

The Effects of Phenoxybenzamine and Cold Stress On Tyrosine Hydroxylase Enzyme Activity and Expression of Tyrosine Hydroxylase Gene in the Rat Adrenal Medulla

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Abstract: The effects of cold exposure and cold plus phenoxybenzamine treatment on tyrosine hydroxylase (TH) activity and TH mRNA levels were investigated in the adrenal medulla of Sprague Dawley (SD) rats. SD rats were exposed to cold (4 °C) for 48 hours. Phenoxybenzamine was injected intraperitoneally (i.p) at 20 mg/kg, which was prepared in 5% ethanol containing 0.9 % NaCl

TH activity was measured by monitoring the formation of $^3\text{H}_2\text{O}$ as a product of L-(^3H)-tyrosine hydroxylation, the formation of L-(^3H)-dopa from L-(^3H)-tyrosine. Total adrenal RNA was isolated and blotted onto a nylon membrane and the immobilized RNA was then hybridized from TH cDNA probe. TH mRNA was assayed by densitometric scanning of the autoradiograms with a videodensitometer.

TH activity and mRNA levels were found to be significantly increased in the adrenal medulla of SD rats exposed to cold for 48 hours and treated with cold plus phenoxybenzamine ($P<0.01$).

Key Words: Cold exposure, adrenal medulla, phenoxybenzamine, tyrosine hydroxylase tyrosine hydroxylase mRNA

Phenoxybenzamine ve Soğuk Stresinin Sıçan Adrenal Medulla Tirozin Hidroksilaz Enzim Aktivitesi ve Tirozin Hidroksilaz Gen İfadesi Üzerine Etkileri

Özet: Soğuk uygulaması ve soğuğa ek olarak phenoxybenzamine uygulamasının, tirozin hidroksilaz (TH) ve TH mRNA seviyesi üzerindeki etkileri Sprague Dawley (SD) sıçanlarının adrenal medullasında araştırıldı. SD sıçanları 48 saat 4 °C'de soğuğa maruz bırakıldı. Phenoxybenzamine uygulaması % 0.9 NaCl içeren % 5 etanol içinde hazırlanarak 20 mg/kg olacak şekilde periton içine (i.p) yapıldı.

TH aktivitesi, L-(^3H)-tirozinden L-(^3H)-dopa oluşurken, L-(^3H)-tirozin'in hidroksilasyon ürünü olan $^3\text{H}_2\text{O}$ 'nun izlenmesi ile ölçüldü. Total adrenal RNA izole edildi ve naylon membrana bağlandı, bu şekilde immobilize olan RNA daha sonra TH cDNA probu ile hibritleştirildi. TH mRNA miktarı, otoradyogramlarının videodensitometre kullanılarak densitometrik taraması yapılarak saptandı.

SD sıçanlarının adrenal medullasında TH aktivitesi ve TH mRNA seviyelerinin, 48 saat soğuk uygulaması ve soğuğa ek olarak phenoxybenzamine verilen gruplarda önemli derecede arttığı saptandı ($P<0.01$).

Introduction

Exposure to cold and the administration of antihypertensive drugs are known to proceed the synthesis and release of catecholamines in the adrenal medulla (1-8). Tyrosine hydroxylase is the rate limiting step in the biosynthesis of catecholamines (TH; tyrosine-3-monoxygenase, EC: 1, 14, 16, 2). TH catalyses the formation of DOPA (dihydroxy-phenylalanine) from L-tyrosine (9-13). The rate of synthesis of norepinephrine and epinephrine is controlled by the activity of tyrosine hydroxylase. Catecholamines are synthesized in the adrenal medulla, hypothalamus substantia nigra, locus cereleus, putamen, striatum, cortex cerebri and neuron terminals. Experimental studies in pharmacology, physiology and biochemistry have clearly shown that catecholamines play neuroendocrine and endocrine roles in mediating a variety of autonomic functions in the periphery. Normal catecholamine function is critical for maintaining homeostasis (14). Physical activity, psychological stress, drug treatment and generalized allergic reactions enhance the synthesis and release of catecholamines in organisms. Changes in TH enzyme activities have been investigated in the periphery and adrenergic nervous system after some pharmacological and physiological treatment (15, 16).

