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Ornithine Decarboxylase Activity: Control by Cyclic Nucleotides

Abstract. *Both exposure to cold and administration of aminophylline result in rapid increases in cyclic adenosine monophosphate (cyclic AMP) in the adrenal medulla and adrenal cortex. These increases are followed by dramatic increases in ornithine decarboxylase activity. Inhibitor studies suggest that the increase in ornithine decarboxylase activity is due to new enzyme synthesis. The data suggest that the decarboxylase activity is regulated by an increase in cyclic AMP.*

Polyamine biosynthesis is one of the earliest events that occurs during tissue growth. This increased synthesis takes the form in a variety of tissues of dramatically elevated levels of ornithine decarboxylase (ODC), the initial enzyme in the polyamine biosynthetic pathway. Activity of ODC is highest in those tissues with high rates of growth or protein synthesis. In contrast, ODC activity in static or nongrowing tissues is very low. When induced in mammalian liver by partial hepatectomy, ODC activity rises rapidly and remains elevated for 2 days (*1*). Also, ODC activity can be increased by all those hormones that affect growth processes (*2*). Following administration of inhibitors of protein synthesis, ODC activity returns to the control level, with a half-life of 10 to 20 minutes (*3*). This ability of ODC activity to fluctuate rapidly in response to the introduction or withdrawal of stimuli suggests that ODC elevation constitutes an important step in the control of cellular mechanisms. Because cyclic nucleotides are considered the second messenger in a chain of events between the receptor site on the cell membrane and the subsequent increase in biosynthetic activity, we thought that it was important to ascertain whether adenosine 3′,5′-monophosphate (cyclic AMP) might mediate the activity of ODC. We have therefore studied the relationship between cyclic AMP and ODC in the adrenal medulla of the rat. Within 30 minutes of exposure to cold (4°C), in response to cholinergic nerve impulses, the intracellular cyclic AMP increases 10- to 20-fold in the adrenal medulla of the rat (*4*). Within 2 hours this elevation disappears, and the cyclic AMP concentration returns to normal. Exposure to cold leads to the stimulation of the splanchnic nerve and results in the transynaptic induction of tyrosine hydroxylase (*4*), the regulating enzyme in catecholamine synthesis. Because of the general relation between an elevation in ODC activity and subsequent increases in biosynthetic activity, we thought that an increase in ODC activity might be intermediate between the rise in cyclic AMP in the adrenal medulla and the delayed induction of tyrosine hydroxylase. Therefore, we studied the relation between cyclic AMP and ODC in the adrenal medulla and adrenal cortex after cold exposure and in another group after aminophylline injection.

All experiments were performed with male Sprague-Dawley rats (125 to 150 g). For cyclic AMP determinations, the animals were decapitated and the adrenal glands were removed and frozen within 10 seconds. The adrenal medulla was rapidly separated from the adrenal cortex at 0° to 4°C with the aid of a dissecting microscope. Cyclic AMP was purified from the other acid-soluble nucleotides (*5*), and its concentration was determined by a protein kinase assay (*6*).

The ODC activity was measured by the release of $^{14}CO_2$ from *dl*-[1-^{14}C]-ornithine (7.66 mc/mmole, New England Nuclear) as described (*1*), with minor modifications. The buffer used was 0.05*M* sodium potassium phosphate, *p*H 7.2, containing 1 m*M* dithiothreitol (DTT). Monolaterally denervated (by splanchnicotomy) animals were purchased (Zivic-Miller, Allison Park, Pa.) and used 6 to 8 days after they underwent operation.

We substantiated the finding that within 30 minutes of subjecting the animals to cold, cyclic AMP in the adrenal medulla increased 10- to 20-fold (Fig. 1A). The cyclic AMP concentration returned to normal within 2 hours. Ornithine decarboxylase activity is elevated within 1 hour of cold exposure, becoming 10 to 20 times that of the control within 4 hours. Splanchnicotomy abolishes both the increase in

Table 1. Cyclic AMP concentration and ODC activity in the adrenal medulla and adrenal cortex of rats after cold exposure. Animals were exposed to cold (4°C, 2 hours) as described in Fig. 1. The cyclic AMP concentrations and ODC activities were determined at 0.5 and 4.5 hours, respectively, relative to the initiation of cold exposure. The experiments were performed with monolaterally denervated animals. The data are represented also relative to values obtained from control animals not subjected to cold exposure. The values are the mean ± the standard error.

