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Abstract: The effects of antihipertensive phenoxybenzamine were investigated on tyrosine hydroxylase (TH) enzyme activity and TH mRNA levels. In the present study 5 months male sprague dawley (SD) rats were used. Phenoxybenzamine was injected i.p as 20 mg/kg which prepared in the 0.9 % NaCl and 5% ethanole. TH activity was measured by detecting of formation of ^3H_2O as a formation of dopa from ^3H-tyrosine by radioisotope technique total adrenal RNA was isolated and hybridized wit ^{32}P labeled cDNA. TH mRNA was assayed by densitometric scanning of the autoradiograms using a densitometer.

TH activity and TH mRNA levels were found to be significantly increased by the effcet of phenoxybenzamine ($P<0.01$).

Key Words:: Phenoxybenzamine, tyrosine hydroxylase, adrenal medulla, TH mRNA

Introduction

Tyrosine hydroxylase (TH) is thought to be rate-limiting enzyme in biosynthesis of catecholamines. Dopamine, norepinephrine (NE), epinephrine are the catecholamines which synhthesized from tyrosine amino acid. The synthesis and releases of catecholamines have occurred in brain, kromaffin cells, sympathetic ganglia and heart (1-6). TH activity is controllled by negative feedback in catecholamine biosynthesis. First step is hydroxylation of tyrosine and biopterin cofactor required. Various stressors have been shown to increase in TH activity. Constant cold exposure, hypertension, neurochemical alterations, aging, antihypertensive drug treatments are well known to increase TH activity in the adrenal medulla and sympathetic neurons. Tyrosine hydroxylase comprise a family of enzymes known as the aromatic amino acid hydroxylases. This enzyme is iron-containing mixed function oxidases which require a reduced pterin cofactor and molecular oxygen (7-9). TH (EC 1.14.16.2) catalyses the formation of L-dihydroxyphenylalanine (Dopa) from L-tyrosine. The accumulation of norepinephrine by sympathetic nerves of tissues made it possible to examine the effect of drugs in blocking its uptake. The following drugs were found to block the uptake of norepinephrine: cocaine, imipramine, amphetamine, tyramine and pnenoxybenzamine (10). Phenoxybenzamine binds covalently to alpha receptors, causing irreversible blockade of long duration (14-48 hours). The drug inhibits reuptake of released norepinephrine by presynaptic adrenergic nerve terminals (11, 12). The pharmacological actions of phenoxybenzamnie are primarily related to antagonism of alpha-receptor mediated events. Most importantly, phenoxybenzamine blocks catecholamine induced vasoconstriction. Phenoxybenzamine competes with the catecholamines for alpha receptor sites and neuronal uptake is blocked. The pharmacological consequence of blocking neuronal uptake is to increase the actions of norepinephrine by blocking inactivation by neuronal uptake (13). In the present study Epinephrine and norepinephrine are the most known catecholamines and neurotransmitters. Their concentrations depend on TH enzyme activity in the catecholamine biosynthesis pathway. The aim of the present study is to investigate of effects of phenoxybenzamine on TH enzyme activity and TH mRNA levels.

Methods

Ten males Sprague Dawley (SD) rats, 3 months old, were used in the present study. Rats were housed individually in cages with food and water ad libitum. Temperature was 26°C. Five rats were maintained at 26°C for control animals. Phenoxybenzamine was prepared in the 0.9% NaCl of 5% Ethanol and injected other 5 rats as 20 mg/kg i.p. 0.9% NaCl of 5% ethanol was injected in to control animals. In-

Table 1. Amount of solutions in assay of TH enzyme activity

	Assay number					
Solutions	20	30	40	50	60	Final Moles
0.5 M PIPES	100	150	200	250	300	50 mM
1mg/ml catalase	40	60	80	100	120	40 mg/ml
2 mM tyrosine	50	75	100	125	150	100mM
1 mM DTT	5	7.5	10	12.5	15	5 mM
d H_2O	245	367.5	490	612.5	735	-
1mM$Fe(NH_4)(SO_4)_2$	10	15	20	25	30	10 mM
30 mMH_4 6MPH_4	50	75	100	125	150	1.5 mM
Final Volume(μl)	500	750	1000	1250	1500	-