Cold stress stimulates the hypothalamic-pituitary-adrenal (HPA) axis, and it is important to consider the role of glucocorticoids in regulating norepinephrine function in the brain (17). Phenoxybenzamine inhibits reuptake of released norepinephrine by presynaptic adrenergic nerve terminals. The pharmacological actions of phenoxybenzamine are primarily related to antagonism of alpha-receptor mediated events. Phenoxybenzamine blocks catecholamine-induced vasoconstriction (18-27). It also increases blood flow to the skin, mucosa and abdominal viscera, and lowers both supine and erect blood pressures. It has no effect on the parasympathetic system.

The aim of this study was to compare differences in TH activity and TH mRNA levels between cold-exposed and cold+phenoxybenzamine-treated animals. In addition, the ability of the catecholaminergic system of SD rats to respond to cold exposure and cold plus phenoxybenzamine was investigated.

Materials and Methods

Ten male 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. The temperature was 26 °C. Five rats were maintained at 26 °C as control animals. For cold exposure, 5 rats were placed in a 4 °C chamber (Revco Cryo-frig.). Phenoxybenzamine was prepared in 5% ethanol containing 0.9% NaCl and injected in cold-exposed rats as 20 mg/kg i.p. 0.9% NaCl of 5% ethanol was injected in control animals (LD_{50} for phenoxybenzamine was approximately 2000 mg/kg in rats) (18, 20). animals were anaesthetized with pentobarbital and adrenal glands were quickly removed and rapidly frozen in liquid nitrogen. Tissues were stored at -80 °C until use. Tissues were homogenized in phosphate buffer (pH=7.0, 2 mM 1% triton-x) for 15 seconds in an ice bucket in the sonicator. TH activity, total protein and TH mRNA were determined in aliquots of the sample. Total protein was quantified according to Bradford (28). TH activity was meas-

ured using radioenzymatic assay, as described by Reinhard et al. (29). TH activity was measured using radioenzymatic assay, as described by Reinhard et al. (29). TH activity was determined by monitoring the formation of $^3\text{H}_2\text{O}$ as a product of L- [^3H]-tyrosine hydroxylation, the formation of L- [^3H]-dopa from L- [^3H]-tyrosine. Determination of TH enzyme activity was as follows: 25 μl homogenate was analyzed at pH 7.0 in the presence of 6-MPH₄ (DL-6 Methyl-5,6,7,8-tetrahydropterine; DL-2-amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydropteridine, Sigma M4758) and [3,5, ^3H]tyrosine (100 μM , 1 μCi) (Sigma) in a total volume of 50 μl for 15 min at 37 °C. Total adrenal medullary RNA was isolated by using RNazolB (Biotecx, Friendswood, TX). Total RNA was quantified spectrophotometrically at 260 nm (4,30). Diluted RNA samples were blotted onto nylon membrane (Gene Screen, New England Nuclear, Boston, MA) using a slot blot apparatus. The filters were baked at 80 °C for 2-4 h, then prehybridized with 50 μl denatured salmon testes DNA. Following incubation for 14-16 h at 42 °C, filters were hybridized with a ^{32}P TH.36 cDNA probe (supplied by Dr. Karen O'Malley Washington University, School of Medicine, 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. 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 μg of total RNA. Means and SEMs were calculated from values obtained from a pair of adrenal medulla. Comparison of means among control and treatment groups was made by Student's t-test.

Results and Discussion

TH activity and TH mRNA were tested in cold-exposed and phenoxybenzamine-treated animals. Table 1 gives the weight of adrenal glands, the amount of total protein and the TH enzyme activity in the control and treatment rats. Total protein and tissue weight was higher treatment and cold-exposed animals than in control animals. TH enzyme activity was 31 ± 0.79 nmol/mg protein/h in control rats, 49.32 ± 3.65 nmol/mg protein/h in cold-exposed rats and 63.17 ± 3.53 nmol/mg protein/h in phenoxybenzamine-treated rats (Figure 1). Cold exposure and cold+phenoxybenzamine altered TH enzyme activity, which was significantly higher ($P < 0.01$) in the cold-exposed and treatment groups than in the control group (Table 1). TH mRNA was significantly higher in the adrenal medulla of the cold-exposed and treatment groups ($P < 0.01$) than in that of the control group. The total RNA and TH mRNA levels are shown in Table 2.

