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EFFECTS OF CHRONIC NICOTINE ADMINISTRATION ON THE DENERVATED RAT ADRENAL MEDULLA

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1 The effects of chronic nicotine administration (1 or 10 mg/kg, s.c., twice daily) were studied in intact and denervated rat adrenal glands to determine the relative roles of central input and direct actions on catecholamines.

2 Catecholamine depletion was obtained in the intact glands from 1–7 days of treatment with 10 mg/kg, with recovery by 14 days of treatment; catecholamines were not decreased in denervated adrenal glands.

3 Catecholamine depletion was accompanied by a decline in functional storage vesicles (determined by [^{3}H]-adrenaline uptake per gland) in the intact side, while no change was seen in the denervated side; the proportion of newly synthesized vesicles increased markedly during 1–7 days of treatment with 10 mg/kg in the intact side, while a much smaller increase of shorter duration was seen in the denervated adrenal gland.

4 Chronic nicotine administration at either dose level induced tyrosine hydroxylase in both intact and denervated glands, but the increase occurred more slowly in the denervated glands.

5 Dopamine β-hydroxylase levels increased similarly in both sides during treatment with nicotine (10 mg/kg).

6 These studies suggest that although long-term adrenal denervation eliminates the catecholamine depletion caused by chronic administration of nicotine, the mechanisms for induction of catecholamine synthesizing enzymes are still capable of responding to the drug.

Introduction

The actions of nicotine on the adrenal medulla are thought to comprise at least three distinct effects: stimulation via central firing of the splanchnic nerve (Patrick & Kirshner, 1971), direct stimulation of chromaffin cells via nicotinic receptors (Schneider, 1969), and to a lesser extent hormonal stimulation via the adrenal cortex (Rubin & Warner, 1975). The loss of adrenal catecholamines resulting from intense stimulation by nicotine and other drugs (morphine, reserpine, insulin) is accompanied by compensatory induction of the catecholamine synthesizing enzymes, tyrosine hydroxylase and dopamine β-hydroxylase, and by accelerated synthesis of catecholamine storage vesicles (Thoenen, Mueller & Axelrod, 1969; Viveros, Arqueros, Connett & Kirshner, 1969; Slotkin & Seidler, 1975; Anderson & Slotkin, 1975). As a result, chronic treatment of rats with nicotine eventually results in a return of adrenal catecholamine levels to normal despite continued drug administration (Slotkin & Seidler, 1975); the high levels of catecholamine synthesizing enzymes and storage vesicle synthesis can thus replace the amines being lost through accelerated secretion.

It is unclear, however, to what extent adrenal catecholamine depletion and compensatory changes during chronic nicotine administration can be accounted for on the basis of direct effects *versus* neurally-mediated stimulation of the tissue, or whether the two types of stimulation produce different biochemical changes. In the current study, rats with denervated adrenals have been used in order to determine the role of central input in the effects of nicotine, and to evaluate ways in which catecholamine stores are maintained in the absence of normal splanchnic activity.

Methods

Male Sprague-Dawley rats (Zivic-Miller) had their left adrenal glands denervated surgically by splanchnic section 10 days before the start of the experiment, and completeness of denervation established by the absence of a secretory response to insulin (Slotkin & Kirshner, 1973a). Rats were given either 1 or 10 mg/kg nicotine subcutaneously twice daily for two

weeks; control rats received the same surgical procedure, but were administered 0.9% w/v NaCl solution (saline). Animals were killed by decapitation at 4 h, 24 h, 7 days and 14 days after initiation of drug administration, and except for the first time point, decapitation occurred 12 h after the last nicotine injection.

Individual glands were removed, cleaned of fat and homogenized (glass-to-glass) in 2.5 ml of 0.3 M sucrose containing 0.025 M Tris (pH 7.4) and 10^{-5} M iproniazid; 0.1 ml was removed and deproteinized with 1.9 ml of 3.5% perchloric acid and centrifuged for 10 min at 26,000 *g*. The supernatant was analyzed for catecholamines by the trihydroxyindole method using an autoanalyzer (Merrills, 1963). Duplicate 0.2 ml portions of the homogenate were added to 0.2 ml of water containing 400 u of beef catalase (Sigma) and assayed for dopamine β-hydroxylase (Friedman & Kaufman, 1965), using 10 μM [^{3}H]-tyramine as substrate; para-hydroxymercuribenzoate (optimal concentration, 0.5 mM) was used to inactivate endogenous inhibitors.

