxidation when asbestos is incubated with either rat liver or lung microsomes. Using spin trapping techniques, Weitzman and Graceffa [1984] have demonstrated that asbestos catalyzes the generation of hydroxyl and superoxide radicals from H$_2$O$_2$. Interestingly, in the tracheal epithelial system O$_2^-$ is produced in the absence of inflammatory cells. In fact, the role of an inflammatory process in the development of bronchogenic carcinoma is uncertain [Netteschein et al, 1981], although asbestos engenders pulmonary inflammation. Endotracheal lavage of mice 1 y after exposure for 75 days showed populations of lymphocytes and PMNs elevated 3- and 9-fold, respectively, compared to unexposed mice [Bozelka et al. 1983]. Additionally, human PMNs respond to coin-cubation with asbestos with phagocytosis and generation of chemiluminescence [Gau-mer et al, 1979]. The chemiluminescent response to different forms of asbestos correlates with in vivo fibrogenic and carcinogenic potentials. Asbestos also elicits a vigorous chemiluminescent response in guinea pig alveolar macrophages [Hatch et al, 1980]. However, analogous to the situation with TPA in mouse skin, the importance of oxidant production by inflammatory cells to the promoting action of asbestos is essentially unexplored.

There are a number of enzymes localized to mitochondria, peroxisomes, endoplasmic reticulum, and cytosol, which catalyze the univalent or divalent reduction of molecular oxygen [Chance et al, 1979]. In several instances, tumor promoters are known to modify the biochemical action of these organelles, such that output of O$_2^-$ and H$_2$O$_2$ are enhanced. Whether these effects directly relate to promotion is unresolved.

Hyperoxia induces an oxidant stress that is manifested primarily in the lung. Low-level chemiluminescence is increased in perfused lung as well as in perfused and in situ liver under conditions of hyperbaric oxygenation [Chance et al, 1979]. High oxygen tension increases O$_2^-$ formation by lung submitochondrial particles [Turrens et al, 1982a] and H$_2$O$_2$ release by intact lung mitochondria and microsomes [Turrens et al, 1982b] by 2–10-fold. Hyperoxia also appears to have a promoting effect. Heston and Pratt [1956] and DiPaulo [1959] have shown an enhancement in the number of pulmonary tumors by placing mice under increased oxygen tension following treatment with dibenz[a,h]anthracene or urethan, respectively. Dettmer et al [1968] have reported that exposure of rats to hyperoxia for 6 wk following 400 rads whole-body irradiation diminished the latency period and enhanced the incidence of mammary tumors.

Hepatocarcinogenesis proceeds in qualitatively distinct stages similar to the initiation-promotion process of skin carcinogenesis. Peraino et al [1971] originally demonstrated the enhancing effects of dietary phenobarbital exposure on hepatic tumorogenesis in rats previously fed 2-acetylaminofluorene and an expanding array of hepatic tumor promoters have since been enumerated [Pitot et al. 1980; Periera, 1982;
Jensen et al., 1982]. A common feature of many, but not all, of these agents (ie. phenobarbital, DDT, TCDD, polychlorinated, and polybrominated biphenyls, and di(2-ethylhexyl)phthalate) is their pronounced inductive effect on hepatic mixed function oxidase activity. The mixed function oxidase system, in the presence of pyridine cofactors, generates reactive oxygen metabolites [White and Coon, 1980; Trager, 1982; Ingelman-Sundberg and Hagbjork, 1982]. Microsomes induced with phenobarbital or pregnenolone-16α-carbonitrile generate \( H_2O_2 \) at a 5-fold greater rate than noninduced microsomes [Hildebrandt et al., 1973]. However, considerations that the elaboration of reactive oxygen species in response to enzyme induction is an important component of the action of these chemicals as tumor promoters must be tempered by the observations that not all inducers of hepatic mixed function oxidase activity are promoters (ie. amobarbital) [Peraino et al., 1975]. Additionally, Leonard et al [1982] and Jensen et al [1983] have recently reported that induction of hepatic mixed function oxidase enzymes is neither a prerequisite for nor an indicator of tumor promotion ability in a study utilizing brominated biphenyls.

