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STUDIES ON THE MECHANISM OF TOLERANCE TO NICOTINE-INDUCED ELEVATIONS OF URINARY CATECHOLAMINES*

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Abstract—The mechanism of the return to normal of the elevated urinary catecholamines observed after the chronic administration of nicotine has been investigated. Three possible mechanisms leading to this phenomena were explored: (1) that continual nicotine exposure leads to an induction of enzyme activity resulting in an increased metabolism of the alkaloid, (2) that nicotine exposure results in some alterations in the storage or release of catecholamines, and (3) that some alteration in the inactivation of the amines occurred. The data indicate that after chronic nicotine (1 mg/kg, s.c., twice daily up through 14 days), (1) there was no increase in the metabolism of nicotine itself determined from measurements of nicotine oxidase activity, (2) there was no alteration in the endogenous tissue levels of catecholamines determined by fluorometric analysis, (3) there was no alteration in the tissue uptake of [^{3}H]norepinephrine, and, as previously reported, (4) no alteration in the turnover of norepinephrine determined by measuring the decline of [^{3}H]norepinephrine in the heart following i.v. administration. On the other hand, there was a significant increase in the monoamine oxidase activity of the heart and liver and an increase in the catechol-*o*-methyl transferase activity of the liver. It is concluded that tolerance to nicotine induced elevations of urinary catecholamines is due to increased metabolic enzyme activity resulting in faster metabolism of the catecholamines released from the adrenal medulla and adrenergic nerve terminals.

NICOTINE has been one of the most useful pharmacological tools available in helping to gain a better understanding of the autonomic nervous system.[1] In addition, it is also a very important psychopharmacological agent as evidenced by its wide spread use in the form of tobacco smoking. One of the most prominent of the pharmacological actions of this drug is its ability to stimulate the adrenal medulla and autonomic ganglia.[2–4] Adrenal medullary stimulation with a resultant release of catecholamines is thought to play a very important part in contributing to the various cardiovascular responses that are observed after the administration of this alkaloid.[5–8] In addition, nicotine has recently been shown to produce a vasoconstrictor effect which is due to a direct action on adrenergic nerve terminals.[9,10]

We have previously observed that if rats are treated daily with nicotine there is an elevation in the urinary excretion of catecholamines which reaches a peak following 3 days of treatment. However, after 7 days of continuous administration there is a marked fall-off in the urinary levels and by 14 days the urinary catecholamine excretion is essentially normal.[5,11] The purpose of this present investigation was to study the mechanisms of the return to normal of the elevated urinary catecholamines observed

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during chronic nicotine administration. Three possible mechanisms leading to this phenomena have been studied: First, that continuous nicotine exposure leads to an induction of the enzymes responsible for the metabolism of the alkaloid, therefore reducing the amount of nicotine available to stimulate the adrenal medulla and autonomic ganglia; secondly, that nicotine treatment results in a decreased release of catecholamines because the stores had been depleted, or because of a decrease in neural impulses arriving at adrenergic nerve terminals and/or at the adrenal and thirdly, that nicotine produces an increase in the processes responsible for the inactivation of catecholamines once released from storage sites (reuptake and/or enzymatic metabolism by monoamine oxidase and/or catechol-*o*-methyl transferase).

MATERIALS AND METHODS

All studies were carried out on male Sprague–Dawley rats with weights ranging from 200 to 300 g. Nicotine (Eastman Organic) was administered in various doses as the pure base by subcutaneous (s.c.) administration. Saline served as control vehicle in all experiments.