Splanchnicotomy	Cold exposure	Cyclic AMP		ODC activity	
		Amount (pmole per mg of protein)	Percent of control	Amount (pmole $^{14}CO_2$/30 min per mg of protein)	Percent of control
		Medulla			
No	No	41 ± 5	100 ± 12	20 ± 4	100 ± 25
No	Yes	426 ± 22	1040 ± 55	150 ± 8	750 ± 40
Yes	No	39 ± 6	100 ± 15	24 ± 7	100 ± 29
Yes	Yes	89 ± 23	230 ± 60	43 ± 6	180 ± 25
		Cortex			
No	No	11 ± 3	100 ± 27	18 ± 3	100 ± 16
No	Yes	72 ± 19	650 ± 180	146 ± 19	810 ± 105
Yes	No	11 ± 2	100 ± 18	22 ± 4	100 ± 18
Yes	Yes	68 ± 13	620 ± 115	181 ± 12	825 ± 55

cyclic AMP concentration and the rise in ODC activity normally seen after exposure to cold (Table 1), suggesting that ODC activity also is increased in response to transynaptic stimulation. In the adrenal cortex after exposure to cold, there is an early, less dramatic increase in cyclic AMP, that is, four- to fivefold 30 minutes after exposure (Fig. 1B). In the cortex, ODC activity also increases but does not rise as early as in the medulla since an increase is not detectable until 3 to 4 hours after the initiation of cold exposure, and maximal stimulation occurs within 6 hours (Fig. 1B). These increases cannot be abolished by denervation of the adrenal medulla (Table 1). The observed elevations in cyclic AMP and ODC in the adrenal cortex are probably mediated by adrenocorticotropic hormone (ACTH) since ACTH is known to be released during times of stress and since injection of ACTH increases cyclic AMP concentration and ODC activity in the adrenals (*7*).

Aminophylline, an inhibitor of phosphodiesterase activity, increases cyclic AMP (under most conditions) by inhibiting its degradation (*8*). After a single injection of aminophylline (200 μmole/kg, injected intraperitoneally), cyclic AMP in the adrenal medulla increases to a level 10- to 15-fold above that of controls, an increase similar to that after exposure to cold (Fig. 1C). Subsequently, ODC activity also increased in a manner similar to that after cold exposure (Fig. 1C). The temporal increases in cyclic AMP and ODC activity in the adrenal cortex after treatment with aminophylline are analogous to those found after cold exposure (Fig. 1D), with a greater increase in the amount of cyclic AMP after treatment with the drug. These data suggest that ODC activity is regulated by the rapid increase in cyclic AMP.

Several mechanisms are possible by which cyclic AMP, through the activation of cyclic AMP–dependent protein kinase, could mediate the increase in ODC activity. The most likely are (i) phosphorylation of ribosomal proteins, allowing transcription of stored messenger RNA (mRNA) from ODC (*9*); (ii) phosphorylation of nuclear histone and nonhistone proteins which would allow transcription of specific mRNA (*10*); and (iii) direct phosphorylation of ODC.

Administration of drugs whose major effects are on DNA-dependent RNA synthesis and protein synthesis (Table 2) suggest that the most likely mechanism is (ii) phosphorylation of nuclear proteins which results in new mRNA that is specific for ODC synthesis. Either cycloheximide or actinomycin D administered 30 minutes before exposure to cold abolishes the response of ODC to this exposure in both the adrenal cortex and the adrenal medulla without affecting the early increase in cyclic AMP. Inhibition of RNA synthesis would not be predicted to interfere with the subsequent increase in ODC activity if ODC were directly phosphorylated (iii) or if the synthesis of new mRNA were not needed (i). Cycloheximide administered 3.5 hours after the initiation of cold exposure results in a rapid decrease in ODC activity to a level lower than that of the control. This would be predicted if de novo synthesis were responsible for the increase in ODC activity since this enzyme has a very short half-life (*3*).