The remainder of the homogenate was centrifuged at 800 *g* for 10 min and 1 ml of the supernatant used for the determination of adrenaline uptake (*vide infra*). The rest of the 800 *g* supernatant was centrifuged at 26,000 *g* for 10 min to sediment the catecholamine storage vesicles and the supernatant used for duplicate determinations of tyrosine hydroxylase activity by the method of Waymire, Bjur & Weiner (1971), using 100 μM [^{14}C]-tyrosine as substrate.

The abilities of the storage vesicles to incorporate [^{3}H]-adrenaline were determined by standard techniques as described previously (Slotkin & Kirshner, 1973a). For each adrenal preparation, duplicate tubes were prepared containing 0.5 ml of the 800 *g* supernatant, 5 μmol of ATP and Mg^{2+}, 5 μCi of [^{3}H]-adrenaline, 0.1 μmol of unlabelled adrenaline and sucrose-Tris in a final volume of 1 ml. Samples were incubated at 30°C for 30 min while duplicates were kept on ice to serve as blanks. Uptakes were stopped by the addition of 2 ml of ice-cold sucrose-Tris and the labelled vesicles sedimented at 26,000 *g* for 10 minutes. The supernatant was deproteinized with an equal volume of 7% perchloric acid, centrifuged at 26,000 *g* for 10 min and assayed for catecholamines and radioactivity; this enabled determination of the specific activity of the labelling medium. The vesicular pellet was washed with fresh sucrose-Tris and recentrifuged twice, and then suspended in 3 ml of 3.5% perchloric acid, centrifuged and the supernatant analyzed for catecholamines and radioactivity. Although contaminating particles are present, under these conditions labelling occurs solely in the storage vesicles (Slotkin & Kirshner, 1971, 1973b). The temperature-dependent uptake was calculated as described previously (Slotkin & Kirshner, 1973a) and expressed as uptake per gland (a composite measure reflecting the number of functional vesicles as well as the uptake capability of each vesicle) and as uptake per unit of catecholamines (a measure of the abilities of individual vesicles to incorporate [^{3}H]-adrenaline relative to endogenous content, independently of the number of vesicles present). Nicotine *in vitro* has no direct effect on uptake (Carlsson, Hillarp & Waldeck, 1963).

In order to compare the effects of nicotine after surgical denervation with those after nicotinic receptor blockade, other experiments were done in which rats were pretreated with a blocking agent (chlorisondamine, 7.5 mg/kg s.c.) 30 min prior to receiving a single injection of nicotine (1 or 10 mg/kg s.c.). Three and 24 h after the second injection, animals were killed and the adrenals were homogenized in 2 ml of 0.15 M KCl. Aliquots (0.1 ml) were withdrawn for catecholamine analysis and the remaining homogenate either centrifuged at 26,000 *g* and the supernatant used for tyrosine hydroxylase determinations, or else diluted with an equal volume of 10 mM Tris (pH 7.2), centrifuged and the supernatant used for assay of ornithine decarboxylase according to Anderson & Schanberg (1975), with 12 μM [^{14}C]-ornithine as substrate.

Results are expressed as mean ± s.e., and levels of significance calculated by Student's *t*-test (Wine, 1964).

(±)-Adrenaline-[7-^{3}H], tyramine-[G-^{3}H], L-tyrosine-[1-^{14}C] and DL-ornithine-[1-^{14}C] were purchased from New England Nuclear Corp. (−)-Adrenaline bitartrate and nicotine were obtained from Winthrop Laboratories and Sigma Chemical Corp., respectively, and chlorisondamine from Ciba Pharmaceuticals.

Results

Administration of low doses of nicotine (1 mg/kg) had little effect on catecholamine levels in either innervated or denervated adrenal glands (Figure 1). With the higher dose (10 mg/kg) however, catecholamines in the intact glands were markedly depleted 4 to 24 h after beginning drug administration, while in the denervated side there was no change. Catecholamines in the innervated glands had returned to control levels by day 14 despite continued drug administration; at no time were the levels in the denervated glands significantly different from those of denervated controls.