jections were performed every 20 minute. Rats were anaesthetized with pentobarbital (90 mg/kg) and adrenal glands were removed quickly and rapidly frozen in liquid nitrogen. Tissues were stored at -20˚C until use. TH activity, total protein and TH mRNA were determined in aliquots of the same sample. Total protein was quantified by the method of Bradford (14). TH activity was measured using the radioenzymatic assay as described by Reinhard et al (15). TH activity was determined by monitoring the formation of ^3H_2O as a by product of L-[^{3}H]-tyrosine hydroxylation the formation of L-[^{3}H]-dopa from L-[^{3}H]-tyrosine. Determination of TH enzyme activity as follows; 25 μl homogenate was analyzed at pH 7.0 in the presence of 6-MPH_4 and [3,5-^{3}H]-tyrosine in a total volume 50 μl for 15 min. at 37˚C. Total adrenalmedullary RNA was isolated by using RNAzolB (Biotec, Friendswood, TX). Total RNA was quantified spectrophotometrically at 260 nm (5,25). Diluted RNA samples were blotted onto nylon membrane (Gene Screen, New England Nuclear, Boston, MA) using a slot blot apparatus. The filters baked at 80˚C for 2-4 h, then prehybridized with 50 μl denatured salmon testes DNA. After incubation for 14-16 h 42˚C, filters hybridized with a ^{32}P TH.36 cDNA probe (supplied by Dr. Karen O'Malley Washington University, School of Medicine and phenoxybenzamine supplied by Dr. Nihal Tümer, University of Florida, Pharmacology Department). The resulting ^{32}P labeled RNA-DNA hybrids were detected by autoradiography using Kodak x-ray films (16). TH mRNA was assayed by densitometric scanning of the autoradiograms, using a densitometer (Bio-Rad, 620 video densitometer). The amount of TH mRNA was expressed as OD units per mg of total RNA. Means and SEMs were calculated from values obtained from a pair adrenal medulla. Comparisons of means among control and treatment groups were made by Student's t-test. The Solutions and radiochemicals that used in the present experiment are given Table 1 and 2.

Table 2. Amount of solutions in TH mRNA assay

Solutions	Volume (μl)
5X Random primer buffer	10
4 mg/μl BSA	5
DNTP (dCTP, dGTP, Dttp)	3
[a-32P]-dATP	5
TH probe	3

Results

The changes of TH enzyme activity and TH mRNA levels were investigated in adrenal medulla of phenoxybenzamine injected and control animals. Adrenal medulla weight, total protein and TH activity are given in Table 3 and Figure 1. Total RNA and TH mRNA levels are shown Table 4 and Figure 2.

Table 3. The amount of tissue weight, total protein and TH activity in phenoxybenzamine treated and control rats.

Group	Adrenal Medulla (mg)	Total protein (mg/μl)	TH activity (nmol.mg $prot^{-1}.hour^{-1}$)
Control	29.23	34.76	27.63±2.08*
Phenoxybenzamine	42.24	44.16	59.73±4.72*

* P<0.01 for difference with control

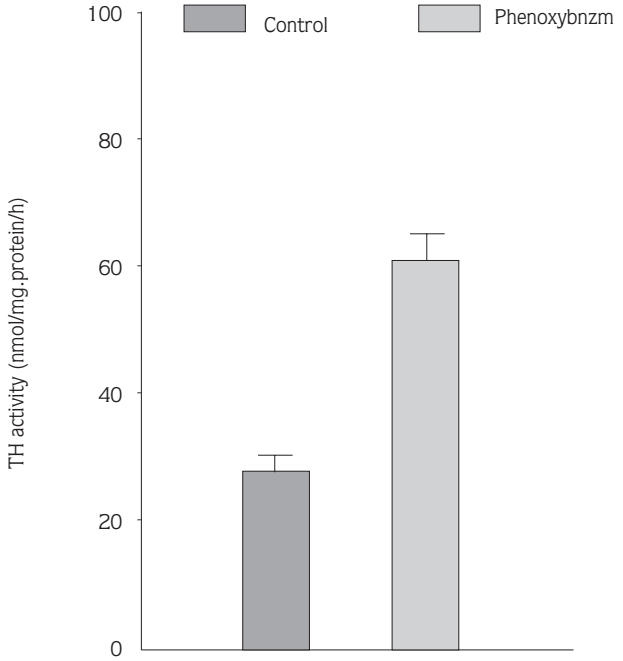

Figure 1. TH activity in control and phenoxybenzamine (phenoxybnzm) treated rats.
*Significantly different from control, P<0.01).

TH activity was significantly found to be elevated in adrenal medulla depends on phenoxybenzamine (Table 3, Figure 1) (P<0.01). TH enzyme activity was 27.63±2.08 nmol.mg $prot^{-1}.hour^{-1}$ and 59.73±4.72 nmol.mg $prot^{-1}.hour^{-1}$ in control and phenoxybenzamine treated rats respectively. There was a difference among tissue weight and total protein between control and treated animals, but statistical analysis was not performed. Adrenal medulla weight was 29.93 mg in control and 42.24 mg in treated animals. The amount of total protein in control rats was 34.76 (mg/µl) and phenoxybenzamine treated rats was 44.16 (mg/µl). As seen in Table 4 and Figure 2, total RNA and TH mRNA levels were increased in phenoxybenzamine treated animals compared with control (P<0.01). The amounts of total RNA were 1.883 (mg/µl) and 3.414 (mg/µl) in control and treated animals respectively. TH mRNA levels were 0.286±0.057 (OD Unit/mg RNA) in control and 1.570±0.163 (OD Unit/mg RNA) in treated rats (Table 4, Figure 2).