Table 2. The effect of inhibitors of RNA and protein synthesis on the increase in ODC activity after cold exposure. Actinomycin D (6 mg/kg) and cycloheximide (50 mg/kg) were injected into rats intraperitoneally in 0.9 percent NaCl at doses sufficient to block adrenal RNA and protein synthesis (*13*). The ODC was assayed 4.5 hours after the initiation of the 2-hour exposure of the rats to cold as described in Fig. 1. Injection of either drug prior to cold exposure did not affect the subsequent rise in cyclic AMP. (The values shown are the averages of five determinations not differing by more than 15 percent.) The ODC activity is expressed as picomoles of $^{14}CO_2$ per 30 minutes per milligram of protein.

Cold exposure (4°C, 2 hr)	Time of injection* (hr)	ODC activity	
		Medulla	Cortex
	Saline		
No	−0.5	21	18
Yes	−0.5	360	307
	Actinomycin D		
Yes	−0.5	42	27
Yes	+1.0	320	60
	Cycloheximide		
Yes	−0.5	17	10
Yes	+3.5	10	24

* Time of injection is shown relative to the initiation of exposure to cold for 2 hours.

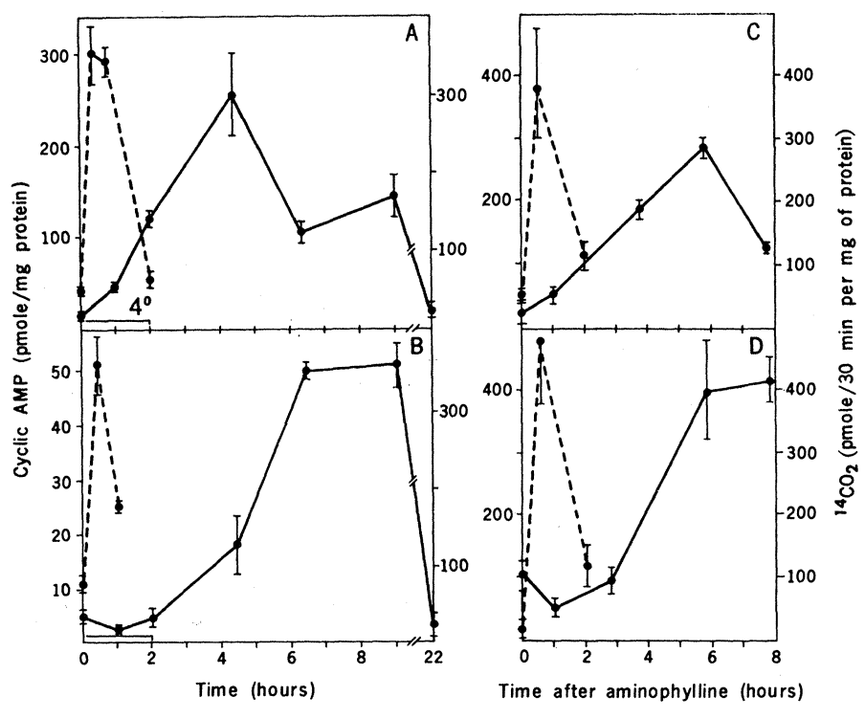

Fig. 1. Changes in cyclic AMP concentration (- - - -) and ODC activity (———) in the rat after exposure to cold and aminophylline injection. Animals were immersed in water for 1 minute and placed in individual cages at 4°C for 2 hours, after which they were removed from the cold and maintained at room temperature. Cyclic AMP concentrations and ODC activity were determined at the times indicated in the adrenal medulla (A) and cortex (B). In a separate series of experiments, animals were injected with aminophylline (200 μmole/kg, intraperitoneally), and ODC activity and cyclic AMP concentrations were determined in the adrenal medulla (C) and cortex (D). The ODC assays were performed at an L-ornithine concentration of 0.32 m*M* in a total volume of 100 μl. Assays performed in the presence of saturating levels of substrate (+ 2 m*M*) yielded results similar to those reported. (Each point represents the mean ± the standard error of five to ten determinations.)