The effects of nicotine on adrenal tyrosine hydroxylase activity are shown in Figure 2. After nicotine (1 mg/kg), tyrosine hydroxylase was elevated at 24 h in the intact gland while there was no significant increase in the denervated side until 7 days. With the high dose of nicotine, tyrosine hydroxylase activities in both sides were elevated by 24 h and remained so for the entire course of the study,

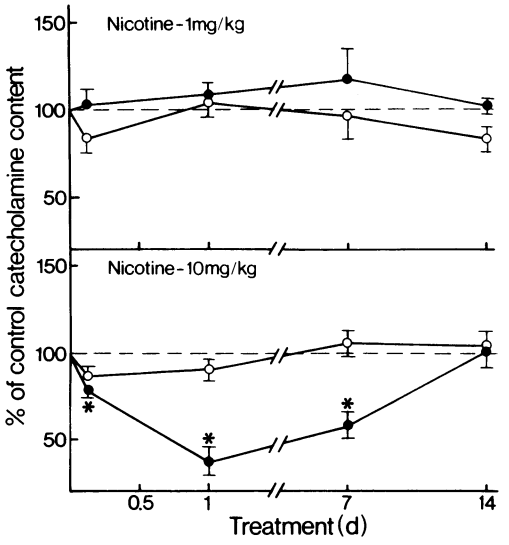

Figure 1 Effects of nicotine (1 mg/kg or 10 mg/kg twice daily) on catecholamine content in innervated (●) and denervated (○) rat adrenal glands. Points and bars represent means ± s.e. of 5 animals expressed as percentage of control catecholamine content; * denotes significant differences from control ($P < 0.05$). Control values were 8.74 ± 0.46 μg/gland in the innervated side, and 7.66 ± 0.46 μg/gland in the denervated side (20 animals).

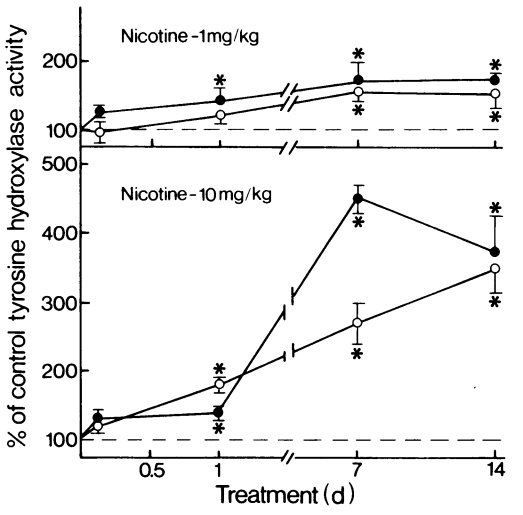

Figure 2 Effects of nicotine (1 mg/kg or 10 mg/kg twice daily) on tyrosine hydroxylase activity in innervated (●) and denervated (○) rat adrenals. Points and bars represent means ± s.e. of 5 animals expressed as percentage of control; * denotes significant differences from control ($P < 0.05$). Control values were 12.3 ± 0.8 nmol $^{14}CO_2$ evolved/h per gland in the innervated side, and 10.9 ± 0.3 nmol/h per gland in the denervated side (20 animals).

reaching levels as high as 4 times control in the intact glands and 3 times control in the denervated glands; the denervated glands required a longer period of treatment to reach the maximum level.

Adrenal dopamine β-hydroxylase activity in intact glands showed only minor changes during administration of the low dose of nicotine (Figure 3). However at the high dose, dopamine β-hydroxylase was elevated in both sides after 7 days, reaching levels twice to three times control.

To determine whether nicotine-induced changes in dopamine β-hydroxylase reflected alterations in storage vesicle synthesis, the abilities of isolated vesicles to incorporate [^{3}H]-adrenaline were evaluated. Uptake per gland remained approximately at control levels in both sides with the low dose of nicotine (Table 1). However at the high dose, uptake per gland was markedly reduced in the intact side between 1 and 7 days of drug administration, while in the denervated side there was little change.