A gradual increase in catecholamine biosynthesis may be caused by a prolonged increase in the activity of sympathetic nerves through the administration of reserpine, phenoxy-benzamine, prazosin or 6-hydroxydopamine, drugs which lower blood pressure. These drugs were found to increase the activity of tyrosine hydroxylase in the adrenal gland (7, 31). There are two explanations of the increase in TH activity and TH mRNA levels of cold-exposed and cold -plus-phenoxybenzamine-treated animals. The first is that peripheral thermoreceptors are stimulated by an increase in splanic nerve activity or the stimulation of baroreceptors. The second is that the hypothalamus-pituitary-adrenal axis and glucocorticoids play such a role.

Table 1. Effects of cold exposure and cold plus phenoxybenzamine treatment on TH activity in SD rats

	Tissue Wt. (mg)	Total protein ($\mu\text{g}/\mu\text{l}$)	TH Activity (nmol/mg protein/h)
Control	26.16 \pm 1.53	41.32 \pm 3.74	31.98 \pm 0.79
Cold exposed	30.98 \pm 1.09	50.53 \pm 1.83*	49.32 \pm 3.65*
Cold+ Phenoxybenzamine	42.24 \pm 2.85**	44.16 \pm 2.97	63.17 \pm 3.53**

* Significantly different with cold exposure P<0.01

** Significantly different with cold+phenoxybenzamine P<0.01

	Total RN mg/ml	ODUnit/mg RNA
Control	1.90 \pm 0.13	0.35 \pm 0.07
Cold exposed	5.67 \pm 0.92*	3.57 \pm 0.63*
Cold+Phenoxybenzamine	3.36 \pm 0.24**	1.31 \pm 0.18**

*Significantly different with cold exposure P<0.01

**Significantly different with phenoxybenzamine P<0.01

Table 2. Amount of total RNA and TH mRNA in cold exposed and cold plus phenoxybenzamine treated rats

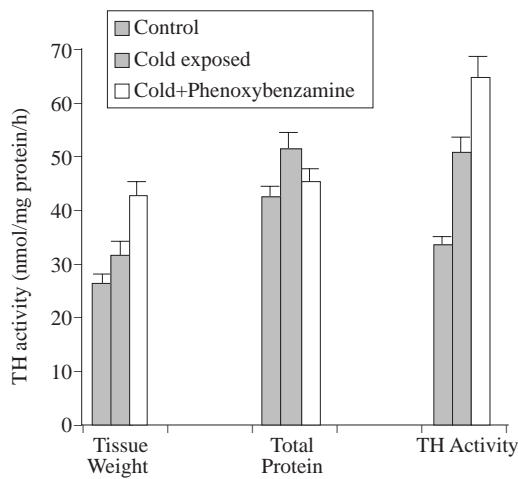


Figure 1. The effects of cold exposure and cold plus phenoxybenzamine treatment on the adrenal TH activity of SD rats.

A similarity is found in the increase of TH activity and TH mRNA levels when treated animals are compared with controls. The rate of TH activity was 1.5-fold in cold-exposed and 2-fold in cold-plus-phenoxybenzamine-treated rats. Furthermore, TH mRNA levels were 10-and-3.5 fold in cold-exposed and cold+phenoxybenzamine-treated animals, respectively (Figure 2).

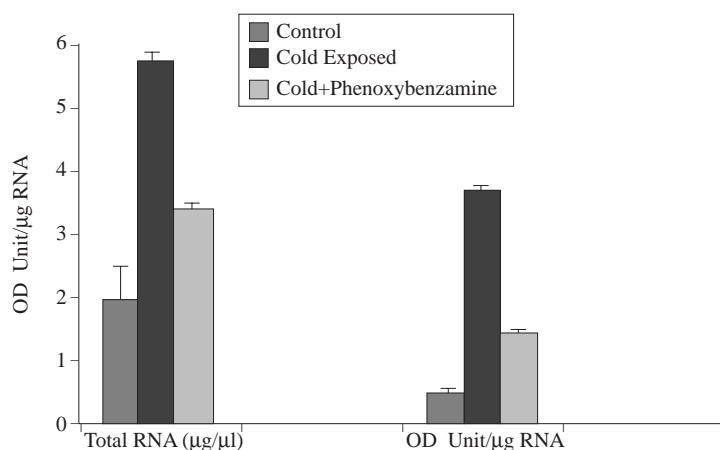


Figure 2. The effects of cold exposure and cold plus phenoxybenzamine treatment on the TH mRNA levels of adrenal medulla of SD rats.

