Inhibition of Trans-synaptically Increased Tyrosine Hydroxylase Activity by Cycloheximide and Actinomycin D

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SUMMARY

Reserpine produces a neurally mediated increase in tyrosine hydroxylase activity in the adrenal medullae and sympathetic ganglia of the rat. This increase in enzyme activity can be prevented by the administration of actinomycin D or cycloheximide. These results suggest that the synthesis of tyrosine hydroxylase is regulated either by changes in the release or turnover of catecholamines, or by the direct effect of a neurotransmitter. Reserpine increases the incorporation of ^{3}H-leucine into hepatic and adrenal protein, and this incorporation is inhibited by a dose of cycloheximide that prevents the reserpine-initiated increase in tyrosine hydroxylase activity.

INTRODUCTION

The activity of tyrosine hydroxylase, the rate-limiting enzyme in catecholamine biosynthesis (1), can be increased in the adrenal gland, sympathetic ganglia, and brain stem by administration of reserpine (2). A similar increase in adrenal tyrosine hydroxylase has been observed after the administration of 6-hydroxydopamine and phenoxybenzamine (3). The latter drugs interfere with postganglionic sympathetic transmission by different mechanisms (4–6) and, as a consequence of this interference, produce a reflex increase in preganglionic sympathetic nerve activity (7, 8). Since the increase in adrenal tyrosine hydroxylase produced by these drugs can be abolished by cutting the splanchnic nerve (9) and the increase in superior cervical ganglion enzyme activity initiated by reserpine can be prevented by section of the preganglionic sympathetic trunk (3), it has been proposed that the increased amount of tyrosine hydroxylase is the result of an increase in efferent nerve activity.

Kinetic studies indicated that the increased adrenal enzyme activity was probably the result of an increase in the number of active enzyme sites (2). This could be the result of an unmasking of "hidden" sites or an increase in the amount of enzyme protein. In order to determine whether the elevation in tyrosine hydroxylase activity by reserpine might be due to an increased amount of enzyme, the effects of inhibitors of protein and ribonucleic acid synthesis were examined. The present study shows that cycloheximide and actinomycin D prevent the increase in adrenal and ganglionic tyrosine hydroxylase observed after reserpine administration.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 100–150 g were obtained from Hormone Assay, Chicago, 3 days before use. Animals received 2.5 mg/kg of reserpine (Serpasil,

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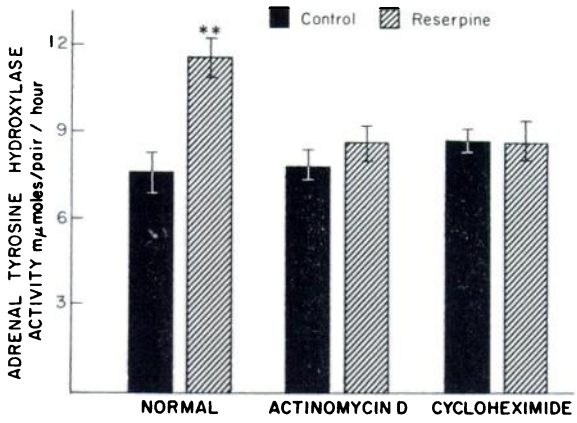

FIG 1. *Inhibition of reserpine-induced increase in adrenal tyrosine hydroxylase activity by actinomycin D and cycloheximide*

Each column represents the mean ± standard error (brackets) of five to seven observations. Reserpine (2.5 mg/kg) was given subcutaneously 18 hr before the animals were killed. Actinomycin D (0.6 mg/kg) and cycloheximide (0.9 mg/kg) were given subcutaneously 5 min, 6 hr, and 12 hr after reserpine. Each enzyme assay was done at a concentration of 1.14 mM reduced pteridine cofactor and 27.7 μM 3,5-ditritiotyrosine.
** $p < 0.01$.

Ciba) subcutaneously 18 hr before being killed by decapitation. Actinomycin D, kindly supplied by Dr. H. B. Wood (National Cancer Institute), and cycloheximide, a gift of Dr. J. T. Correll (Upjohn Company), were dissolved in 0.9% NaCl so that each animal received 1 ml/kg containing the amounts of drug indicated in the figure legends. Pairs of adrenal glands and superior cervical ganglia were placed in 2.0 ml and 0.5 ml, respectively, of 0.25 M sucrose at 4°, and homogenized in ground-glass homogenizers. After centrifugation at 27,000 × *g* for 20 min, the supernatant solution was examined for tyrosine hydroxylase activity by a slight modification (2) of the method of Levitt *et al.* (10), and for protein content by the method of Lowry *et al.* (11). Adrenal catecholamines were determined by methods described previously (12, 13).