Uptake per 100 μg of endogenous catecholamine showed little or no consistent change in either side with the low dose of nicotine, but with the high dose of nicotine at 24 h there was a marked enhancement in the innervated side with a smaller increase in the denervated side (Table 1). Uptake per 100 μg of

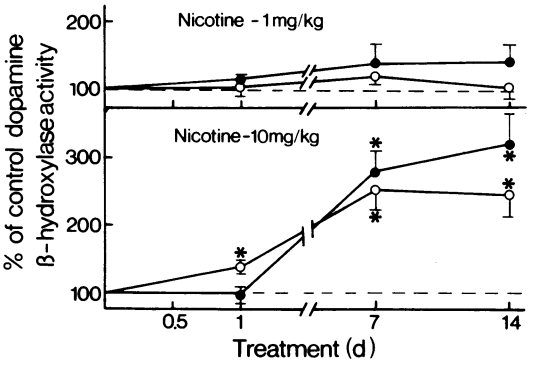

Figure 3 Effects of nicotine (1 mg/kg or 10 mg/kg twice daily) on dopamine β-hydroxylase activity in innervated (●) and denervated (○) rat adrenals. Points and bars represent means ± s.e. of 5 animals expressed as percentage of control; * denotes significant differences from control ($P < 0.05$ or better). Control values were 0.681 ± 0.037 nmol [^{3}H]-octopamine formed/h per gland in the innervated side, and 0.520 ± 0.050 nmol formed/h per gland in the denervated side (20 animals).

Table 1 Effects of nicotine on uptake of [^{3}H]-adrenaline into isolated storage vesicles from intact and denervated rat adrenals

Nicotine	*Time after start of treatment*	*[^{3}H]-adrenaline uptake* per gland		per 100 μg endogenous catecholamines	
		innervated	*denervated*	*innervated*	*denervated*
1 mg/kg	4 h	108 ± 15	99 ± 16	105 ± 8	124 ± 12
	24 h	126 ± 9*	106 ± 15	115 ± 6	98 ± 9
	7 d	101 ± 9	97 ± 14	90 ± 7	98 ± 6
	14 d	102 ± 2	87 ± 4	101 ± 6	107 ± 6
10 mg/kg	4 h	82 ± 5	95 ± 6	103 ± 4	111 ± 9
	24 h	67 ± 9**	120 ± 7	199 ± 13****	132 ± 9***
	7 d	73 ± 7**	109 ± 6	128 ± 9**	101 ± 6
	14 d	84 ± 11	83 ± 7	112 ± 8	80 ± 4***

Nicotine was given subcutaneously twice daily at the dosages indicated. Data represent means ± s.e. of 5 rats per group, as percentages of control. Control values were: innervated gland, 1.96 ± 0.16 nmol/gland, 22.1 ± 1.1 nmol/100 μg of endogenous catecholamines; denervated gland, 1.56 ± 0.15 nmol/gland, 20.0 ± 1.0 nmol/100 μg catecholamines (20 animals).
Significant differences from control are indicated by *($P < 0.05$), **($P < 0.02$), ***($P < 0.005$) or ****($P < 0.001$).

Table 2 Effects of chlorisondamine and nicotine on rat adrenal catecholamines, ornithine decarboxylase and tyrosine hydroxylase activities.

First injection	*Second injection (30 min later)*	*3 h after second injection*		*24 h after second injection*	*n*
		Catecholamine (μg/gland)	*Ornithine decarboxylase (nmol $^{14}CO_2$/h per gland)*	*Tyrosine hydroxylase (nmol $^{14}CO_2$/h per gland)*	
Saline	*Saline*	10.2 ± 0.5	0.00 ± 0.09	5.95 ± 0.34	12
Chlorisondamine (2.5 mg/kg)	*Saline*	10.2 ± 0.3	0.60 ± 0.12****	6.77 ± 0.63	6
Chlorisondamine (7.5 mg/kg)	*Saline*	9.48 ± 0.39	2.85 ± 0.72****	7.31 ± 0.40**	12
Chlorisondamine (20 mg/kg)	*Saline*	9.01 ± 0.82	2.18 ± 0.65***	8.84 ± 0.21****	6
Saline	*Nicotine* (1 mg/kg)	9.39 ± 0.72	0.54 ± 0.12***	7.54 ± 0.63*	6
Saline	*Nicotine* (10 mg/kg)	7.31 ± 0.37****	7.09 ± 1.16****	10.7 ± 0.7****	6
Chlorisondamine (7.5 mg/kg)	*Nicotine* (1 mg/kg)	9.27 ± 0.78	0.53 ± 0.14**	8.79 ± 0.90**	6
Chlorisondamine (7.5 mg/kg)	*Nicotine* (10 mg/kg)	8.95 ± 0.47	3.72 ± 0.75****¶	7.45 ± 0.40**§	6