It has been shown that an increase in TH activity depends on increased TH mRNA levels following administration of reserpine or exposure to cold (5, 38, 33). Sharma and Khanna have reported increased catecholamine levels following cold stress. This is the result of hypothalamic stimulation (34). Stachowiak et al. have reported that cold stress increases TH enzyme activity in catecholamine biosynthesis (33). Baruchin et al. have shown that rats subjected to 5 °C cold exposure for 1 hour had maximal increases of 300-400 %, levels that the controls took 3-6 hours to reach, and that levels remained constant for the duration of cold exposure (35).

These findings provide a molecular approach to the adaptation of the catecholaminergic system during cold stress and administration of drugs. Our results suggest this phenomenon.

Organisms may be exposed to more severe stressors during their lives, for cold stress, hard exercise, drug treatment and physiologic stress. Further investigations are required to understand the underlying mechanisms directing the catecholaminergic system of organisms for responding and adapting to more than two stressors.

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References

1. Thoenen H., Induction of tyrosine hydroxylase in peripheral and central adrenergic neurons by cold exposure of rats. *Nature* 228, 861-862, 1970.
2. Kvetnansky R. Gerwitz G. P., Weise V K. and Kapin I J., Catecholamine-synthesizing enzymes in the rat adrenal gland during exposure to cold. *Am. J. Physiol.* 220, 928-931, 1971.
3. Fluharty S J., Synder G. L., Stricker E. M. and Zigmond M. J., Short and long term changes in adrenal tyrosine hydroxylase activity during insulin-induced hypoglycemia and cold stress. *Brain Res.* 267, 364-387, 1987.
4. Tümer N., Hale C., Lawler J. , and Strong R., Modulation of tyrosine hydroxylase gene expression in the rat adrenal gland by exercise; effect of age. *Mol. Brain Res.*, 14 51-56, 1992.
5. Tank A. W., Lewis E. W., chikaraishi D. M., and Weiner N., Elevation of RNA coding for tyrosine hydroxylase in rat adrenal gland by reserpine treatment and exposure to cold. *J. Neurochem* 45, 1030-1033, 1985.
6. Richard F. Faucon-Biguet N., Labatut R., Rollet D., Mullet J. and Buda M., Modulation of tyrosine hydroxylase gene expression in rat brain and adrenals by exposure to cold. *J. Neurosci. Res.* 20, 32-37, 1988.
7. Fregly M. J., Rossi F., Sun Z., Tümer N., Cade R., Rollet D., Heagland D., and Yürekli M., Effect of chronic treatment with prazosin and L-arginine on the elevation of blood pressure during cold exposure. *Pharmacology*, 49, 351-362, 1994.
8. Tümer N., LaRochelle J. S., Yenice S., Yürekli M., Effect of cold exposure and age on tyrosine hydroxylase gene expression. *Endocrinology*, 31 (Suppl); p 77, 1993.
9. Nagatsu T., Levitt M., Udenfriend J., Tyrosine hydroxylase., *J. Biol. Chem.*, 237, 2910-2917, 1964.
10. Coyle J. T., Tyrosine hydroxylase in rat brain cofactor requirements, regional and subcellular distribution. *Biochem. Pharm.* 21, 1935-1944, 1972.
11. Masserano J. M., Weiner N., Tyrosine hydroxylase regulation in the central nervous system. *Molecular and Cellular Biochemistry*, 53/54 129-152, 1983.
12. Weiner N., *The basic neuroscience the nervous system* (Brady R. O., ed.) 1, 341-354, Raven Press New York 1975.
13. Levitt M., Sector S. Sjöderma A., and Udenfriend J., Elucidation of the ratelimiting step in norepinephrine biosynthesis in the perfused guinea-pig heart. *J. Pharmacol. Exp. Ther.*, 148, 1-8, 1965.
14. Roberts J., and Tümer N., Age related changes in autonomic function of catecholamines. In: *Review of Biological Research in Aging.*, (M. Rothstein, Alan R., Liss., Eds, New York), Vol., 3 pp 257-298, 1987.
15. Guidotti A., and Costa E., Transsynaptic refulation of tyrosine 3-monooxygenase biosynthesis in rat adrenal medulla. *Biochem. Pharmacol.*, 26, 817-823, 1977.
16. Zigmond R. E., The long term refulation of ganglionic tyrosine hydroxylase by preganglionic nerve activity. *Fed. Proc.*, 39, 3003-3008, 1980.
17. Smith M. A., Brady L. J., Glowa J., Gold P. W., and Herkenham M., Effects of stress and adrenalectomy on tyrosine hydroxylase mRNA levels in the locus cereleus by in situ hybridization. *Brain Res.* 544, 26-32, 1991.

