DCA is due to deficient activity of the medium-chain acyl-CoA dehydrogenase, the enzyme responsible for initiating the β-oxidation of C_6–C_{10} fatty acyl-CoA's. To our knowledge, DCA is the first inborn error of metabolism in man that has been found to be caused by a deficiency of one of the enzymes of the β-oxidation cycle. This study underscores the utility of using purified mitochondrial preparations from normal diploid fibroblasts to study the biochemistry of human metabolic diseases. The use of such mitochondrial preparations from fibroblasts or other cultured human cells should allow us to pursue detailed investigations of the biochemistry of DCA, as well as other human disorders involving mitochondrial function, such as glutaric aciduria type II, ethylmalonic-adipic aciduria (*13, 15*), and the etiologically heterogeneous group of human mitochondrial myopathies (*18*).

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25 October 1982; revised 30 December 1982

Inhibition of Tumor Promotion by a Biomimetic Superoxide Dismutase

Abstract. *A low molecular weight, lipophilic, copper coordination complex with superoxide dismutase-mimetic activity inhibited biochemical and biological actions of a tumor promoter in mouse epidermis. Such inhibitory effects implicate reactive oxygen species in the tumor promotion process.*

Tumor promoters are noncarcinogenic compounds that cause the development of tumors when applied repeatedly after initiation with a single subthreshold dose of a carcinogen. The phenomenon of two-stage carcinogenesis (initiation followed by promotion) has been studied most thoroughly in mouse skin, where esters of the tetracyclic diterpene, phorbol, are the most potent promoters. Topical application of phorbol esters to mouse skin results in numerous biochemical changes that lead to enhanced cell proliferation and that are associated with the promotion process (*1*). Reactive oxygen, that is, free oxygen radicals may play important roles as mediators of these pleiotropic responses.

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_2O_2), the hydroxyl radical (OH·), and finally water. The intermediates of oxygen reduction to water are highly reactive and present a challenge to the integrity of cells. As either an oxidant or a 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, cleave DNA, and kill cells (*2*). In peripheral white cells, the formation of O_2^-, triggered by phorbol esters, produces extensive single-strand breakage of DNA (*3*). However, not all reactions of oxygen radicals are necessarily destructive to biological macromolecules. H_2O_2 in particular appears to be involved in the activation of guanylate cyclase and glucose transport (*4*). The possible role of O_2^- and H_2O_2 in the regulation of cell division has been recently reviewed by Oberley *et al.* (*5*).

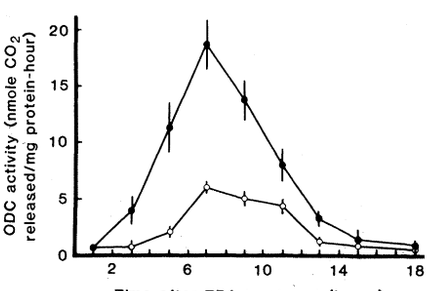

Fig. 1. Effect of treatment with CuDIPS on the induction of epidermal ODC activity by TPA. Mice were shaved with surgical clippers 2 days before use and only those in the resting phase of the hair growth cycle were used. Animals were treated topically with 5 μmole of CuDIPS (in 0.2 ml of diethyl ether) (●) 30 minutes before the application of 17 nmole of TPA (in 0.2 ml of acetone) and killed at the indicated times after TPA treatment. Epidermis was isolated and ODC activity determined in the 12,000*g* supernatants of epidermal homogenates (*13*). Each point represents the mean ± standard error of triplicate determinations done on each of three mice.

The involvement of reactive oxygen in tumor promotion is suggested by several recent observations. Leukocytes, macrophages, and lymphocytes respond to promoting agents, such as phorbol esters, by generating O_2^- and chemiluminescense, and antipromoting agents such as retinoids, protease inhibitors, and anti-inflammatory drugs inhibit these oxygen-related responses (*6*). Furthermore, benzoyl peroxide, lauroyl peroxide, and *m*-chlorobenzoic acid, all free radical–generating compounds, promote skin tumors in mice (*7*), whereas antioxi-

dants, which act as terminators of free radical chain reactions, antagonize the process (*8*). Moreover, phorbol esters provoke a rapid and sustained decrease in murine epidermal superoxide dismutase (SOD) and catalase activities (*9*), the foremost defenses against oxygen radical toxicity. These findings prompted us to ask what effect replacement of the diminished SOD activity would have on the promotion process. Native SOD, a metalloprotein of high molecular weight, does not penetrate well into cells (*10*); however, this limitation may be overcome by the use of low molecular weight, lipophilic, copper complexes with SOD-mimetic action, such as copper(II) (3,5-diisopropylsalicylic acid)$_2$ (CuDIPS) (*11*).