Discussion

Rats treated with phenoxybenzamine had a significant increasing of TH enzyme activity and TH mRNA levels their adrenal medulla. In addition the weight of adrenal medulla and total protein were increased significantly (Table 3,4). These observations suggest that phenoxybenzamine blocks reuptake of noradrenaline and adrenaline. Tyrosine hydroxylase is the rate-limiting enzyme and controlled by negative feedback mechanism. There is an elevation of TH mRNA level and TH enzyme activity. Also norepinephrine and epinephrine level increase depend on increased TH activity. Norepinephrine and epinephrine do not effect presynaptic neuron because their reuptake is blocked by phenoxybenzamine. Also phenoxybenzamine and catecholamines compete for alpha receptors. Phenoxybenzamine caused irreversible blockade of long duration such as 14-48 hours and blocks catecholamine-induced vasoconstriction (11,12).

Table 4. Effects of phenoxybenzamine on Total RNA and TH mRNA level.

Group	Total RNA (mg/µl)	TH mRNA (OD Unit/mg RNA)
Group	1.883	0.286±0.057*
Phenoxybenzamine	3.414	1.570±0.163*

* P<0.01 for difference with control

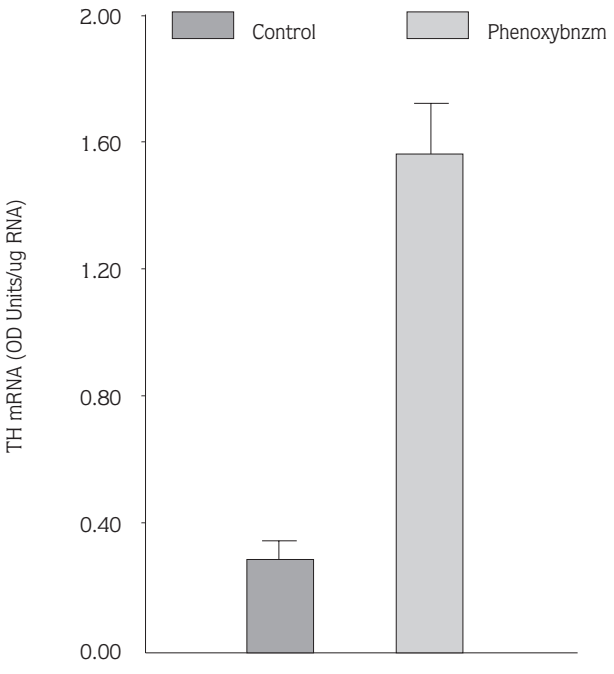

Figure 2. TH mRNA levels in control and phenoxybenzamine (Phemoxybnzm) treated rats.
*Significantly different from control, P<0.01.

The alterations of TH activity and TH mRNA levels were assessed in control and phenoxybenzamine treated animals. TH mRNA was significantly ($P<0.01$) increased by 5 fold in adrenal medulla from phenoxybenzamine treatde rats compared with control. This was similar to the increase in TH activity in phenoxy benzamine treated rats. Even though increased catecholamines caused to vasoconstruction, elevation of blood pressure etc., vasoconstruction or elevation blood pressure are not observed. Because reuptake of catecholamines was blocked by phenoxybenzamine

Catecholamine biosynthesis can be prolonged in the activity of sympathetic nerves. The administration of reserpine, phenoxybenzamine, or 6-hydroxydopamine results in an increased firing of sympathetic nerves. These drugs were found to increase the activity of tyrosine hydroxylase in the adrenal gland (10).

There is some evidence that the increased TH activity following the administration of the adrenergic antagonist which called prazosin. It has been shown that TH activity and TH mRNA levels in the adrenal medulla have ben incrased in prazosin treated animals (17). Also it has been shown that, TH activity and TH mRNA levels were increased after administration of reserpine in preipheral adrenergic tissues. The relative increase in mRNA levels was two fold compared with the TH activity (18).

These findings are confirmed by our data. In he present study TH activity and TH mRNA levels have been increased after phenoxybenzamine treatmant.

Catecholamine biosynthesis are governed by neuronal and hormonal control. Hypothalamus plays more important role in the control of biosynthesis of catecholamine. Also tyrosine hydroxylase is rate-limiting enzyme in the biosynthesis of catecholamines and its activity is an important regulatory step in this pathway. The effects of phenoxybenzamine and other adrenergic antagonist on TH activity and TH mRNA levels in the brain are planned follow-up studies.

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