For studies of amino acid incorporation,

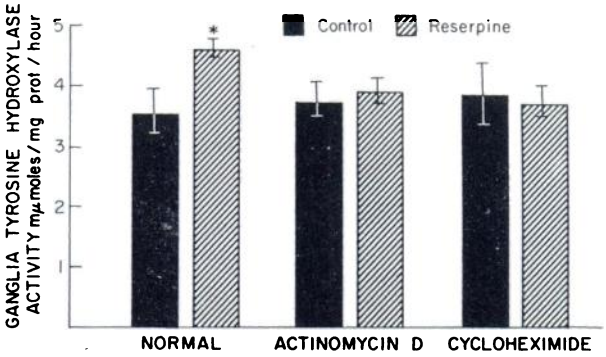

FIG. 2. *Inhibition of reserpine-induced increase in superior cervical ganglionic tyrosine hydroxylase activity by actinomycin D and cycloheximide*

Each column represents the mean ± standard error (brackets) of five to seven pairs of ganglia. Reserpine (2.5 mg/kg) was given 18 hr before the animals were killed. Actinomycin D (0.6 mg/kg) and cycloheximide (0.9 mg/kg) were given subcutaneously 5 min, 6 hr, and 12 hr after reserpine. The concentration of reduced pteridine cofactor was 0.74 mM; that of 3,5-ditritiotyrosine was 18.6 μM.
* $p < 0.05$.

each rat received 0.25 ml of 0.9% NaCl which contained 107 μCi (experiment 1) or 68 μCi (experiment 2) of L-leucine-4,5-^{3}H, 22.2 μCi/mmole (Amersham-Searle), via a tail vein while momentarily restrained. Thirty minutes later, the animals were stunned by a blow on the head, a neck incision was made, and blood was collected in heparinized tubes. Then both adrenals and a small piece (100–150 mg) of liver were rapidly removed and placed in ground-glass homogenizers containing 0.5 ml of 0.25 M sucrose. After homogenization at 4°, an aliquot was removed for determination of total protein. After precipitation of protein by the addition of 100 μl of 4 N $HClO_4$ each sample was centrifuged, the supernatant solution was discarded, and the pellet was homogenized again in 1 ml of 0.4 N $HClO_4$. This sequence was repeated three times, and the final pellet was dissolved with 1.0 ml of a protein solubilizer solution ("NCS," Amersham-Searle). The final supernatant fraction was analyzed for $HClO_4$-soluble tritium to ensure complete washing. The heparinized blood was centrifuged, and an aliquot of plasma was removed and precipitated with 9.0 ml of 0.4 N $HClO_4$. A portion of the deproteinized plasma supernatant and one of the NCS tissue digests were counted at 10% efficiency in a Packard liquid scintillation spectrometer after the addition of a toluene phosphor solution containing 4 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene per liter.

RESULTS

Effect of cycloheximide and actinomycin D on increase in tyrosine hydroxylase elicited by reserpine in adrenal glands and sympathetic ganglia. Preliminary experiments in which various doses of cycloheximide and actinomycin D were employed showed that the mortality due to these inhibitors of protein and ribonucleic acid synthesis was considerably greater in reserpine-treated animals than in animals which had not received reserpine. For this reason, it was necessary to use the lowest dose of reserpine and the shortest time after reserpine administration which would produce a significant elevation in both adrenal and ganglionic tyrosine hydroxylase. Reserpine (2.5 mg/kg) produced a 50% increase in adrenal tyrosine hydroxylase activity within 18 hr (Fig. 1). Previous experiments had shown that repeated administration of this dose of reserpine at daily intervals produces a 3–4-fold increase in adrenal and ganglionic enzyme activity. The increase of tyrosine hydroxylase was prevented by administration of cycloheximide or actinomycin D 5 min after reserpine and at 6-hr intervals thereafter. Although a lower dose (0.5 mg/kg) of cycloheximide produced a significant ($p < 0.01$) increase in adrenal tyrosine hydroxylase activity, 0.9 mg/kg or larger doses of this inhibitor did not. Lower doses of actinomycin D (0.4 mg/kg or less) did not completely prevent the increase in enzyme activity observed after reserpine administration.

Cycloheximide and actinomycin D also prevented the increase (30%; $p < 0.05$) in superior cervical ganglionic tyrosine hydroxylase activity produced by reserpine (Fig. 2).