Actinomycin D given 1 hour after the rat is exposed to cold does not affect the subsequent rise in ODC activity in the adrenal medulla, whereas it does in the adrenal cortex. Concurrently, the rise in ODC activity is later in the cortex (Fig. 1, A and B) than in the medulla. This delay in the increase in ODC activity in the cortex as compared to the medulla appears to reflect a temporal difference in the synthesis of new mRNA. The proposed mechanism for the mediation of ODC activity by cyclic AMP involves the activation of protein kinase. Guidotti and Costa have recently observed that there is a rapid stimulation of cyclic AMP–dependent protein kinase activity in the adrenal medulla after cold exposure (*11*). These data plus the previously reported observations that injection of dibutyryl cyclic AMP results in large increases in ODC activity in the adrenal glands (*7*) and in the liver (*12*) of the rat suggest that ODC activity may be regulated by cyclic AMP.

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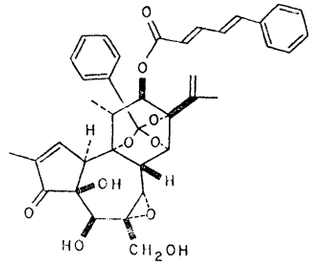
Mezerein: Antileukemic Principle Isolated from *Daphne mezereum* L.

Abstract. *An alcohol-water extract of* Daphne mezereum *L., a plant widely used in folk medicine for treating cancers, showed antileukemic activity against the P-388 lymphocytic leukemia in mice. Systematic fractionation of the extract has led to isolation and characterization of mezerein as the principal antileukemic component.*

Daphne mezereum L. and other *Daphne* species (family Thymelaeaceae) have been used to treat cancers from the time of Aphrodisias (circa A.D. 200), and references to their use in folk medicine have appeared in the herbal literature of many countries (*1*).

In the course of our search for tumor inhibitors of plant origin, an alcohol-water (1 : 1) extract of the seeds of *Daphne mezereum* L. from Italy (*2*) showed significant inhibitory activity when tested in mice against the P-388 lymphocytic leukemia (*3*). We report herein the fractionation of an active extract and the characterization of the principal antileukemic component, which is identified as mezerein. Mezerein shows significant inhibitory activity, at dosages of 50 μg per kilogram of body weight, against the P-388 and L-1210 leukemias in mice (*3*).

Successive solvent partition of the chloroform extract of the ground seeds of *D. mezereum* led to concentration of the antileukemic (P-388) activity in the chloroform layer of a chloroform-water partition and the aqueous methanol layer of a 10 percent aqueous methanol–Skellysolve B partition. Column chromatography of the residue from the aqueous methanol solution on SilicAR CC-7 and subsequent thin-layer chromatography (TLC) on Chromar were guided by testing for antileukemic (P-388) activity and goldfish toxicity. This procedure led to the concentration of the active principle (or principles) into a single TLC-homogeneous fraction (0.05 percent of the plant weight). Crystallization from dichloromethane–diethyl ether afforded the principal active constituent as colorless prisms with melting point (mp) 258° to 262°C and specific optical rotation at 27°C for the sodium D line ($[\alpha]_D^{27}$) + 125° in chloroform. The mass spectrum showed the molecular ion (M^+) at *m/e* (mass/charge) 654. Ultraviolet absorption maxima in ethanol [and log extinction (log ε)] were 314 (4.63), 240.5 (4.23), 233.5 (4.26), and 227 (4.23) nm. A comparison of the mp, $[\alpha]_D$, ultraviolet (UV), infrared (IR), nuclear magnetic resonance (NMR), and mass spectra with those described for mezerein (*4*)



indicated that the active constituent was mezerein. Confirmatory evidence was secured by methanolysis of the active compound, which yielded 12-hydroxydaphnetoxin, identified by comparison of its spectra with published data (*4*, *5*), and methyl 5-phenyl-2,4-pentadienoate (*6*), identified by comparison (mixture mp, gas-liquid chromatography, and IR, UV, and NMR spectra) with a sample prepared by synthesis.

Also, in pursuing the antileukemic principles of several *Gnidia* species (Thymelaeaceae), we have recently characterized three new agents from *G. lamprantha* Gilg (gnididin, gniditrin, and gnidicin) which are close chemical relatives of mezerein (*7*). In view of our earlier findings (*7*, *8*), it will be of interest to determine the significance of the ester, the epoxide, the cyclopentenone, the orthoester, and of other structural features for the antileukemic activity of mezerein-like compounds.

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