Biochemical Pharmacology, Vol. 37, No. 8, pp. 1637–1639, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00
Pergamon Press plc

SHORT COMMUNICATIONS

Centrally mediated activation of tyrosine hydroxylation in rat adrenals by thyrotropin-releasing hormone

(*Received* 3 *June* 1987; *accepted* 27 *October* 1987)

Thyrotropin-releasing hormone (TRH) has been reported to increase the release and turnover of noradrenaline in rat brain tissues [1–3], and to increase blood noradrenaline [4]. This effect of TRH appears to be due to activation of tyrosine hydroxylase (TH) [5], which is the rate-limiting step of noradrenaline biosynthesis in the peripheral sympathetic nervous system [6]. Brown [7] found that TRH acts within the brain to increase plasma catecholamine levels which are associated with an increase in plasma glucose, suggesting an involvement of central TRH in regulating the autonomic nervous system.

Recently, we observed that intraperitoneal administration of TRH caused a rapid and marked increase of 3,4-dihydroxyphenylalanine (DOPA) accumulation in various regions of the brain and in the adrenals of mice treated with 3-hydroxybenzylhydrazine (NSD-1015), an inhibitor of aromatic L-amino acid decarboxylase, indicating activation of *in vivo* tyrosine hydroxylation by TRH [8].

It was the aim of the present study to prove whether the action of TRH on adrenal TH is a centrally mediated effect. For this purpose, we measured tyrosine hydroxylation both in adrenal slices [9] and in the adrenals *in vivo* [10], after intraperitoneal or intracisternal administration of TRH, by high performance liquid chromatography (HPLC) with electrochemical detection (ECD).

Materials and methods

Materials. Male Wistar rats weighing 150–200 g were used for all experiments. NSD-1055 (4-bromo-3-hydroxybenzyloxyamine) and NSD-1015 (3-hydroxybenzylhydrazine) were obtained from Nakarai Chemical Co. (Kyoto, Japan). Nucleosil $7C_{18}$ was purchased from the Macherey-Nagel (Duren, F.R.G.). TRH was a gift from the Takeda Chemical Industries (Osaka, Japan). All other reagents were of the highest purity commercially available. TH was homogeneously purified from rat adrenals by the procedure as described previously [11].

Measurement of in vivo *TH activity in rat adrenal glands. In vivo* TH activity was estimated based on the accumulation of DOPA after inhibition of aromatic L-amino acid decarboxylase in rats treated intraperitoneally with NSD-1015 (100 mg/kg), by high-performance liquid chromatography with electrochemical detection (HPLC-ECD) with a column of Nucleosil $7C_{18}$ according to the previously reported procedure [10].

Measurement of TH activity in adrenal slices. Enzyme activity in adrenal slices was measured by the assay method as described previously [9]. The adrenal slices were incubated with 1 mM NSD-1055 and 0.1 mM L-tyrosine in Krebs-bicarbonate solution saturated with 95% O_2–5% CO_2 (pH 7.2) for 30 min at 37°. After incubation, formed DOPA in the slices was estimated by HPLC-ECD.

Measurement of TH activity in vitro. TH activity of pure enzyme was determined with 6-methyl-tetrahydropterin (6-MPH_4) as a cofactor by measuring L-DOPA formed from L-tyrosine by HPLC-ECD [12].

Results and discussion

TRH elicited a substantial increase in DOPA accumulation *in vivo* in rat adrenals 30 min after intraperitoneal administration. As shown in Table 1, a substantial effect was observed at 40 mg of TRH/kg. At 80 mg/kg of a maximal dose, the effect was about the same as that produced by 40 mg/kg of TRH. The increased DOPA accumulation induced by TRH was accompanied with a slight but substantial decrease of catecholamine levels in the adrenal glands.