A number of structurally divergent xenobiotics, including hypolipidemic drugs and phthalate plasticsizers, have been described as peroxisome proliferators in rodent liver. Peroxisomes are single membrane-limited cytoplasmic organelles that have been implicated functionally in gluconeogenesis, lipid metabolism, and the detoxification of \( H_2O_2 \). Sustained peroxisome proliferation has been associated with an increased occurrence of hepatocellular carcinoma and it appears that peroxisome proliferators as a class are carcinogenic [Reddy and Azarnoff, 1980; Warren et al., 1982]. Included in this grouping is the ubiquitous environmental contaminant, di(2-ethylhexyl)phthalate (DEHP), which produces hepatocellular carcinomas in F344 rats and B6C3F1 mice following long-term dietary exposure [Kluwe et al., 1982]. Ward et al [1983] have recently reported that DEHP exhibits promoting activity, and an apparent absence of initiating activity, in a B6C3F1 mouse liver assay using diethyl-nitrosamine as an initiating agent and that DEHP is a stage II promoter in mouse skin [Diwan et al., 1984]. Similarly, Reddy and Rao [1978] and Mochizuki et al [1982] have reported that the hypolipidemic drugs WY-14,643 and clofibrate promote the appearance of hepatocellular carcinomas following initiation of F344 rats by diethyl-nitrosamine. The antioxidants ethoxyquin and BHA inhibit the hepatic tumorigenic effect of another peroxisome proliferator, ciprofibrate [Rao et al., 1984], although peroxisome proliferation itself is unaffected [Lalwani et al., 1983]. The underlying mechanism of the promoting activities of these compounds may relate to the production of active oxygen species. Peroxisomes of liver and other organs contain a number of \( H_2O_2 \)-generating enzymes, including some flavoproteins, D-amino acid oxidase, L-α-hydroxyacid oxidase, fatty acyl CoA-oxidase and urate oxidase [Chance et al., 1979]. Clofibrate and other hypolipidemic drugs cause an 11-18-fold increase in the capacity of rat liver to oxidize long-chain fatty acids [Lazarow, 1977; Osumi and Hashimoto, 1978]. Increased \( H_2O_2 \) generation has been observed in the livers of rats administered peroxisome proliferators [Lalwani et al., 1981]. Catalase activities are also increased, but in a manner disproportionately small compared to the increase in peroxisome volume [Moody and Reddy, 1976] and \( H_2O_2 \)-generating fatty acid \( β \)-oxidation [Lazarow and DeDuve, 1976]. Prolonged exposure to peroxisome proliferators results in the excessive accumulation of autofluorescent lipofuscin in the liver [Reddy et al., 1982] providing indirect evidence for the increased production of biologically damaging free radicals. Although the exact nature of the fluorescent
chromophores in lipofuscin is poorly defined, it is ascribed to products of lipid peroxidation.

In addition to peroxisomal effects, phthalic acid esters also inhibit electron and energy transport in rat liver mitochondria [Takahashi, 1977]. Inhibition of mitochondrial respiration can result in enhanced $H_2O_2$ production [Boveris, 1977]. An inhibitor of mitochondrial electron transport, rotenone, is a hepatocarcinogen [Gosalvez, 1983]. Interestingly, Backer et al [1982] have shown that TPA inhibits mitochondrial respiration in C3H10T1/2 mouse fibroblasts at nanomolar concentrations. Although the nonpromoter 4-O-methyl TPA does not affect oxygen consumption, the promoters phorbol-12,13-dibutyrate and teleocidin are also inhibitory. Tangeras and Malviya [1983] find that micromolar concentrations of TPA have no effect on respiration or $H_2O_2$ production by isolated rat liver mitochondria. On balance, although mitochondria may be the major source of intracellular $O_2$ and $H_2O_2$, the importance of altered mitochondrial function in carcinogenesis remains undefined.