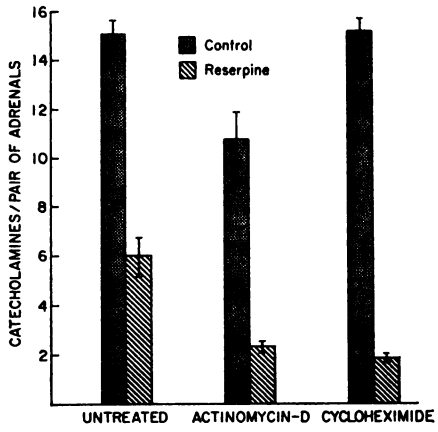

FIG. 3. *Effect of actinomycin D and cycloheximide on reserpine-initiated adrenal catecholamine depletion*

Each column represents the mean ± standard error (brackets) of total catecholamines (epinephrine + norepinephrine), micrograms per pair of adrenal glands. Reserpine (2.5 mg/kg) was given subcutaneously 18 hr before the animals were killed. Actinomycin D (0.6 mg/kg) and cycloheximide (0.9 mg/kg) were given subcutaneously 5 min, 6 hr, and 12 hr after reserpine.

TABLE 1

Effect of cycloheximide on ^{3}H-leucine incorporation into adrenal and hepatic protein 13 hr after reserpine administration

Reserpine-treated rats received 2.5 mg/kg of reserpine subcutaneously 13 hr before intravenous administration of 107 μCi of ^{3}H-leucine. Cycloheximide (0.9 mg/kg) was given subcutaneously 13 hr, 7 hr, and 1 hr before ^{3}H-leucine. All animals were killed 30 min after administration of ^{3}H-leucine. The tritium contents of adrenal and hepatic protein and of the non-protein fraction of the plasma were determined as described in the text. Individual values are presented.

Group	Rat	Adrenal	Liver	Plasma
		cpm ^{3}H/mg protein		*(cpm ^{3}H/ml) × 10^3*
Control	1	3033	2081	78.4
	2	3180	2733	95.2
	3	3647	2800	102.5
Reserpine	4	6020	4708	91.4
	5	5444	3396	74.5
Cycloheximide	6		310	216.0
	7	928	317	172.0
	8	1001	339	167.0
Reserpine + cycloheximide	9	1331	397	167.0
	10	1187	184	238.0
	11	1291	187	188.0

Effect of cycloheximide and actinomycin D on adrenal catecholamine depletion produced by reserpine. Cycloheximide and actinomycin D in the dosage schedule which prevented the reserpine-initiated increase in tyrosine hydroxylase activity not only failed to inhibit but tended to increase the reserpine-induced depletion of adrenal catecholamines by 18 hr (Fig. 3). The dosage schedule of actinomycin D selected did lower total adrenal catecholamines to 70% of the control value ($p < 0.05$) when used alone.

Effect of cycloheximide on incorporation of ^{3}H-leucine into liver and adrenal protein. Studies on the effect of the dosage schedule of cycloheximide on incorporation of ^{3}H-leucine into adrenal and liver protein were made for two different time periods. In the first experiment (Table 1), ^{3}H-leucine was given 1 hr after the third dose of cycloheximide, when any inhibitory effect

TABLE 2

Effect of cycloheximide on ^{3}H-leucine incorporation into adrenal and hepatic protein 5 hr after reserpine administration

Rats received 2.5 mg/kg of reserpine or 0.9 mg/kg of cycloheximide plus reserpine subcutaneously 5 hr before administration of 68 μCi of ^{3}H-leucine intravenously. Animals were killed 30 min after receiving ^{3}H-leucine. The tritium contents of adrenal and hepatic protein and of the non-protein fraction of the plasma were determined as described in the text. Each value represents the mean ± standard error of four determinations.

Group	Adrenal	Liver	Plasma
	cpm ^{3}H/mg protein		*(cpm ^{3}H/ml) × 10^3*
Control	1671 ± 167	974 ± 48	22 ± 1
Reserpine	2676 ± 338[a]	1269 ± 98[a]	26 ± 2
Reserpine + cycloheximide	1310 ± 97[a]	576 ± 30[a]	63 ± 6[b]

[a] $p < 0.05$.
[b] $p < 0.001$.

should have been maximal. In the second experiment (Table 2), ^{3}H-leucine was given 5 hr after the first dose of cycloheximide, when the inhibitory effect might have been at a minimum level. Both tables show that the dose of reserpine used in this study produced a pronounced increase in the incorporation of tritium into perchloric acid-insoluble material in liver and adrenals. Cycloheximide significantly ($p < 0.05$) inhibited amino acid incorporation at both time intervals. The plasma tritium activity in cycloheximide-treated rats was at least double that of control animals in both studies.

DISCUSSION

Our results indicate that the increase in adrenal and sympathetic ganglionic tyrosine hydroxylase activity which occurs within 18 hr of reserpine administration can be prevented by concurrent injection of cycloheximide or actinomycin D.

Cycloheximide inhibits protein synthesis in mammals *in vivo* (14, 15), although the mechanism of inhibition of hepatic protein synthesis *in vivo* remains unsettled (16). The dosage schedule of cycloheximide used in our study inhibited the incorporation of ^{3}H-leucine into perchloric acid-insoluble protein of both liver and adrenal gland.

