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EFFECTS OF α-DIFLUOROMETHYLORNITHINE ON POLYAMINE BIOSYNTHESIS AND TYROSINE HYDROXYLASE INDUCTION IN THE ADRENAL GLAND OF THE RAT SUBJECTED TO STRESS OR APOMORPHINE

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Abstract—Ornithine decarboxylase (ODC) and tyrosine hydroxylase (TH), the first enzymes in the polyamine and catecholamine biosynthetic pathways, respectively, are induced in the adrenal gland of the rat through the application of stressors or dopamine agonists. In the present work, following exposure of rats to cold, application of bodily restraint, or administration of apomorphine (APM), adrenal putrescine increased in proportion to the induction of ODC. Spermidine content increased by 60% after APM and about 30% after immobilization. Spermine was unaffected. To test whether the increases of ODC (and polyamines) are necessary to the slower and later induction of TH, induction of ODC *in vivo* was undertaken. α-Difluoromethylornithine (α-DFMO), an irreversible inhibitor of ODC, given orally or subcutaneously, almost completely abolished the induction of ODC by APM or immobilization, and inhibited the increase of putrescine in both cases, but did not affect spermidine after APM. Repeated administration of α-DFMO over several days did not affect the induction of adrenal TH. The results question whether increases of adrenal ODC activity and of putrescine are essential for the induction of TH in that gland.

The polyamines spermidine and spermine and the diamine putrescine are organic cations found ubiquitously in living cells. High concentrations of these substances occur during cellular growth. They are known to stabilize nucleic acids and to play a role in the cellular events leading to protein synthesis [1].

Ornithine decarboxylase (ODC), the first enzyme in the polyamine biosynthetic pathway, is known to be induced by a wide variety of hormones, mitogens and drugs [2–4]. This enzyme is characterized by an extremely short half-life, viz. about 12 min [5]. In the adrenal medulla of the rat, ODC is induced by exposure of the animal to cold [6, 7], immobilization [7], cholinergic agents [8, 9], reserpine [7, 8], and apomorphine [10]. The induction of the adrenocortical enzyme is also brought about by these means [6, 7, 11], as well as by aminophylline, a phosphodiesterase inhibitor [12], and (unlike its medullary counterpart) by ACTH [13].

In the studies that we now report, we have examined the relation of adrenal ODC induction to the polyamine content of the gland and the influence of α-difluoromethylornithine (α-DFMO), a potent and specific enzyme-activated irreversible inhibitor of mammalian ODC [14], on adrenal ODC activity and polyamine concentrations following exposure of rats to stressors or administration of apomorphine. We have also examined the effect of repeated administration of α-DFMO over several days on the induction of tyrosine hydroxylase (TH), another adrenomedullary enzyme that responds to the same stressors and pharmacological agents as ODC but whose induction proceeds much more slowly than that of ODC.

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MATERIALS AND METHODS

Drugs. Apomorphine was purchased from F. E. Cornell & Co. (Montreal, P.Q.). L-[1-^{14}C]Ornithine, sp. act. 58.9 mCi/mmole, was purchased from the New England Nuclear Corp. (Boston, MA). DL-α-Difluoromethylornithine was a gift from Merrell-International Laboratories (Strasbourg, France). All other chemicals were purchased from standard commercial sources.

Animals. Male Sprague–Dawley rats, weighing 185–210 g, were used throughout this work. Animals were purchased from Canadian Breeding Farms and Laboratories (St. Constant, P.Q.). They were maintained in individual wire cages under a light–dark cycle of 12:12 hr with a diet of tap water and Purina checkers *ad lib.* unless otherwise specified.