**OXYGEN RADICAL-BIOMOLECULE INTERACTIONS AND CARCINOGENESIS**

A variety of chemicals, including tumor promoters, can create a cellular prooxidant state either directly by elaborating oxy radicals or indirectly by disrupting the homeostasis that normally exists between the rate of cellular radical generation and the rate of radical dissipation. Although cells have various defense and repair mechanisms, this perturbation often leads to cellular injury and death [Halliwell, 1978; Bus and Gibson, 1979; Holtzman 1981; Trush et al, 1982; Hochstein, 1983; Sies and Cadenas, 1983; Thaw et al, 1983]. While it may be that similar reactive oxygen-target interactions are involved in both chemical-induced acute toxicity and carcinogenesis, the alterations resulting in the latter are likely to be more subtle and selective. Membrane lipids, free amino acids, nucleic acids, polysaccharides, enzymes, receptors, and structural and transport proteins have all been shown to be altered by oxy radicals [Demopoulos et al, 1980, 1983; Fridovich, 1981]. Many of these investigations have been conducted in well-defined chemical and/or isolated biochemical systems. However, the critical macromolecular target(s) and the cellular processes involved in reactive oxygen toxicity and carcinogenicity remain unresolved. The following paragraphs examine some of these interactions and how they may be related to carcinogenesis.

The interaction of reactive oxygen metabolites with DNA is well characterized [Brawn and Fridovich, 1981; Floyd, 1982; Schmidt and Borg, 1976; Schulte-Frohlinde, 1983; Van Hemmen and Mueling, 1975; Von Sonntag et al, 1981] and may result in chromosome aberrations, mutagenesis and modulation of gene expression. Tumor promoter-stimulated PMNs produce a series of DNA damaging oxidant species that are derived in part from oxy radicals [Birnboim, 1982a,b, 1983; Birnboim and Biggar, 1982; Emerit and Cerutti, 1981, 1982, 1983; Cerutti et al, 1983]. DNA strand breaks are detectable in PMNs 5 minutes following TPA exposure and are maximal by 45 min. Nonphorbol epidermal tumor promoters such as dihydroteleocidin B, anthralin, benzoyl peroxide, $H_2O_2$, iodoacetic acid, and canthardin are also effective inducers of DNA strand breaks in PMNs [Birnboim, 1983]. An extensive evaluation by Birnboim [1982b] of inhibitors of TPA-mediated DNA damage suggests a mechanism involving $O_2^-$ and $H_2O_2$, but the nature of the ultimate damaging radical remains unclear. SOD and catalase block the response;
however, 'OH scavengers such as DMSO and glycerol are not very effective and ¹⁰₂ scavengers produce no decrease. O₂⁻ generated from a xanthine oxidase/xanthine system also produces DNA strand breaks in unstimulated PMNs [Birnboim, 1983].

Emerit and Cerutti [1981, 1982, 1983] have shown that exposure of PMNs, monocytes, or phytohemagglutinin-stimulated lymphocytes in the presence of monocytes or platelets to TPA, but not its weakly promoting derivatives, induces chromosomal aberrations such as breaks, gaps, fragments, dots, double minutes, interchanges, and pulverization. This clastogenic action of TPA, which is inhibited by SOD, is indirect and is mediated by secondary stable products which are formed by the cell in response to the interaction with the tumor promoter. In addition to active oxygen species, intermediates of arachidonate metabolism are involved in the formation and action of clastogenic factor because phospholipase A₂ inhibitors as well as inhibitors of the cyclooxygenase and lipoxygenase pathways are anticlastogenic [Cerutti et al, 1983; Emerit et al, 1983]. Preliminary chromatographic evidence suggests that the clastogenic factor is composed of lipid hydroperoxides, aldehydic break-down products, and free arachidonic acid. Cerutti and coworkers have proposed a model for "membrane mediated chromosomal damage" in which membrane-active agents such as some tumor promoters and complete carcinogens (eg, aflatoxin B₁) elicit an oxidative burst and stimulate the arachidonate cascade to perturb membrane integrity so that phospholipids are more susceptible to autooxidation and form a DNA-damaging clastogenic factor [Cerutti et al, 1983]. In addition to the clastogenic factors, Weiss et al [1983] have found that TPA-activated PMNs produce a stable oxidant that has sufficient potential to modify sulfhydryl and thioether containing molecules. The elaboration of these phagocyte-derived N-chloramines is inhibited by catalase, but not SOD. The differential susceptibilities to inhibitors suggests that these three DNA damaging and oxidant factors are probably distinct.