Actinomycin D inhibits protein synthesis both by interfering with messenger ribonucleic acid synthesis (17) and by a more direct mechanism at higher doses (18). The data of Prosky *et al.* (19) indicate that the dose of actinomycin used in our studies would inhibit hepatic ribonucleic acid synthesis much more completely than protein synthesis. Similar data on the adrenal medulla are not available; therefore the inhibition of tyrosine hydroxylase induction by actinomycin D could be due to inhibition of either ribonucleic acid or protein synthesis, or both.

Since both inhibitors prevented the elevation of tyrosine hydroxylase activity observed after reserpine administration, an increased amount of enzyme protein could be responsible for the enhanced enzyme activity. A rise in the amount of enzyme could result from an increase in its rate of synthesis or a decrease in its rate of degradation. The rate of change in amount of enzyme after cessation of its synthesis provides an approximation of its turnover rate. No decrease in enzyme activity at 18 hr was noted in the experiments with cycloheximide, and this would imply a turnover half-time of at least several days. If a stimulus which increases or maintains adrenal tyrosine hydroxylase levels does so by increasing the synthesis of the enzyme, removal of that stimulus would abolish the increased synthesis, and the rate of return to the new steady state would provide a measure of enzyme turnover. The stimulus to increase adrenal tyrosine hydroxylase after reserpine administration is carried via the splanchnic nerve (9). If the splanchnic nerve is cut 3 days after reserpine administration, when the adrenal enzyme activity has just reached its maximal increase (2), the rate of decrease in enzyme activity indicates a $t_{1/2}$ of about 6 days. Hypophysectomy combined with splanchnic nerve section also produces a fall in adrenal tyrosine hydroxylase activity exhibiting a $t_{1/2}$ of about 8 days. Therefore, if one assumes that none of these procedures alters the rate of enzyme degradation but only changes the rate of enzyme synthesis by removing a stimulus, the shortest half-life so far obtained is 6 days. An exact determination of degradation rate would require measurement of the rate of decay of labeled enzyme.

Since the turnover of adrenal tyrosine hydroxylase is rather slow, the increased enzyme concentration observed after reserpine administration appears to be due to an increase in the rate of enzyme synthesis. It is possible that the protein synthesis required for the neurally mediated reserpine effect on postsynaptic tyrosine hydroxylase is due to increased synthesis, not of the enzyme itself, but of a noncatalytic modulating protein.

The increased incorporation of ^{3}H-leucine into adrenal and liver protein at both 5 hr and 13 hr after reserpine admin-

istration could have been due to alterations in ^{3}H-leucine distribution or cytoplasmic penetration rather than to an actual increase in the rate of protein synthesis. However, Viveros *et al.* (20) have observed that reserpine produces a neurally dependent decrease in dopamine β-oxidase, which is followed by a return of this enzyme to levels greater than the control. Thus there is an active synthesis of adrenal dopamine β-oxidase and tyrosine hydroxylase after reserpine administration. Perhaps the increased amino acid incorporation observed here in reserpine-treated animals reflects this increased synthetic activity.

Recently Weiner and Rabadjija (21) reported that the increase in norepinephrine synthesis from tyrosine in the guinea pig vas deferens observed after interval stimulation at a rate of 30/sec for 60 min was markedly reduced by puromycin. They concluded that the post-stimulation increase in norepinephrine synthesis was most probably due to an increase in tyrosine hydroxylase. However, Thoa and Kopin[3] did not find an increase in tyrosine hydroxylase activity *in vitro* under similar experimental conditions, although they confirmed the increase in norepinephrine synthesis and its reduction by puromycin. The latter findings are consistent with our previous observation that the increase in tyrosine hydroxylase activity represents a long-term adaptation and occurs only after 12 hr of a drug-induced increase in nerve activity.

It has been proposed that actinomycin D prolongs the myocardial catecholamine depletion produced by reserpine as a result of inhibiting the replenishment of catecholamine synthesis or storage constituents inactivated by reserpine (22). The present experiments have shown that in the sympathetic ganglia, as in the adrenal medulla, there is a neurally mediated increase in the production of tyrosine hydroxylase, and that this increased production can be blocked by inhibition of protein synthesis. The effects of inhibition of this increased synthesis of enzymes or catecholamine-binding sites by actinomycin D are manifested in the nerve endings several days later as a retarded recovery of normal catecholamine content.

The amount of postsynaptic adrenal medullary or sympathetic ganglion tyrosine hydroxylase is regulated by the activity of the efferent nerves. This regulation could be either a direct effect of the neurotransmitter on enzyme synthesis or an indirect effect resulting from changes in the release or turnover of catecholamines.

[3] N. G. Thoa and I. J. Kopin, personal communication.

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