Drug treatments. Apomorphine was dissolved in 0.9% NaCl and was given subcutaneously in a dose of 10 mg/kg four times a day at 2-hr intervals, starting at 9:00 a.m. Animals were killed 1 hr after the last injection (i.e. at 4:00 p.m.), unless otherwise stated. In some experiments this procedure was followed for 1 day only ("7-hr experiment"). In another, the treatment was applied on 3 consecutive days, the animals being killed the following morning ("4-day experiment"). Controls received only vehicle. When α-DFMO was to be injected, it was dissolved in 0.9%

NaCl. The solution was titrated to pH 6.5 with dilute NaOH. α-DFMO was also administered orally for the 4-day experiment as a 2% (Series I) or 3% solution (Series II).

Application of stress. For cold exposure rats were immersed for 1 min in cold water and then placed in individual cages in a room maintained at 4°. After 3 hr, they were removed to the laboratory (ambient temperature: 20–21°). They were killed 3 hr after their removal from the cold room. In restraint studies, rats received saline or α-DFMO, 200 mg/kg (s.c.), 1 hr before being immobilized in a cast of plaster bandage (Johnson & Johnson, Montreal, P.Q.). After a 3-hr period of restraint, they were returned to their individual cages and were killed after an additional period of 3 hr. All rats remained without food during the experimental procedure but had access to water.

Tissue preparation. The animals were killed by decapitation. Adrenals were quickly removed, cooled, freed from capsular tissue, and weighed. The separation of the adrenal medulla from the cortex was done at 4° with fine scissors under a magnifying lamp.

ODC activity. For the determination of ODC activity, the tissue was homogenized in 0.05 M sodium–potassium phosphate buffer, pH 6.8. ODC activity was determined by a method that combines elements of the assays described by Russell and Snyder [15] and Jänne and Williams-Ashman [16], with some minor modifications [9, 13]. The activity of ODC was expressed as pmoles CO_2 produced per mg protein during a 45-min incubation period at 37°. Protein content of the supernatant fraction was estimated by the method of Lowry *et al.* [17].

Adrenal TH activity (ATHA). For determination of TH activity, adrenals were homogenized in 0.5 ml of ice-cold 0.9% NaCl solution with an Artek–Fisher dismembranator model 150 (Farmingdale, NY) set at about 60 acoustical W for 2 min. The TH activity was determined in 100 μl portions of the homogenate according to the method of Nagatsu *et al.* [18], as modified by Gauthier *et al.* [19]. The final concentrations of 6,7-dimethyltetrahydropterin and of brocresine were 1.0 and 0.2 mM respectively. The radioactivity of the tritiated water produced by the enzymic reaction was measured in 10 ml Biofluor (New England Nuclear, Lachine, P.Q.). ATHA was expressed as nmoles of L-3,4-dihydroxyphenylalanine (DOPA) formed at 30° per hour per single adrenal. All determinations were done in duplicate.

Polyamine determinations. The content of putrescine, spermidine and spermine ("polyamines") in the adrenal glands was determined by high performance liquid chromatography (HPLC) of their dansylated derivatives. Each adrenal gland was homogenized in 0.4 ml of 0.4 M perchloric acid and centrifuged for 10 min at 12,000 *g*. A known amount of 1,6-diaminohexane was added to 150 μl of the supernatant extract as internal standard in a screw-capped tube. The mixture was evaporated under a gentle stream of dry air. To the residue were added 50 μl of a saturated Na_2CO_3 solution and 100 μl of a solution of dansyl chloride, 5 mg/ml, in acetone. The tubes were vigorously shaken and heated at 70° for 20 min. After the tubes had cooled, a few drops of a saturated proline solution were added to destroy excess reagent, and the contents were evaporated. Water (0.5 ml) and 2 ml of toluene were added to the residue. After vortexing for 1 min followed by centrifugation, the organic layer was removed and evaporated to dryness. The residue was dissolved in 2 ml of acetone, 5 μl of which was injected into the chromatographic port.