The elaboration of oxygen radicals by PMNs also mediates genotoxicity in other cells. PMNs stimulated by phagocytosis increase reversion frequency in Salmonella typhimurium strain TA100 [Weitzman and Stossel, 1981] and induce dark mutants of the luminous bacteria Photobacterium fischeri to revert to heredity stable luminescent forms [Barak et al, 1983]. Phorbol ester-stimulated PMNs produce cytogenetic changes in mammalian cells as evidenced by a dose-dependent increase in sister chromatid exchange in CHO [Weitberg et al, 1983] and V-79 (Popescu NC, Trush MA and Kensler TW, unpublished observations) fibroblasts. Mutagenesis can also be induced in CHO cells by potassium superoxide and is inhibited by SOD [Cunningham and Lokesh, 1983]. Birnboim [1983] reports that when TPA-stimulated PMNs are cocultured with erythroleukemia cells appreciable DNA strand breakage is observed in the secondary target cells, but in this instance SOD is not protective, although catalase remains so. Collectively, these studies demonstrate that diffusible species act as mediators of inflammatory cell-induced genotoxicity. Addition of TPA to primary cultures of epidermal cells results in substantial increases in chromosomal aberrations [Fusenig et al, 1982]. However, Gensler and Bowden [1983] have shown that DNA strand scission is unrelated to irreversible late stage promotion by TPA in JB-6 mouse epidermal cells although TPA does induce DNA single strand breaks in primary mouse epidermal cells cocultured with macrophages [Dutton and Bowden, 1984]. Thus the importance of DNA damage in promotion certainly requires further clarification. Birnboim [1983] has presented several hypotheses suggesting how reactive
oxygen-mediated DNA damage might be interlinked with oncogene activation and expression of a malignant phenotype. For example, studies by Sher and Friend [1978] and Terada et al [1978] suggest that DNA strand breaks induced by a variety of stimuli may trigger derepression of genes required for hemoglobin synthesis in erythroid leukemia cells. Aft and Mueller [1983] have recently proposed that hemin may implement changes in gene expression associated with erythroid differentiation by the generation of localized reactive oxygen species that produce DNA nicks.

DNA damage by chemical carcinogens, radiation, or ultraviolet light is subject to enzymatic repair [Hanawalt et al., 1979; Kimball, 1979]. While these systems have been extensively characterized in bacterial systems, their existence in mammalian cells are implicated as a result of defective DNA repair in cell lines established from individuals with ataxia telangiectasia and xeroderma pigmentosum [Patterson et al., 1983]. DNA repair-deficient bacteria strains are hypersensitive to \( \text{H}_2\text{O}_2 \) [Demple et al., 1983] and it has been demonstrated that the rec\(^+\) gene product may be more important than SOD and catalase in protecting \( E.\text{coli} \) against \( \text{H}_2\text{O}_2 \) toxicity [Carlson and Carpenter, 1980]. A newly described component of DNA repair processes is poly(ADP-ribose) polymerase. This enzyme catalyzes the formation of poly(ADP-ribose) from \( \text{NAD}^+ \) in a reaction that requires histones and damaged DNA [Yamada et al., 1971]. Although the exact cellular function of poly(ADP-ribose) is not known, there appears to be a relationship between its synthesis and DNA damage and repair in mammalian cells. Inhibition of poly(ADP-ribose) polymerase enhances DNA strand breakage and sister chromatid exchange induced by alkylating agents [Park et al., 1983] and potentiates cell killing by ultraviolet light [Durrant and Boyle, 1982]. Sugimura and Miwa [1983] have recently summarized the relationship of poly(ADP-ribose) to various aspects of cancer. Studies with alloxan suggest a relationship between poly(ADP-ribose) synthesis, \( \text{NAD}^+ \) levels and oxidant-mediated toxicity. Alloxan undergoes both enzyme-catalyzed and enzyme-independent redox cycling resulting in reactive oxygen generation [Malaisse, 1982] and SOD and catalase protect against alloxan-induced DNA strand breaks and inhibition of proinsulin synthesis in alloxan-exposed pancreatic islets [Yamamoto et al., 1981; Uchigata et al., 1982]. This latter effect is attributed to the depletion of \( \text{NAD}^+ \) resulting from its utilization by nuclear poly(ADP-ribose) polymerase. Increased oxygen tension also reduces cellular nicotinamide adenine dinucleotide levels through oxygen-mediated inactivation of the enzymes involved in \( \text{NAD}^+ \) biosynthesis [Brown et al., 1979]. Thus, depletion of \( \text{NAD}^+ \) by this mechanism would also influence the subsequent repair of DNA damage by the poly(ADP-ribose) system.