To estimate the action of TRH on TH-regulating systems in the adrenals, adrenal slices were incubated with TRH in the presence of the inhibitor of aromatic L-amino acid decarboxylase for 30 min at 37°. As can be seen in Fig. 1, there was no significant increase in DOPA formation by incubation with TRH at 10^{-5} and 10^{-4} M. It is well known that cholinergic mechanism is involved in some of the central action of TRH. Yarbrough [13] has found that TRH potentiates the excitatory response to iontophoretically applied acetylcholine on rat cortical neurones. Schmidt [14] showed that TRH was able to block the ability of pentobarbital to decrease cholinergic function in hippocampus and cortex. In the present studies, we showed that the depolarizing agents such as high K^+ (52 mM) and carbachol (0.1 mM) caused a substantial and significant increase of DOPA formation in adrenal slices. This evidence has led us to speculate that intraperitoneal administration of TRH to rats may cause the potentiation of the acetylcholine responses in the adrenals, leading to activation of TH. Therefore, we examined the action of TRH on tyrosine hydroxylation enhanced by carbachol treatment in adrenal slices. However, there was no influence of TRH on enhanced tyrosine hydroxylation by carbachol in adrenals. The activity of TH purified from rat adrenal gland was not affected by TRH at 10^{-4} M. Thus, TRH had no direct effect on TH-regulating systems in the adrenals.

Table 1. Effect of TRH on tyrosine hydroxylation *in vivo* in rat adrenal glands

	DOPA accumulation (pmol/adrenal)	% of control
Control (saline)	46.74 ± 5.23 (10)	100
TRH (20 mg/kg)	50.21 ± 2.97 (6)	107
TRH (40 mg/kg)	87.24 ± 14.75 (4)*	187
TRH (80 mg/kg)	71.10 ± 6.82 (9)*	152

NSD-1015 (100 mg/kg) and TRH were dissolved in saline, and both injections were made intraperitoneally 30 min before killing. The values are means ± SEM. Values in parentheses indicate the numbers of individual experiments. Key: *statistical difference between control and treated groups ($P < 0.05$), calculated according to Student's *t*-test.

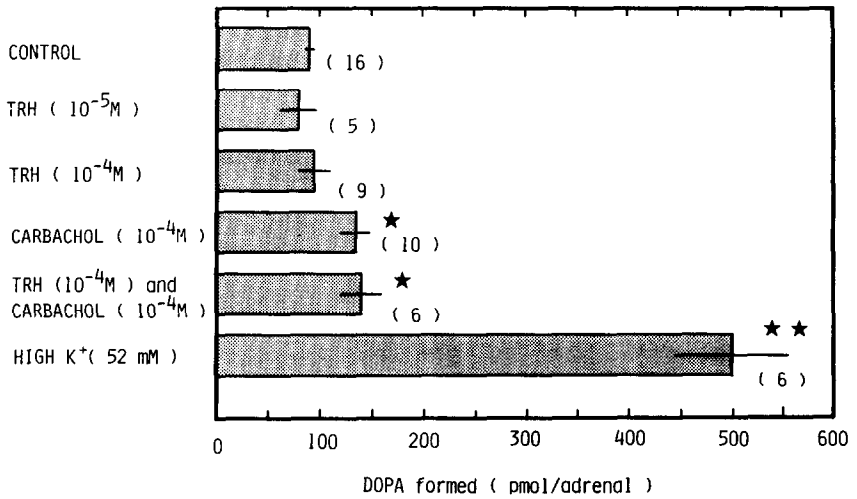

Fig. 1. Effects of TRH on tyrosine hydroxylation in rat adrenal slices. After equilibration at 37° for 30 min in a Krebs-bicarbonate solution, adrenal slices were incubated for 30 min at 37° in the presence of 100 μM L-tyrosine and 1 mM NSD-1055 at pH 7.2. DOPA formed is expressed as picomoles per one adrenal. The numbers in parentheses indicate the numbers of individual experiments. Data are plotted as the means, and vertical bars illustrate the SEM. Key: *P < 0.05, **P < 0.01, statistical difference between the control and treated groups, calculated according to Student's *t*-test.