Sleeping time experiments. In order to gain some information on the possible enzyme inducing properties of nicotine, sleeping time measurements with the test drugs, hexobarbital (Winthrop Laboratories) and zoxazolamine (McNeil Laboratories) were carried out according to the procedures suggested by Conney, *et al.*[12] Rats were injected with nicotine (1 mg/kg, s.c., twice daily) or saline (0·2 ml, twice daily) for 7 or 14 days prior to the tests. Phenobarbital (60 mg/kg, i.p., once daily for 7 days) and 3-methylcholanthrene (Mann Research Laboratories, Inc.) (40 mg/kg, i.p., 24 hr prior to testing) were included as additional controls. Hexobarbital was administered in a dose of 125 mg/kg, i.p., and zoxazolamine in a dose of 100 mg/kg, i.p. The dose of nicotine was administered in the form of the pure base, whereas the other drugs were in the form of their salts. The onset of sleeping time was determined by the disappearance of the righting reflex, and duration of sleeping time, the time required for an animal to elicit his righting reflex spontaneously.

Nicotine oxidase assay. Nicotine oxidase activity in the liver of nicotine or saline treated rats was assayed in order to determine more directly whether or not nicotine administration can increase the activity of enzymes which metabolize the alkaloid. A modified version of the method described by Hucker *et al.*,[13] was used. Rats were killed by decapitation and preparation of the tissue samples carried out at 3°. Upon removal, the liver was homogenized in a glass conical homogenizer in 1·15% cold potassium chloride. The homogenate was centrifuged at 10,000 *g* and the supernatant fraction taken as the enzyme source. A 3-ml aliquot was incubated in a mixture containing 500 mg/ml nicotine; 0·25 μmole DPN, 0·25 μmole TPN, 6 μmoles glucose-6-phosphate, 75 μmoles $MgCl_2$, 2 μmoles ATP, 120 μmoles nicotinamide and 0·2 M phosphate buffer, pH 7·4. The incubation was carried out at 37° in a Dubnoff shaker under oxygen for 1 hr.

Following incubation, three ml of the incubation mixture were pipetted into a 50-ml glass-stoppered centrifuge tube containing 2 ml of 0·1 N NaOH and 30 ml of heptane containing 1·5% isoamyl alcohol and shaken for 20 min. Following centrifugation, 20 ml of the heptane phase were transferred to another 50 ml glass-stoppered centrifuge tube containing 5 ml 0·1 N HCl. The solution was shaken for 5 min, centrifuged

and the organic phase removed by aspiration. Three ml of the acid phase was transferred to a cuvette and the optical density measured at 259 mμ in a spectrophotometer. Reagent blanks run through the procedure was used as the zero setting and solutions of known amounts of nicotine served as standards. Enzyme activity was measured by circulating the amount of nicotine metabolized.

Norepinephrine levels. Animals were killed by decapitation at various periods of time after nicotine administration and tissues removed, washed in saline, weighed and homogenized in 5% trichloroacetic acid in an Ultra-Turrax homogenizer. Adrenal glands were homogenized by means of a mortar and pestle and a small amount of ground sand. Following homogenization, the samples were filtered, adsorbed on alumina and catecholamine determinations carried out according to the trihydroxyindole method of Euler and Lishajko.[14] The mean recovery of catecholamines added to tissue homogenates and carried through the procedure was 78 per cent for norepinephrine and 75 per cent for epinephrine. All reported tissue values have been corrected for recovery.

Uptake of norepinephrine. Hearts were removed and placed in an Anderson–Craver coronary perfusion apparatus (Metro Scientific Company). The normal perfusion medium contained the following composition in millimoles per liter: NaCl, 119·8; KCl, 5·63; $CaCl_2$, 2·16; $MgCl_2$, 2·10; dextrose 10·0 and $NaHCO_3$, 25·0. Ascorbic acid (10 mg/100 ml) and EDTA (10 mg/l.) were added to the perfusion medium to prevent the oxidation of norepinephrine. The solution was bubbled with 95% O_2–5% CO_2; the temperature was 38 ± 1°. Hearts were perfused at a constant rate of 5·0 ml/min ± 0·25 ml/min. Hearts were perfused for 30 min with a normal medium and then for 10 min with a medium containing 0·1 μc of [^{3}H]norepinephrine per ml. This was followed by a 3 min perfusion with norepinephrine-free medium to wash out extracellular norepinephrine. At the end of the perfusion, hearts were removed, extracted in 5% TCA and following alumina column chromatography, assayed for norepinephrine by fluorometry and scintillation spectrometry.