Table 1. Effects of stressors and drugs on adrenal ODC activity and polyamine content*

Expt.	Treatment	No. of rats	ODC activity (pmoles CO_2/45 min per mg protein)	Polyamine concentrations: No. of rats	Putrescine (nmoles/g)	Spermidine (nmoles/g)	Spermine (nmoles/g)
1	Control	3	30 ± 5	6	<10	323 ± 17	1009 ± 24
	Cold	4	138 ± 20†	6	69 ± 8	319 ± 13	928 ± 33
2	Control			4	<10	238 ± 17	850 ± 31
	Restraint			4	144 ± 40	326 ± 26‡	880 ± 49
3	Control	4	54 ± 14	4	<10	375 ± 12	1128 ± 23
	Restraint	3	752 ± 189§	3	143 ± 6	459 ± 12†	1116 ± 50
	Restraint + α-DFMO	4	157 ± 15†	4	27 ± 6	406 ± 15‖	993 ± 29‡
4	Control	3	44 ± 24	5	<10	260 ± 12	869 ± 40
	Apomorphine	4	1914 ± 254§	6	229 ± 29	418 ± 12§	809 ± 31
	α-DFMO	4	27 ± 4	7	<10	250 ± 12	817 ± 27
	Apomorphine + α-DFMO	4	103 ± 12¶**	6	<10	426 ± 26¶	849 ± 39

* Expt. 1: Rats were exposed to 4° for 3 hr and then removed to the laboratory. They were killed 3 hr later. Expt. 2: Rats were immobilized for 3 hr and then returned to their cages. They were killed 3 hr later. Expt. 3: α-DFMO (200 mg/kg) was given s.c. 1 hr before the animals were immobilized. Expt. 4: Rats received four consecutive injections of apomorphine (10 mg/kg) given s.c. every 2 hr. They were killed 1 hr after the last injection. α-DFMO (200 mg/kg) was given s.c. 1 hr before and 3 hr after the first apomorphine injection. ODC activity was not determined in Expt. 2.

†–§ Significantly different from corresponding controls: †$P < 0.01$; ‡$P < 0.05$ and §$P < 0.001$.

‖ Significantly different from immobilized group: $P < 0.05$.

¶ Significantly different from α-DFMO-treated group: $P < 0.001$.

** Significantly different from apomorphine-injected group: $P < 0.001$.

Dansylated polyamines were separated on a C18 μBondapak column (Waters Associates, Milford, MA) coupled to an M45-M45G solvent delivery system, a 660 solvent programmer (Waters Associates) and an FS 970 LC Fluorometer (Schoeffel Instrument Corp., Westwood, NJ). Gradient elution was started at 70% methanol: 30% water, increasing to 90% methanol in 10 min (curve No. 7 on the solvent programmer). After elution of all peaks (17 min), the gradient was reversed to initial conditions over a period of 5 min and the column equilibrated for an additional 5 min.

The concentrations of polyamines were determined by comparing the peak height ratio of polyamine to the internal standard 1,6-diaminohexane, with a polyamine standard curve. All determinations were made in duplicate.

Statistical analysis. Values in all tables are expressed as mean ± standard error. Comparison of paired means was done by Student's *t*-test [20].

RESULTS

Effect of stressors and apomorphine on adrenal polyamines. As previously reported for separated medulla and cortex [7, 10], ODC activity was increased in the whole adrenal gland following cold exposure, bodily restraint or apomorphine administration (Table 1, Expts. 1, 3 and 4). Apomorphine in the dosage schedule used was the most potent of the three inducers. Cold exposure produced the smallest induction of ODC, whereas immobilization produced an intermediate degree of induction.