The induction of ornithine decarboxylase (ODC) activity is a prominent event after exposure to epidermal tumor promoters (*12*), and the quantification of ODC activity offers a useful first approach to the identification and characterization of potential antagonists of the tumor promotion process. The topical application of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) to mouse epidermis results in a rapid and transient induction of epidermal ODC activity. As shown in Fig. 1, peak ODC activity (18.6 nmole per milligram of protein per hour) occurred 7 hours after application of TPA, and enzyme activity returned to near basal levels by 18 hours. Treatment of mouse skin with 5 μmole of CuDIPS 30 minutes before TPA application resulted in a 70 percent reduction in the peak induction of ODC activity. There was no evidence of an altered time course in TPA-induced ODC activity after CuDIPS treatment. Topical administration of CuDIPS caused a dose-dependent inhibition of TPA-induced ODC activity over the range of 0.1 to 10 μmole (Fig. 2A). The dose required to inhibit the induction of ODC by 50 percent was approximately 1 μmole. While application of 10 μmole of CuDIPS essentially abolished the induction of ODC by TPA, application of equimolar 3,5-diisopropylsalicylic acid or cupric acetate engendered only 15 and 35 percent inhibition, respectively, underscoring the specificity of the action of CuDIPS. Inhibition of the induction of ODC activity was strongly dependent on the time of application of CuDIPS (Fig. 2B). Maximum inhibition occurred when CuDIPS was applied immediately after TPA. Whereas substantial inhibition (> 50 percent) was maintained if CuDIPS was given within ±2 hours of TPA, earlier or later applications were only minimally effective. Two conclusions are suggested by this finding. First, the inhibitory effect of CuDIPS is not a manifestation of cell toxicity since in that case inhibition at all time points would be expected, and second, CuDIPS acts as an early antagonist of the phorbol ester–induced response. Phenolic antioxidants produce a similar pattern of inhibition of ODC induction (*13*), suggesting a rapid elaboration of reactive oxygen or free radical species in the skin after TPA treatment.

Table 1. Effect of CuDIPS on tumor promotion. Female CD-1 mice (7 to 9 weeks old) were shaved with surgical clippers 2 days before treatment and only those mice not exhibiting hair growth were used. Eighteen mice were used for each treatment group. All mice received a single topical application of 0.2 μmole of 7,12-dimethylbenz[*a*]anthracene dissolved in 0.2 ml of acetone. Beginning 10 days after initiation, mice were treated with 4 nmole of TPA twice weekly for 20 weeks. Mice were also treated with either 0.5 or 2 μmole of CuDIPS, 4 μmole of 3,5-diisopropylsalicylic acid (DIPS), or 2 μmole of cupric acetate 30 minutes before each TPA application; control mice received the appropriate vehicle only. The number and incidence of papillomas were recorded weekly.

Group	Treatment		Number		
	Modifier (μmole)	TPA (nmole)	Mice alive at 20 weeks	Papillomas per mouse at 20 weeks	Mice with papillomas at 20 weeks
1	CuDIPS (0.5)	TPA (4)	18	6.9*	17
2	CuDIPS (2)	TPA (4)	18	0.9*	6†
3	Diethyl ether	TPA (4)	18	12.3	16
4	Cupric acetate (2)	TPA (4)	17	11.3	16
5	DIPS (4)	TPA (4)	17	7.5	13
6	Acetone and H_2O	TPA (4)	17	9.4	15
7	Diethyl ether	Acetone	18	0	0
8	CuDIPS (2)	Acetone	18	0	0

*Differs from control ($P < .05$ for group 1 and $P < .01$ for group 2) by Duncan's new multiple-range test on data transformed by $\sqrt{x + 1}$ to stabilize the variance. Kruskal-Wallis test followed by Dunn's multiple comparison procedure gave essentially the same results. †Differs from control ($P < .01$) by χ^2 test.