Monoamine oxidase activity. Monoamine oxidase activity was carried out, according to the method of Wurtman and Axelrod.[15] Animals were sacrified by decapitation and 200 mg of liver, or the whole heart, taken and homogenized in cold isotonic KCl. Tissues were rehomogenized so that final tissue concentration of liver was 2 mg/ml. Tissue homogenates were incubated in a reaction mixture containing 0·1 M phosphate buffer and [^{14}C]tryptamine (New England Nuclear, S.A. 10 mc/m-mole) for 20 min at 37°. The reaction was stopped by the addition of 2 N NCl and the [^{14}C]indoleacetic acid formed was extracted into toluene and counted in a scintillation counter. Blanks were prepared by placing them in a boiling water bath for 3 min.

Catechol-O-methyl transferase activity. COMT activity was measured according to the method of Krakoff *et al.*[16] One g of tissue (heart or liver) was homogenized in 4 ml of 1·15% KCl and centrifuged for 10 min at 10,000 *g*. An aliquot of the supernatant fraction was then added to an incubation mixture containing 0·5 M phosphate buffer, 2 M $MgCl_2$, 50 μg of epinephrine and 1 μg of 5-adenosyl-L-methionine-methyl-^{3}H (New England Nuclear) (S.A. 384 mc/m-mole). The samples were incubated at 37° for 1 hr and the reaction stopped by the addition of 0·5 M borate buffer. [^{3}H]metanephrine was extracted into 6 ml of a toluene: isoamylalcohol mixture by shaking and an aliquot taken for radiometric analysis and counted in a scintillation counter.

RESULTS

It can be seen from Table 1 that both phenobarbital and 3-methylcholanthrene resulted in a significant decrease in the sleeping time to zoxazolamine while only phenobarbital produced a reduction in the sleeping time to hexobarbital. On the contrary, pretreatment of similar rats with nicotine for 7 or 14 days did not significantly

TABLE 1. INFLUENCE OF DRUGS ON THE "SLEEPING TIME" TO HEXOBARBITAL OR ZOXAZOLAMINE IN THE RAT

Treatment	Length of treatment (days)	"Sleeping time" (min) Hexobarbital* Mean ± S.E.M.	*n*	Zoxazolamine† Mean ± S.E.M.	*n*
Saline	7	32 ± 2	12	329 ± 55	15
Nicotine‡	7	45 ± 7	14	292 ± 42	15
Saline	14	38 ± 4	13	367 ± 41	13
Nicotine‡	14	40 ± 2	15	380 ± 42	16
Phenobarbital§	7	6 ± 0·4	10	83 ± 10	10
3-MC‖	1	35 ± 6	11	24 ± 3	11

* 125 mg/kg, i.p.
† 100 mg/kg, i.p.
‡ 1 mg/kg, s.c., twice daily.
§ 60 mg/kg, i.p.
‖ 40 mg/kg, i.p.

alter the sleeping times to either test drug. In order to determine more directly whether or not chronic nicotine administration can increase the activity of enzymes which metabolize the alkaloid, nicotine oxidase activity was measured. The results are shown in Fig. 1 and it can be seen that treatment with nicotine does not alter the enzyme activity *in vitro*.

The influence of nicotine on the tissue concentration of catecholamines is depicted in Table 2. In this experiment, nicotine was injected four times a day and there was

TABLE 2. TISSUE CATECHOLAMINE LEVELS FOLLOWING NICOTINE ADMINISTRATION IN THE RAT

Saline or days of nicotine administration*	Adrenal E (μg/g) ± S.E.M. (*n*)	Adrenal NE (μg/g) ± S.E.M. (*n*)	Heart NE (μg/g) ± S.E.M. (*n*)
Saline	120 ± 10 (12)	40 ± 5 (12)	0·74 ± 0·03 (25)
Nicotine:			
1 day	118 ± 15 (10)	43 ± 9 (10)	0·72 ± 0·05 (12)
4 days	115 ± 18 (11)	50 ± 6 (11)	0·75 ± 0·06 (14)
7 days	122 ± 19 (9)	42 ± 5 (9)	0·70 ± 0·05 (12)

* 1 mg/kg, s.c., four times daily.