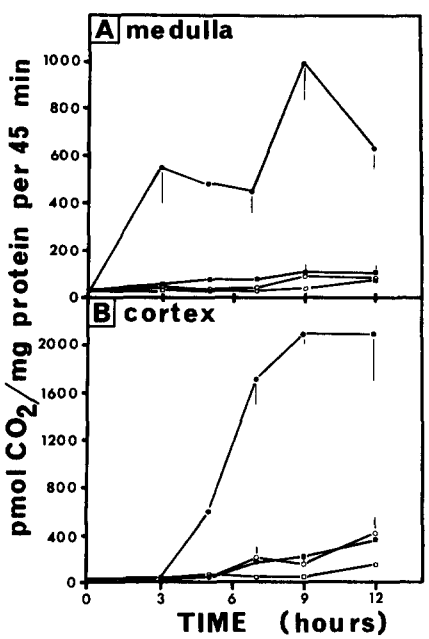

Fig. 1. Time course of the stimulation of adrenomedullary (A) and adrenocortical (B) ODC activity in response to apomorphine with or without pretreatment with α-DFMO. Apomorphine was administered s.c. in a dose of 10 mg/kg, at 0, 2, 4 and 6 hr. α-DFMO was administered s.c. in a dose of 200 mg/kg at −1 hr. Each point represents the mean ± S.E.M. for four to six animals. The following symbols have been used: (■) control; (●) apomorphine; (□) α-DFMO; and (○) apomorphine + α-DFMO.

Adrenal putrescine was also increased by apomorphine, immobilization or cold exposure (Table 1), from essentially undetectable amounts to readily measurable quantities. These increases ranked in the same order as for ODC induction. Thus, the increase in adrenal putrescine reflects the increase in ODC activity. Spermidine responded less strongly in this respect: apomorphine produced a 60% increase in the adrenal content of this polyamine, and immobilization about half as much (Table 1). None of the treatments significantly affected spermine concentration.

Effect of α-DFMO on immobilization- and apomorphine-induced changes in adrenal polyamines. To determine the extent to which increases in adrenal putrescine and spermidine are due specifically to the induction of ODC, inhibition of ODC *in vivo* with α-DFMO was undertaken. Figure 1 describes the effect of α-DFMO on the time course of adrenal ODC induction by apomorphine. Administration of apomorphine in the dosage schedule described under Materials and Methods produced increases in adrenomedullary ODC that appeared at t = 3 hr and attained a peak at t = 9 hr (Fig. 1A). A single injection of α-DFMO, given 1 hr before the beginning of the apomorphine treatment did not affect significantly the ODC activity of control rats but impaired by at least 90% the apomorphine-induced increase in ODC throughout the 12-hr period.

Administration of apomorphine also increased the activity of ODC in the adrenal cortex (Fig. 1B). As previously reported [10], the onset of the apomorphine effect was not as rapid as for the adrenal medulla, and the ODC activity of rats receiving the drug was apparently still maximal 12 hr after the beginning of the treatment. ODC activity of control (saline-treated) animals also increased over the period studied, possibly owing to the effect of the mild stress provoked by the injections of the inert vehicle and the sensitivity of adrenocortical ODC to ACTH [13]. Pretreatment of the rats with α-DFMO resulted in a smaller increase in ODC activity in the cortex of saline-treated animals and blocked by at least 95% the increase produced by apomorphine.

In a different experiment, the ODC response of the whole adrenal gland to immobilization or apomorphine was attenuated by 85–90% when α-DFMO was given 1 hr before the rats were immobilized (Table 1, Expt. 3) or 1 hr before and 3 hr after the first apomorphine injection (Table 1, Expt. 4). Besides severe attenuation of the ODC response, α-DFMO also inhibited the increase of putrescine and prevented, at least partially, the increase in spermidine after immobilization. However, it did not prevent the increase in spermidine after apomorphine. The inhibitor was without effect on adrenal polyamine concentrations in saline-treated animals.