These results suggest that the activation of TRH on adrenal tyrosine hydroxylation *in vivo* is a centrally mediated effect, without affecting the adrenals directly. The high doses of intraperitoneal TRH necessary to activate tyrosine hydroxylation in adrenals also suggest the possibility that the primary effect of TRH on adrenal TH is central rather than peripheral. To prove this possibility, the effect of intracisternal injection of TRH on DOPA accumulation *in vivo* in the adrenals were examined. It was shown in Table 2 that the DOPA accumulation in the adrenals was increased by intracisternal administration of TRH, as observed by intraperitoneal administration of TRH.

In agreement with the present results, Yokoo *et al.* [15] reported that TH activity in central dopaminergic regions was increased following repeated DN-1417 treatment (20 mg/kg/day for 7 days, i.p.i.). The circadian rhythm of pineal TH activity was related to increased neuronal activity of the sympathetic noradrenergic neurons that innervate the pineal gland [16].

In summary, the data presented indicate that intraperitoneal administration of TRH substantially increase the *in vivo* TH activity in rat adrenals and that this activation may be caused by a centrally mediated activation of splanchnic nerves without affecting peripheral regulating systems of adrenal TH. These evidences were provided by comparing the effects of TRH on tyrosine hydroxylation measured by the three assay methods recently developed in our laboratory, *in vivo* (for animals), *in situ* (for tissue slices) and *in vitro* (for pure TH).

Table 2. Effect of intracisternal administration of TRH on tyrosine hydroxylation in rat adrenal glands

	DOPA accumulation (pmol/adrenal)	% of control
Control (saline)	71.8 ± 3.0 (6)	100
TRH	130.7 ± 12.9 (8)*	182

NSD-1015 and TRH were dissolved in saline. TRH (250 μg/kg) was administrated intracisternally, and NSD-1015 (100 mg/kg) intraperitoneally. Both injections were made 30 min before killing, under light anesthesia with ether. The values are means ± SEM. Values in parentheses indicate the numbers of individual experiments. Key: *statistical difference between control and TRH groups (P < 0.01), calculated according to Student's *t*-test.

Acknowledgements—This work was supported in part by a grant from the Ministry of Health and Welfare of Japan.

Department of Biochemistry
Nagoya University School of Medicine
Nagoya 466, Japan, and
**Department of Life Chemistry*
Graduate School at Nagatsuta
Tokyo Institute of Technology
Yokohama 277, Japan

AKIFUMI TOGARI*†
HIROSHI ICHINOSE
KOHICHI KOJIMA*‡
YOKO HIRATA
TOSHIHARU NAGATSU§

† Present address: Department of Pharmacology, Aichi-Gakuin University School of Dentistry, Nagoya 464, Japan.

‡ Present address: Hatano Research Institute, Food and Drug Safety Center, Hatano 257, Japan.

§ Send correspondence to T. Nagatsu at Department of Biochemistry, Nagoya University School of Medicine, Nagoya 466, Japan.

REFERENCES

1. H. H. Keller, G. Bartholini and A. Pletscher, *Nature, Lond.* **248**, 528 (1984).
2. W. D. Horst and N. Spirt, *Life Sci.* **15**, 1073 (1984).
3. R. A. Agarwal, R. B. Rastogi and R. L. Singhal, *Res. Commun. Chem. Pathol. Pharmac.* **15**, 743 (1976).
4. M. Tuck, J. E. Morley, D. Rosenblatt and J. M. Hershman, *Clin. Res.* **27**, 261A (1979).
5. T. Nagatsu, M. Levitt and S. Udenfriend, *J. biol. Chem.* **239**, 2910 (1964).
6. M. Levitt, S. Spector and S. Udenfriend, *J. Pharmac. exp. Ther.* **148**, 1 (1965).
7. M. R. Brown, *Life Sci.* **28**, 1789 (1981).