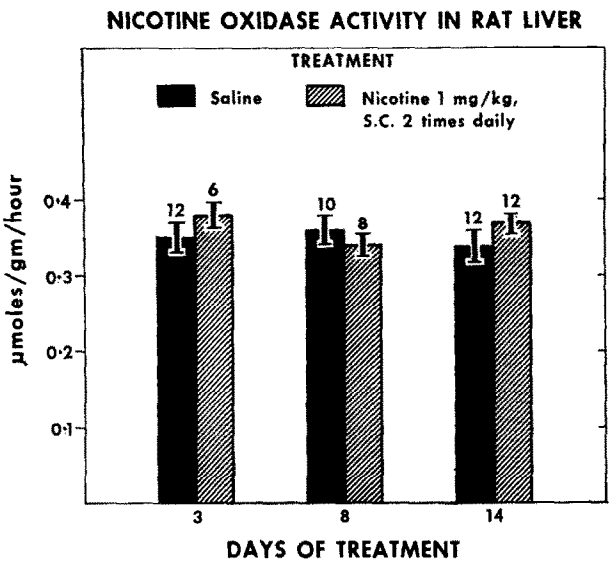

FIG. 1. Depicts the effect of nicotine (hatched bar) administered in a dose of 1 mg/kg, s.c., twice daily, for 3, 8 or 14 days or saline (black bar) 0·2 ml/rat on the nicotine oxidase activity in the liver plotted as μmoles/g/hr $\pm$ standard error of the mean (1). Numbers above the bars refer to number of animals

still no change in the epinephrine or norepinephrine content of the adrenals nor in the norepinephrine content of the heart.

Table 3 shows the effect of nicotine administration on the uptake of [^{3}H]norepinephrine in the perfused rat heart. No significant difference was detected between nicotine or saline treated animals.

Table 4 shows the effect of nicotine treatment on the *in vitro* monoamine oxidase activity of rat heart and liver. Nicotine treatment resulted in a significant increase in the MAO activity of both tissues. The peak effect in the heart was seen after 2 days of treatment and remained elevated through 8 days and finally returned to normal after 10 days of treatment. The effect on liver MAO activity was not as marked and appeared to reach a peak after 4 days of administration. The increased activity in this tissue was also back within the normal range after 10 days of chronic treatment.

The influence of nicotine on catechol-*O*-methyl transferase activity is depicted in Table 5. The data show that there was no change in heart COMT activity, while the activity in liver was significantly elevated over control levels following 2 days of administration and was still elevated after 14 days of treatment.

DISCUSSION

The mechanism of the return to normal of the elevated urinary catecholamines observed after chronic nicotine administration was investigated in the present study. Three possible mechanisms leading to this phenomena were explored. First, that continual nicotine exposure leads to an induction of enzyme activity resulting in an increased metabolism of the alkaloid and therefore to a decrease in the release of

catecholamines due to less nicotine; secondly, that nicotine exposure results in some alterations in the storage or release of catecholamines, or thirdly, that some alterations in the inactivation of the amines occurred which resulted in the appearance of less free amine in the urine.