Finally, to provide a more continuous supply of α-DFMO, we gave this inhibitor to the rats orally in their drinking water. Administration of apomorphine resulted in a 20-fold increase in ODC activity (1046 pmoles CO_2/45 min per mg protein ± 196 compared to control: 53 ± 9; $P < 0.001$). Pretreatment with α-DFMO resulted in 95% impairment of the

Table 2. Effect of apomorphine and α-DFMO on rat adrenal TH activity and polyamine content*

Group	Treatment	No. of rats	ATHA (nmoles DOPA/hr per gland)	Putrescine (nmoles/g)	Spermidine (nmoles/g)	Spermine (nmoles/g)
1	Saline	8	21	16	390	1108
2	Apomorphine	9	48	12	371	1124
3	α-DFMO	8	21	12	324	1107
4	Apomorphine + α-DFMO	8	52	<10	347	1051
S.E. of difference between means:						
Two means of 8			3.4	3.9	23	52
Mean of 8 and mean of 9			3.3	3.8	23	51

* Rats received four consecutive apomorphine injections (10 mg/kg, s.c.) every 2 hr starting at 9:00 a.m. for 3 consecutive days, and were killed on day 4 at 9:00 a.m. α-DFMO was given in the drinking water as a 2% solution (Series I) or a 3% solution (Series II), starting at 16 hr before the first apomorphine injection. The means are shown, and differences between them were judged on the basis of analysis of variance of the data for each variable measured. The treatments (apomorphine × α-DFMO × experiments) yielded eight subgroups, with 7 degrees of freedom, leaving 25 degrees of freedom for error. The F ratios were: for ATHA, 38.147, $P < 0.01$; putrescine, 2.09, $P > 0.05$; spermidine, 5.481, $P < 0.01$; and spermine, 1.204, $P > 0.05$. The mean square for error in the analysis of variance was used in each case to calculate the standard error of difference between means, as shown in the table above.

apomorphine-induced increase in ODC (147 ± 28; $P < 0.001$ compared to apomorphine-treated rats).

Effect of apomorphine and α-DFMO treatment on adrenal tyrosine hydroxylase activity and polyamine content. The effects of apomorphine and α-DFMO were tested in a 4-day experiment, the inhibitor being provided in the drinking water as a 2% solution in series I and as a 3% solution in series II. The experiment involved the administration of apomorphine for 3 days and killing of the rats on day 4, 18 hr after the final injection of the drug. The treatment with apomorphine alone resulted in increased ATHA (Table 2), as found previously [19, 21]. The mean concentrations of the polyamines were not affected significantly by this drug, although for spermidine there was an increase in Series I and a decrease in Series II. This also occurred in rats receiving α-DFMO as well as apomorphine. The reason for the discrepancies in spermidine content in the two series is not immediately apparent. In regard to putrescine, a previous experiment (Table 1, Expt. 3) showed that, when the rats were killed only 1 hr after the last injection of apomorphine, there was a large increase in adrenal putrescine concentration. However, the 18-hr delay after the final apomorphine injection in the experiment of Table 2 apparently not only permitted the ODC activity to return to control levels [10], but also gave time for the excess putrescine to be dissipated in synthetic reactions or metabolic processes.

The mean spermidine content of the adrenal declined by 17% under the influence of α-DFMO ($P < 0.025$), but not if apomorphine was given at the same time (Table 2).

Rats given α-DFMO perorally along with the schedule of apomorphine injections showed the same degree of ATHA induction as rats given apomorphine alone (Table 2). The ODC inhibitor by itself (oral route) did not affect ATHA.

To verify whether the oral administration of α-DFMO results in the impairment of the apomorphine induction of ODC activity, additional rats were treated in the same way and at the same time as those represented in Table 2, with the difference that they were killed 1 hr after the last apomorphine injection. This time of sacrifice was chosen because it corresponds to very high ODC activity in both medulla and cortex. Treatment with α-DFMO resulted in 85% impairment of the apomorphine-induced increase in adrenal ODC activity on day 1 of apomorphine treatment and 90% on day 3 (data not shown).