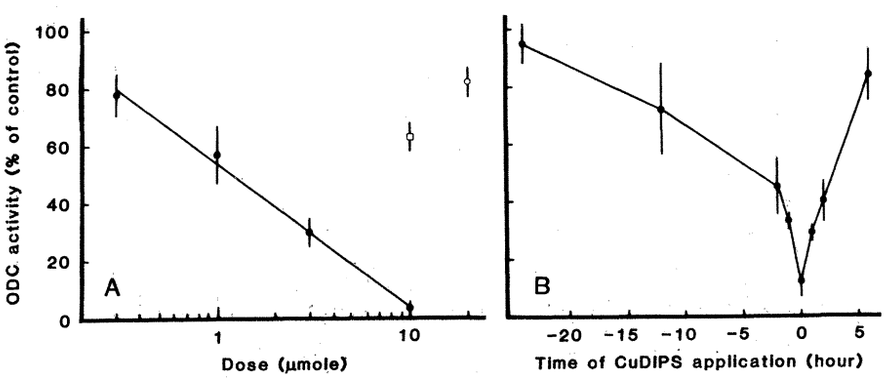

Fig. 2. (A) Effect of dose of CuDIPS on TPA-induced ODC activity. Mice were treated with various doses of CuDIPS 30 minutes before the application of 17 nmole of TPA; epidermal supernatants were prepared 7 hours after TPA treatment. Symbols: (●), indicated doses of CuDIPS; (□), 10 μmole of cupric acetate [$(CH_3COO)_2Cu \cdot H_2O$]; and (○), 20 μmole of 3,5-diisopropylsalicylic acid. Acetone and H_2O (9:1 by volume) was used as the vehicle for cupric acetate and 3,5-diisopropylsalicylic acid. Prior treatment with either diethyl ether or aqueous acetone was without differential effect on maximal ODC induction by TPA. (B) Effect of time of CuDIPS addition on TPA-induced ODC activity (mean ± standard error). All mice received 17 nmole of TPA at time 0 and were killed 7 hours after TPA treatment. Experimental groups of four mice each received 5 μmole of CuDIPS at the indicated times before or after TPA application.

The effect of CuDIPS on the incidence of skin tumors promoted by TPA was also evaluated. As shown in Table 1, application of 0.5 or 2 μmole of CuDIPS 30 minutes prior to each application (twice weekly for 20 weeks) of 4 nmole of TPA to the skin of mice initiated with 7,12-dimethylbenz[*a*]anthracene resulted in a 46 and a 93 percent reduction, respectively, in the number of papillomas per mouse compared to those initiated mice that received vehicle and TPA alone. Initiated mice that received TPA had a tumor incidence of 89 percent, whereas mice that received TPA and 2 μmole of CuDIPS had a tumor incidence of 33 percent. In contrast, application of cupric acetate or 3,5-diisopropylsalicylic acid at doses equimolar to the high dose of CuDIPS was without significant effect

on tumor yield or on the number of mice bearing papillomas. Mice receiving either diethyl ether or CuDIPS and acetone in lieu of TPA developed no tumors, indicating that CuDIPS has no tumor-promoting properties itself.

That the antipromoter effects of CuDIPS in mouse epidermis can be ascribed to its existence as a chelate in vivo with SOD-like chemical reactivity rests on the observed failure of cupric acetate or 3,5-diisopropylsalicylic acid to appreciably inhibit either phorbol ester–induced ODC activity or tumor promotion. Cupric acetate, used as a control for the copper component of CuDIPS, does have the capability of scavenging O_2^-; however, in biological systems, ionizable forms of copper frequently lose their SOD-like activity because they form complexes with protein (*14*). 3,5-Diisopropylsalicylic acid alone has no O_2^- scavenging activity (*11*). This ligand presumably confers a high degree of lipophilicity to copper allowing for the preferential subcellular localization of the copper complex at sites of O_2^- production, accumulation, or reactivity, such as membrane matrices and membrane-cytoplasmic interfaces. By contrast, exogenously applied bovine erythrocyte SOD is excluded from such sites (*10*) because of its size and lack of lipophilic character and as such is an ineffective antagonist of phorbol ester action (*15*).