Since the duration of action of hexobarbital and zoxazolamine are mainly under the control of microsomal enzymes in the liver,[12] they have been frequently employed as test drugs to obtain information on enzyme inducing properties of various drugs. For instance, most agents which increase microsomal enzyme activity appear to decrease the duration of action of one or both of these test drugs. In the present study, the sleeping time to hexobarbital or zoxazolamine was compared in rats receiving saline or nicotine for 7 or 14 days. Phenobarbital and 3-methylcholanthrene, which are well known to alter the sleeping times to the two test drugs, were administered as additional

TABLE 3. UPTAKE OF [^{3}H]NOREPINEPHRINE IN THE PERFUSED RAT HEART FOLLOWING NICOTINE ADMINISTRATION

Days of nicotine* or saline† administration	Saline	Nicotine
	Mean ± S.E.M. (*n*) (dis/min/g × 10^6)	Mean ± S.E.M. (*n*) (dis/min/g × 10^6)
1	1·65 ± 0·17 (5)	1·69 ± 0·21 (5)
3	1·67 ± 0·21 (5)	1·74 ± 0·10 (5)
7	1·68 ± 0·15 (5)	1·70 ± 0·18 (6)
14	1·73 ± 0·23 (4)	1·77 ± 0·21 (5)

* Nicotine, 1 mg/kg, s.c., twice daily.
† Saline, 0·2 mg/rat, twice daily.
[^{3}H]NE (0·1 μc/ml) perfused for 10 min at a constant flow of 5 ml/min.

TABLE 4. EFFECT OF NICOTINE ON RAT HEART AND LIVER MONOAMINE OXIDASE ACTIVITY

Treatment	Length of treatment (days)	Heart MAO activity* ± S.E.M.	Liver MAO activity* ± S.E.M.
Saline	1–14†	0·38 ± 0·04	1·71 ± 0·19
Nicotine	1	0·33 ± 0·02	1·59 ± 0·13
Nicotine	2	0·69 ± 0·07‡	1·63 ± 0·15
Nicotine	4	0·62 ± 0·06‡	2·28 ± 0·21‡
Nicotine	6	0·54 ± 0·07‡	1·80 ± 0·17
Nicotine	8	0·49 ± 0·05‡	2·16 ± 0·19‡
Nicotine	10	0·38 ± 0·06	1·40 ± 0·20
Nicotine	14	0·45 ± 0·06	1·42 ± 0·18

* MAO activity is expressed as micromoles of [^{14}C]indolacetic acid formed from [^{14}C]tryptamine per gram tissue per hour.
† MAO activity in control rats treated with saline for identical periods of time as nicotine did not differ significantly from one another and are therefore pooled together.
‡ $P < 0·05$.

TABLE 5. EFFECT OF NICOTINE ON RAT HEART AND LIVER CATECHOL-*O*-METHYL TRANSFERASE (COMT) ACTIVITY*

Treatment	Length of treatment (days)	Heart COMT activity* ± S.E.M.	Liver COMT activity* ± S.E.M.
Saline	1–14†	14·2 ± 3	67·8 ± 9
Nicotine	1	15·5 ± 4	77·6 ± 5‡
Nicotine	2	10·5 ± 4	89·1 ± 7§
Nicotine	14	12·5 ± 5	86·5 ± 9§

* COMT activity expressed as mμmoles of product formed per hour per gram wet weight of tissue.

† COMT activity in control rats treated with saline for identical periods of time, as nicotine did not differ significantly from one another and are therefore pooled together.

‡ $P < 0.05$.

§ $P < 0.01$.

controls. The data showed that there was no alteration in the sleeping time to either test drug following nicotine administration, although there were marked alterations following either phenobarbital or 3-methylcholanthrene. When the nicotine oxidase activity in the livers taken from nicotine treated rats was measured, there was likewise no indication of induced enzyme activity. Since the major metabolic pathway for nicotine is oxidation to cotinine by nicotine oxidase,[13] the present results have not provided any data to suggest that daily nicotine treatment results in an increased inactivation of the alkaloid; thus a decrease in the amount of nicotine in the circulation does not appear to be the explanation for the return to normal of the elevated urinary catecholamines.