All drugs were administered s.c. Data represent means ± s.e.
*$P < 0.05$ vs. saline–saline; **$P < 0.02$; ***$P < 0.005$; ****$P < 0.001$. ¶ $P < 0.05$ vs. nicotine alone, not significant vs. chlorisondamine alone. §$P < 0.005$ vs. nicotine alone, not significant vs. chlorisondamine alone. n = number of determinations.

catecholamines in the denervated adrenal had declined to normal by 7 days after initiation of the high dose and to slightly below normal by 14 days, while the intact gland remained elevated until 14 days.

The effects of a nicotinic blocking agent on the actions of nicotine are shown in Table 2. In these studies, the activity of ornithine decarboxylase, an enzyme involved in polyamine synthesis, was used as a marker for stimulation of the adrenal gland (Byus & Russell, 1974); the 3 h time point was chosen because in that period, stimulation of the adrenal gland produces an increment in ornithine decarboxylase activity primarily in the medulla as opposed to the cortex (Byus & Russell, 1974). Administration of nicotine (10 mg/kg) alone caused acute catecholamine depletion and marked elevations in ornithine decarboxylase (3 h) and subsequently in tyrosine hydroxylase (24 h). Pretreatment with the blocking agent, chlorisondamine, eliminated the nicotine-induced amine depletion and reduced ornithine decarboxylase and tyrosine hydroxylase activities to those seen after chlorisondamine alone. The partial stimulatory effect of chlorisondamine itself is consistent with previous observations that a number of ganglionic blocking agents also evoke stimulation of the adrenal medulla (Mueller, Thoenen & Axelrod, 1970a; Carlsson & Lindqvist, 1974; Slotkin, Seidler, Lau, Bartolomé & Schanberg, 1976).

Discussion

Agents which either stimulate nicotinic receptors directly or which evoke central stimulation of the sympatho-adrenal axis, cause quantal exocytotic release of adrenomedullary catecholamines (Schneider, 1969; Viveros *et al.*, 1969; Slotkin & Kirshner, 1973a; Anderson & Slotkin, 1975; Slotkin & Seidler, 1975) and a parallel decline in amine levels and in the number of functional adrenal storage vesicles (Viveros *et al.*, 1969; Slotkin & Kirshner, 1973a). In the present study, repeated administration of nicotine (10 mg/kg) produced, as expected, a depletion of catecholamines and also a reduction in the uptake per gland of [^{3}H]-adrenaline, which illustrates the reduced number of functional vesicles. However, in denervated adrenal glands in the same rats, no catecholamine depletion was observed and there was no decrease in vesicle function, indicating that these actions of nicotine require an intact nerve supply.

Three hypotheses could explain this phenomenon: first, nicotine could act only reflexly via the splanchnic nerve and not directly on the adrenal gland; this is unlikely, since direct nicotine-induced secretion of catecholamines already has been demonstrated (Schneider, 1969; Tsujimoto & Nishikawa, 1974). Second, long-term denervation could alter the number and/or function of nicotinic receptors in the adrenal gland such that stimulation by nicotine can no longer occur; this is also unlikely, since nicotine was able to increase activities of both tyrosine hydroxylase and dopamine β-hydroxylase, indicating adrenomedullary stimulation by the drug (Thoenen, 1974). Third, denervation could alter the processes participating in stimulus-secretion coupling, such that nicotine produces a smaller secretory response; since there is substantial evidence that denervation can indeed change the secretory response to a number of other drugs, this may explain the inability of nicotine to cause significant catecholamine depletion. For example, acute (short-term) surgical or chemical interruption of neural input to the adrenal medulla prevents the immediate secretory effects of drugs which act via splanchnic stimulation (Stewart & Rogoff, 1916; Mueller *et al.*, 1970a; Viveros, Arqueros & Kirshner, 1971; Yoshizaki, 1973) but upon chronic denervation (or in neonatal rats, which do not have a functional nerve supply to the adrenal gland) the secretory response reappears (Yoshizaki, 1973; Anderson & Slotkin, 1976; Bartolomé & Slotkin, 1976); however, under those circumstances, the restored secretory response is different from the normal effect in that it probably does not involve nicotinic receptors (Bartolomé & Slotkin, 1976).