The finding that a low molecular weight, lipophilic, copper chelate with SOD-mimetic activity can inhibit certain phorbol ester–induced biochemical and biological responses strengthens the arguments for an essential role of oxygen radicals in the promotion stage of carcinogenesis and of SOD or SOD-like compounds in the homeostatic prevention of carcinogenesis. The action of oxygen radicals in the stimulation of cell proliferation and progression by tumor promoters may involve a direct or indirect modification of genomic integrity (*3*) or modulation of biochemical processes such as the arachidonate cascade and cyclic nucleotide metabolism (*5*). Although O_2^- is clearly a component of the phorbol ester–induced response, the identification of the pertinent activated oxygen species and their macromolecular targets in the tumor promotion process is an important goal.

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2 December 1982; revised 24 January 1983

Metabolism of Polycyclic Aromatic Hydrocarbon Derivatives to Ultimate Carcinogens During Lipid Peroxidation

Abstract. *Lipid peroxidation triggered by ascorbate or reduced nicotinamide adenine dinucleotide in rat liver microsomes can initiate the epoxidation of 7,8-dihydroxy-7,8-dihydrobenzo*[a]*pyrene. The stereochemistry of epoxidation is indicative of a peroxide-dependent free radical process. Since the epoxides formed may be the most carcinogenic derivatives of benzo*[a]*pyrene yet identified, lipid peroxidation can effect the metabolic activation of proximate carcinogens to ultimate carcinogens.*

Oxidation is a critical step in the metabolic activation of many xenobiotics to toxic, mutagenic, and carcinogenic derivatives (*1*). Benzo[*a*]pyrene (BP), a widespread environmental pollutant, is converted to a number of derivatives by the combined action of oxygenases and conjugating enzymes (*2*). One of the metabolites, 7,8-dihydroxy-7,8-dihydrobenzo-[*a*]pyrene (BP-7,8-diol) (Fig. 1), is further oxidized to dihydrodiol epoxides, which represent ultimate carcinogenic

Table 1. NADPH- and ascorbate-dependent oxidation of BP-7,8-diol. Microsomes were suspended (0.5 mg of protein per milliliter) in 0.1*M* tris buffer (*p*H 7.5) at 37°C. Incubations contained 40 μ*M* BP-7,8-diol and various combinations of the following cofactors: NADPH (1.0 m*M*), ADP (4.0 m*M*), Fe^{3+} (15 μ*M*), EDTA (100 μ*M*), Fe^{2+} (110 μ*M*), and ascorbate (1.0 m*M*). Reactions were initiated by the addition of NADPH or ascorbate, and the incubation period was 20 minutes. Values are means ± standard errors.

Cofactors	TBA-reactive material*	Diol metabolism†	Tetraol formation‡	*Anti/syn* ratio
NADPH	0.30 ± 0.07	6.0 ± 1.9	0.6 ± 0.2	1.1 ± 0.3
NADPH + ADP-Fe^{3+} + EDTA-Fe^{2+}	14.0 ± 1.3	27.0 ± 4.3	19.0 ± 4.4	2.5 ± 0.4
NADPH + ADP-Fe^{3+} + EDTA-Fe^{2+} + EDTA§	0.4 ± 0.1	4.0 ± 0.8	0.4 ± 0.1	0.5 ± 0.1
Ascorbate + ADP-Fe^{3+}	23.0	31	14	2.6
Hematin-ROOH (*8*)				2.5

*Nanomoles of malondialdehyde formed per milligram of protein. †Percentage of total radioactivity eluting from high-pressure liquid chromatography in zones not cochromatographing with the authentic standard of BP-7,8-diol. ‡Percentage of total radioactivity eluting in zone cochromatographing with authentic tetraol standards. §Includes 1 m*M* excess EDTA, which completely inhibits lipid peroxidation under these conditions (*25*).