The lack of enzyme induction following nicotine treatment observed in the present study is in contrast to a report by Yamamoto *et al.*,[17] who reported that nicotine treatment increased the activity of metabolizing enzymes to 2-acetylaminofluorene and 3,4-benzpyrene in rat liver microsomes. However, the doses used by these investigators 5–40 mg/kg was far in excess of what was used in the present study and can certainly be considered within the toxic range for this alkaloid. Also, these investigators observed increased enzyme activity only after single injections of nicotine and when the alkaloid was administered for 2 or 3 days, no increase in enzyme activity was observed. Reports of increased enzyme activity have also been seen following cigarette smoking,[18–19] however, none of these studies have actually determined what the active inducing agent is in the cigarette smoke. Since there are substances such as benzpyrene in tobacco smoke, which are well known to have enzyme inducing properties,[12] it is quite possible that they are the active enzyme inducing agents rather than nicotine itself.

In order to determine whether or not chronic nicotine treatment was interfering with mechanisms which store release or inactive catecholamines several experiments were carried out. Tissue catecholamine levels were measured at various periods of time during chronic nicotine treatment to determine whether depletion of the amines could be a possible explanation for the phenomena under investigation. No alterations in tissue levels were observed which is consistent with observations reported elsewhere.[5,6,11,20,21] Although nicotine stimulates autonomic ganglia and the adrenal

medulla in low doses, it is well known that larger doses will block autonomic ganglia.[1] Under such conditions, there would be a decrease in the output of catecholamines due to a decrease in neural impulses arriving at adrenergic nerve terminals, or at the adrenal. Several recent studies have shown that, in the presence of sympathetic decentralization, ganglionic blocking agents such as chorisondamine and pempidine, or adrenergic neuronal blockers such as bretylium and BW-392060, there is a decrease in the efflux of norepinephrine from adrenergic nerve terminals and a decrease in the turnover of the amine.[22] Previous studies utilizing a measure of norepinephrine turnover to obtain information on whether or not nicotine was preventing the release of amine due to ganglionic blockade or by a bretylium-like action did not reveal any changes in turnover due to nicotine when compared to controls.[20] It is therefore assumed that ganglionic blockade or neuronal blockade of the bretylium type had not occurred and was not contributing to the phenomena under investigation. Since it is well established that uptake of catecholamines into adrenergic nerve terminals is an important mechanism for the inactivation of these amines,[23,24] the possibility that some alteration in this process was contributing to the phenomena was also investigated. No significant difference in the uptake of [^{3}H]norepinephrine into hearts of nicotine treated animals was observed.

To determine whether or not there was a faster metabolism of the catecholamines after they were released from the adrenal gland and adrenergic nerve terminals, monoamine oxidase and catechol-*o*-methyl transferase activities were measured in both heart and liver. In both cases there was a significant increase in the activity of those enzymes. The increase in MAO activity followed closely the elevations in urinary catecholamines returning to normal, approximately 2 days after the amines. COMT activity in liver remained elevated throughout the time course of drug treatment.

Because the MAO activity did not remain elevated, it cannot be responsible for the tolerance observed at least after 10–14 days of nicotine treatment. However, COMT activity did remain elevated and it is probable that it is playing a more important role in contributing to the faster metabolism of the catecholamines. This is consistent with the fact that *o*-methylation is the most important enzymatic mechanism for metabolizing circulating catecholamines. This is also consistent with increased *o*-methylation of [^{14}C]norepinephrine observed in familial pheochromocytoma patients who maintained a normal blood pressure despite elevated catecholamines.[25] The question has been raised from these data of why there was no change in tissue norepinephrine content if metabolism were increased yet synthesis remains constant as reported previously.[20] Since COMT activity increased only in the liver and not the heart, and because the increase in MAO activity was not sustained, it would appear that the increased metabolism is mainly in response to the increased circulating levels of catecholamines released from the adrenal medulla rather than norepinephrine within tissues such as the heart.

These data would suggest, therefore, that repeated nicotine administration with the subsequent release of catecholamines and elevated plasma levels results in an increase in the activity of enzymes, primarily COMT, which metabolize the catecholamines, therefore leading to a decrease in the appearance of the free amines in the urine. It is thought that it is the released catecholamines, rather than nicotine itself which is the contributing agent, since there was no alterations in nicotine oxidase activity.

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