DISCUSSION

Adrenal ODC is induced following apomorphine administration to rats or their exposure to stressors [6, 7, 10]. The present work shows that this induction is accompanied by an increase in adrenal putrescine concentration. Apomorphine and immobilization, which produced the largest rises in ODC activity, also produced significant increases in adrenal spermidine within a short period of time. Putrescine is known to activate *S*-adenosylmethionine decarboxylase [22], one of the enzymes involved in the production of spermidine. A greater availability of putrescine may then have been responsible for the increase in spermidine. In contrast to this, there was no increase of adrenal spermine concentration. This is consistent with the known competitive inhibition of spermine synthase by putrescine [23].

Tyrosine hydroxylase is induced by stressors, cholinergic agents [24] and apomorphine [21] just as in the case of ODC. These inductions are dependent upon intact innervation of the adrenal gland [7, 21] and are cAMP-mediated [24, 25]. The neural pathways involved in the control of the respective enzymes present certain similarities, as extensively studied in this laboratory [19, 26–30], but a major difference between them is that optimal ODC induction is obtained 4–5 hr after the beginning of stress or drug treatment [7, 8] whereas TH activity is still increasing on day 3 [21]. It has been demonstrated that induction of ODC and the increases in poly-

amine concentrations play essential roles in the processes leading to increased activity of certain enzymes, viz. renal cytochrome *c* oxidase and lysosomal enzymes of kidney and skeletal muscle [31, 32], but not in the case of aryl hydrocarbon hydroxylase [33]. Because of the similarities in the regulation of ODC and TH activities, we wanted to test whether there is a correlation of TH induction with increases of ODC and polyamines. The experiment showed (Table 2) that adrenal TH induction by apomorphine proceeded unimpaired even though ODC was inhibited at least 85% by α-DFMO. This suggests that large increases in adrenal ODC activity or in putrescine content are not a necessary step in the process leading to the induction of TH. Slotkin and his colleagues [34] have shown that in the neonatal rat α-DFMO does not impair the development of adrenal TH activity, but rather produces increased activity of the enzyme.

Many reports suggest that spermidine plays an important role in cellular events of growth and development [1]. On the basis of the concept of the adrenal gland as a model of secretion hypertrophy [35], there may still be such a role for spermidine in the inductions described in this paper. The fact that α-DFMO was unable to prevent the augmentation in adrenal spermidine content of rats given apomorphine is in apparent contradiction to the ability of this inhibitor to impair nearly completely the ODC response to apomorphine in both parts of the gland. The results suggest that the regulation of adrenal spermidine formation is more complex than through the simple provision of putrescine as one of its precursors. This is also seen in the fact that complete ODC inhibition by α-DFMO does not prevent an increase in spermidine in the regenerating rat liver following partial hepatectomy [36]. Furthermore, although α-DFMO causes a reduction in the concentration of putrescine, it also increases the activity of *S*-adenosylmethionine decarboxylase in cell cultures [37] and in diaphragm muscle *in vivo* [38]. A similar mechanism operating in rat adrenals would provide an abundant supply of aminopropyl groups and these, together with even the small amount of putrescine formed in the rat given apomorphine and α-DFMO (amounts comparable to the adrenal putrescine content of the normal quiescent animal), could maintain an elevated spermidine level.

Some of the spermidine formed could come from the degradation of spermine. The acetyl transferase which catalyzes the first step in the metabolism of spermidine to putrescine or of spermine to spermidine is induced in the rat liver by the same treatments that induce ODC [39, 40]. But even if this induction of transferase occurs in the adrenal gland, spermine is unlikely to be the main source of spermidine formed during the apomorphine treatment or in immobilization, because in our experiments there was no decrease in adrenal spermine content that could account for the increase of spermidine.

The role of the large ODC induction following stress or apomorphine could be to ensure formation of plentiful putrescine for action in certain processes. Yet the induction of ATHA was not prevented in these experiments by almost total impairment of induction of functional ODC and of the formation of putrescine. This questions, therefore, whether ODC induction is an obligatory preliminary step in the induction of adrenal TH activity, either through a direct action of the ODC molecule on factors implicated in nucleic acid formation (such as RNA polymerase I [41–43]), or through the product of the ODC reaction, putrescine.

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