In his now classic review on cellular mechanisms of oxygen toxicity, Haugaard [1968] emphasized the importance of enzyme inactivation in this process. More recently, Fucci et al [1983] have put forth the concept that oxidant-mediated inactivation of key metabolic enzymes may serve a function in regulating cellular protein turnover. Enzymes shown to be inactivated in this study included various kinases, synthetases and \( \text{NAD(P)}\text{H-dependent dehydrogenases} \). The inclusion of catalase in the admixture prevented these oxidant-mediated processes, no matter whether the oxidant was generated by \( \text{NAP} \)H oxidase, the \( \text{NADPH} \) cytochrome P-450 reductase/ P-450 system, or the ferrous iron-\( \text{H}_2\text{O}_2 \) system. Addition of \( \text{H}_2\text{O}_2 \) to the reduced ferrous form of P-450 destroys this hemoprotein [Guengerich, 1978] and inactivates SOD [Bray et al., 1974]. Willson and co-workers [Packer et al., 1978; Searle and Willson, 1980; Willson, 1977, 1982] have discussed the sensitivity of various amino
acids as targets in the inactivation of enzymes such as lysozyme, ribonuclease, and glutathione peroxidase by \`OH and the trichloromethyl peroxy and thiocyanate radicals.

The relevance of enzyme inactivation at the cellular level has been demonstrated by the studies of Brown et al [1979]. Exposure of \textit{E coli} to hyperbaric oxygen results in the inactivation of one of the key enzymes in NAD\(^+\) biosynthesis, quinolate phosphoribosyl transferase. The inhibition of this enzyme is accompanied by significant decline in total cellular pyridine nucleotide content. Niacin, which enters the NAD\(^+\) biosynthetic pathway beyond this enzyme, prevents oxygen-dependent toxicity in \textit{E coli} and the alterations in pyridine nucleotide content. Niacin also prevents hyperoxia-induced toxicity to alveolar macrophages and paraquat-mediated toxicity in rats [Brown et al, 1981; Pearl and Ruffin, 1983]. As discussed previously, NAD\(^+\) is important, not only as a coenzyme in a large number of cellular reactions, but in the poly(ADP-ribose) DNA repair system as well.

In contrast to the inhibiting effect of a pro-oxidant state on enzymes, guanylate cyclase, a prominent enzyme involved in regulating cell growth and transformation, is activated in the presence of oxidant-generating systems. DeRubertis and Creaven [1982] have recently reviewed the various aspects of guanylate cyclase activity in relationship to the actions of a number of carcinogens. White et al [1976] demonstrated that either the direct addition of H\(_2\)O\(_2\), or its generation by glucose oxidase/glucose, activates this enzyme in lung supernatant. The tumor promoter benzoyl peroxide also activates guanylate cyclase as does O\(_2^-\) resulting from the redox cycling of paraquat [Goldberg et al, 1978; Vesely et al, 1979]. Oberley et al [1981] have discussed how the ratio of cGMP to cAMP may be involved in regulating normal cell division and how an increase in the cGMP/cAMP ratio, along with the attainment of cellular immortality, may be integral to the malignant transformation of cells.

Whereas guanylate cyclase represents an example of direct enzyme activation by an oxidant environment, exposure of cells to a pro-oxidant state also results in the induction of enzyme synthesis. Examples of this process, include induction of manganese-containing SOD in \textit{E coli}, benzo(a)pyrene hydroxylase activity in rat liver cells, and a DNA repair system in \textit{E coli} [Hasson and Fridovich, 1979; Paine and Francis, 1980; Demple and Halbrook, 1983]. In summary then, oxygen radicals may mediate enzymic denaturations and inactivations, activations, and inductions, which could result in pertubations in control of cellular growth and differentiation.