18. Katzung B. G., Basic and Clinical Pharmacology, Fourth Edition, Appleton & Lange, Lange Medical Publications, Connecticut, 1989.
19. Adele L. Dowd, (Index Ed.) Physicians' Desk Reference, Medical Economics Company, Montvale N. J., 1993.
20. Thonen H., Mueller R. A. and Axelrod J., Neuronally dependent induction of adrenal phenylethanolamine-N-methyl transferase by 6-hydroxydopamine. *Biochem. Pharmacol.* 19, 669-673, 1990.
21. Weiner N., Drugs that inhibit adrenergic nerves and block adrenergic receptors. *The Pharmacological Basis of Therapeutics* ed. 6, New Yrk MacMillan Publishing Co., p 179 and 182, 1980.
22. Martin E. W., *Drug Interactions Index 1978/1979*, Philadelphia. J. B. Lippincott Co., pp, 209-210, 1978.
23. Yürekli M., Phenoxybenzamine'nin Sprague Dawley sıçanlarında adrenal medulla Tirozin Hidroksilaz (TH) aktivitesi ve TH mRNA seviyesi üzerindeki etkisi. XIII. ulusal Biyokimya Kongresi (Uluslararası katılımlı) 26-30 Mart, 1996 Antalya-Türkiye.
24. Garcia P. T. and Sevilla J. A., Alpha 2-autoreceptor-mediated modulation of tyrosine hydroxylase in noradrenergic regions of rat brain in vivo. *Naunyn Schmiederbergs Arc. Pharmacol.*, 345(6), 653-660, 1992.
25. Gatón J., Fernandez de la G., Gandaa F., Velasco A., The role of the neurotransmitters acetylcholine and noradrenaline in the pathogenesis of stress ulcer. *Comp. Biochem. Physiol C.*, 106(1), 125-129, 1993.
26. Okuda H., Morimoto C. and Tsujita T., Beta adrenergic receptors in rat fat cells and their relationship with lipolysis. *J. Biochem.*, 119(5), 852-856, 1996.
27. Perrosin L, Rapozzi V., Zozet S. and Gialdi T., Bolckers of adrenergic neurons and receptors tumor progression and effect of rotational stress in mice. *Anti Cancer Res.* 16 (6B) 3409-3413, 1996.
28. Bradford M. M., A rapid and sensitive method for the quantities of protein utilizing the principle of protein dye binding. *anal. Biochem.*, 72, 248-254, 1976.
29. Reinhard J. F., Smith G. K., Nichol C. A., A rapid and sensitive assay for tyrosine-3-monooxygenase based upon the release of $^3\text{H}_2\text{O}$ and adsorption of (^3H)-tyrosine by charcoal. *Life Sci.* 39, 2185-2186.
30. Ausebel F. M., Brent R., Kingston E. R., Moore D. D., Seidman J. G., Smith J. A and Struhl A., *Current Protocols in Molecular Biology.*, Vol. 1-2 Jhon Wiley & Sons, New York, 1992.
31. Axelrod J., The fate of noradrenaline in the sympathetic neuron. *Laboratory of Clinical Science, National Institute of Mental health, Bethesda, Maryland, USA*, 1971.
32. Faucon-Biguet N., Buda M., Lamaurox A., Somolyk D., Mallet J., Time course of changes of TH mRNA in rat brain and adrenal medulla after single injection of reserpine. *EMBO J.*, 5, 287-291, 1986.
33. Stachowiak M. K., Fluharty S. J., Stricker E. M., Zigmond M. J., Kaplan B. B., Effect of chronic cold exposure on tyrosine hydroxylase mRNA in rat adrenal gland. *Brain. Res.* 359, 356-359, 1985.
34. Sharma V. N., and Khanna N. K., Effect of cold stress on epinephrine and norepinephrine and in the rat heart. *End. Jour. Med. Res.*, 57, 877-888, 1969.
35. Baruchin A., Jeisburg E. P., Miner L. L., Ennis D., Zigmond M. J., Kaplan B. B., Effects of cold exposure on rat adrenal trosine hydroxylase; an analysis of RNA, protein, enzyme activity and cofactor levels. *J. Neurochem.*, 564, 1769-1775, 1990.

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