The effects of chronic nicotine administration on tyrosine hydroxylase and dopamine β-hydroxylase activities also were different in denervated and in innervated glands. While either low or high doses of nicotine ultimately produced nearly equivalent induction of tyrosine hydroxylase in both types of gland, the increases in the denervated side lagged behind those in the innervated side, indicating that under normal circumstances there are both direct and neural contributions to nicotine-evoked tyrosine hydroxylase induction. In contrast, dopamine β-hydroxylase induction in denervated adrenals exhibited the same time course as in innervated glands, suggesting that the neural component may be relatively less essential in mediating induction of that enzyme. These results are consistent with previous findings in denervated adrenals that other agents also are able to induce dopamine β-hydroxylase fully but are much less effective for tyrosine hydroxylase (Patrick & Kirshner, 1971; Slotkin *et al.*, 1976; Anderson & Slotkin, 1976).

In innervated adrenal glands, the increase in tyrosine hydroxylase activity and replacement of storage vesicles are thought to be determining factors in maintaining catecholamine stores in the presence of increased stimulation; as a consequence of tyrosine hydroxylase induction and new vesicle formation, amine levels can return to normal despite continued drug-induced stimulation. One index of these changes is the uptake per unit (100 µg) of catecholamines, a

measure of new vesicle formation; since new vesicles initially have abnormally low catecholamine levels, this index tends to rise if exocytotic loss of amines and intact vesicles exceeds the rate of replacement and to fall when resynthesis approximates loss (Slotkin & Kirshner, 1973a, 1973b; Anderson & Slotkin, 1975; Slotkin & Seidler, 1975).

In the present study, the innervated glands showed an initial (24 h to 7 days) rise in uptake per unit of catecholamine, corresponding to accelerated vesicle turnover and a consequent increase in the number of immature vesicles. Despite continued drug administration, uptake per unit of catecholamine returned to normal in the innervated gland, indicating that amine and vesicle replacement were able to overcome the nicotine-induced increase in secretion. In contrast, the denervated side showed a much smaller initial increase in uptake per unit of catecholamine, supporting the hypothesis that secretion is reduced by denervation. Uptake per unit of catecholamine in denervated glands returned to normal earlier (7 days) than in the intact adrenals despite the smaller degree of tyrosine hydroxylase induction in the denervated side; this is not surprising, since the lower degree of secretion would result in less demand for replacement.

It is unclear whether the effects of chronic nicotine administration on denervated adrenals result purely from direct effects on the tissue or whether secondary actions via changes in adrenal steroids could play a role (Weinshilboum & Axelrod, 1970; Gewirtz, Kvetňanský, Weise & Kopin, 1971; Kvetňanský, 1974); nicotine causes ACTH release from the pituitary as well as evoking direct secretion of adrenocortical steroids (Kershbaum, Pappajohn, Bellet, Hirabayashi & Shafiha, 1968; Suzuki, Ikeda & Narita, 1973; Rubin & Warner, 1975). Since it is unlikely that cutting the splanchnic nerve alters nicotine-induced ACTH release, hormonal effects alone cannot account for the loss of the catecholamine-depleting effect of nicotine in denervated adrenals. The increases seen in tyrosine hydroxylase and dopamine β-hydroxylase activities after chronic nicotine are much higher and occur much sooner than those which can be achieved after large doses of ACTH or glucocorticoids (Weinshilboum & Axelrod, 1970; Mueller *et al.*, 1970b); however, a partial contribution of the pituitary-adrenal axis cannot be ruled out. Nicotine-induced adrenal stimulation as determined by catecholamine secretion, ornithine decarboxylase and tyrosine hydroxylase activities, was blocked completely by administration of chlorisondamine, suggesting that the non-neural component represents effects mediated via nicotinic receptors in the adrenal medulla itself and/or nicotinic receptors involved in ACTH secretion.

In conclusion, the effects of nicotine on regulation of catecholamine and storage vesicle secretion and synthesis reflect several sites of action, and long-term denervation produces alterations in the abilities of nicotine to deplete adrenal catecholamines and to induce catecholamine biosynthetic enzymes.

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