A number of carcinogens that are metabolized to radical intermediates result in increased peroxidation of various cell organelles. Reactive oxygen-dependent lipid peroxidation has also been implicated in the mechanism of radiation-induced carcinogenesis [Petkau, 1980], ultraviolet light-induced skin carcinogenesis [Logani and Davies, 1980], and the action of epidermal tumor promoters [Logani et al, 1982; Stohs et al, 1983]. It appears that the hydroxyl radical and iron chelates, which function like a hydroxyl radical, are of particular importance in lipid peroxidation [Lai and Piette, 1977; Tien et al, 1982].

Both nonspecific membrane peroxidation and enzymatically controlled stereospecific lipid peroxidation associated with prostaglandin synthesis can mediate the formation of highly reactive and diffusible aldehyde and carbonyl products and clastogenic factors which can amplify these forms of peroxidation and enhance damage. For example, 4-hydroxynoenal, formed during iron-dependent microsomal peroxidation, has been shown to alter mitochondrial function, polyamine synthesis,
tubulin function, adenylate cyclase activity, and protein and DNA synthesis [Dianzani, 1982]. The effects and manifestations of membrane peroxidation are diverse. Peroxidation of plasma membrane can result in decreased ligand binding to receptor and inhibition of sodium-potassium ATPase or adenylate cyclase activity [Demopoulos et al. 1980; Fourcans. 1974; Maridonneau. 1983; Muakkassah-Kelly et al. 1982]. Such alterations can be attributed to both direct effects on enzymes and indirect effects mediated through changes in the lipid environment. Lipid peroxidation in mitochondria induces swelling and alteration of respiratory function [Aono et al., 1981]. Peroxidation products of the nuclear membrane have been implicated as mediators of DNA damage [Ames et al. 1982]. Nuclear-cytoplasmic communication, such as RNA transport [Yannarelli and Awad, 1982], may also be affected by peroxidation at this site. Endoplasmic reticulum subjected to a peroxidation reaction is functionally modified as characterized by inhibition of microsomal ATP-dependent calcium sequestration and mixed-function oxidase activity [Bidlack and Tapel, 1973; Jones et al., 1983]. Thus, all cell membranes can be subject to radical-mediated modification. While the initial consequences of peroxidation are on membrane structure and function and integrity of cell compartmentalization, the ultimate manifestations, short of cell death, could be reflected in altered cellular metabolic and regulatory activities.

**SUMMARY**

The concept that oxygen radicals are involved in the tumor promotion process is presently based on three groups of experimental evidence. First, it has been demonstrated that different classes of tumor promoters can evoke the elaboration of oxygen radicals by direct chemical generation or through the indirect activation or alteration of cellular metabolic sources. Although the bulk of information concerning the indirect action of promoters deals with effects in non-target cell populations, i.e., inflammatory cells, these cells may be coordinately involved in some aspects of promotion or may serve as a model to presage similar effects in target cells, as suggested by an incipient literature [Fischer and Adams, 1984; Nakamura et al. 1984]. Second, tumor promoters can be shown to modulate cellular antioxidant defenses. For example, epidermal SOD and catalase activities are diminished following promoter exposure, perhaps allowing for amplified oxygen radical-biomolecule interactions. Third, reactive oxygen scavengers and detoxifiers, such as SOD, SOD-mimetics, catalase, and phenolic antioxidants, inhibit biochemical and biological actions of tumor promoters in vitro and in vivo, thus providing strong presumptive evidence for oxygen radical involvement in the later stages of neoplastic development. The critical macromolecular targets for these oxygen radicals remain undefined; however, they may include lipids, DNA, DNA repair systems, and other enzymes, and their effects on these biomolecules and cellular processes are compatible with current theories of carcinogenesis.

**ACKNOWLEDGMENTS**

The authors gratefully acknowledge the financial support of the American Cancer Society, Maryland Division, cancer prevention grant SIG-3, and the National Institutes of Health, grants ES00454, CA36380, and BRS.
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