8. K. Oka, K. Kojima, A. Togari and T. Nagatsu, in *Thyrotropin Releasing Hormone and Spinocerebellar Degeneration* (Ed. I. Sobue), p. 53. Elsevier Science Publishers B.V., Amsterdam (1986).
9. A. Togari, T. Kato and T. Nagatsu, *Biochem. Pharmac.* **31**, 1729 (1982).
10. K. Oka, K. Kojima, A. Togari, T. Nagatsu and B. Kiss, *J. Chromatogr.* **308**, 43 (1984).
11. A. Togari, H. Kano, K. Oka and T. Nagtasu, *Analyt. Biochem.* **132**, 183 (1983).
12. T. Nagatsu, K. Oka and T. Kato, *J. Chromatogr.* **163**, 247 (1979).
13. G. G. Yarbrough, *Nature, Lond.* **263**, 523 (1976).
14. D. E. Schmidt, *Commun. Psychopharmac.* **1**, 469 (1977).
15. H. Yokoo, T. Nakahara, T. Matsumoto, K. Inagaki and H. Uchimura, *Peptides* **8**, 49 (1987).
16. P. Abreu, C. Santana, G. Hernandez, C. H. Calzadilla and R. Alonso, *J. Neurochem.* **48**, 665 (1987).

Biochemical Pharmacology, Vol. 37, No. 8, pp. 1639–1642, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

Inhibition of mouse cytosolic aldehyde dehydrogenase by 4-(diethylamino)benzaldehyde

(*Received* 31 *August* 1987; *accepted* 23 *October* 1987)

Increased NAD-dependent aldehyde dehydrogenase (EC 1.2.1.3) activity (ALDH) has been demonstrated to be a mechanism of antitumor drug resistance to the alkylating agent cyclophosphamide (CP) *in vivo* and to the activated analog 4-hydroperoxycyclophosphamide (4-HC) *in vitro* [1, 2]. CP is a pro-drug requiring cytochrome P-450 hydroxylation for activation. Prior to release of the active alkylating metabolite phosphoramide mustard, CP passes through an aldehyde intermediate, aldophosphamide [3]. ALDH catalyzes the oxidation of aldophosphamide to the inactive metabolite carboxyphosphamide [4, 5]. Tumor cell resistance to CP is thus conferred by an increased expression of ALDH activity. This mechanism has been best characterized in the murine L1210 leukemia cell lines which are sensitive (L1210/0) and resistant (L1210/CPA) to CP.

One attempt to reverse the drug-resistant phenotype has been the use of ALDH-specific inhibitors. Disulfiram (DS), diethyldithiocarbamate (DDTC), cyanamide, and 1-aminocyclopropanol have been shown to inhibit ALDH activity in rat [6] and human [7] liver. DS and cyanamide appear to act as irreversible inhibitors *in vivo* and are currently used in alcohol aversion therapy as they elicit elevated blood acetaldehyde levels following ethanol ingestion [8, 9]. These inhibitors have been used experimentally to revert L1210/CPA cells to a CP-sensitive phenotype in soft agar survival and alkaline elution assays [10] and to identify murine and human hematopoietic progenitor cells whose mechanism of resistance to oxazaphosphorines may be due to high ALDH levels [11, 12].

It has been proposed in the case of the human liver E1 (cytosolic) isozyme [13] that DS, as it attacks Cysteine-302 [14], is initially cleaved into two DDTC molecules, one of which forms a mixed disulfide adduct on the enzyme. This bound DDTC is then displaced by another enzymic sulfhydryl, resulting in an inactivated enzyme containing an intramolecular disulfide bond. DS also has been shown to inhibit dopamine β-hydroxylase [15] and may alter other proteins or enzymes by acting as a general sulfhydryl oxidizing agent [16]. The *in vivo* and *in vitro* cellular toxicities due to the nonspecific interactions of DS and DDTC have limited their use as ALDH inhibitors. Though cyanamide does not elicit many of the same toxicities as DS, its use is dependent on the presence of catalase activity for formation of the active inhibitory species [17].

Therefore, an ALDH specific, competitive type (reversible) inhibitor not requiring enzymatic activation would be preferred for *in vivo* inhibition of ALDH. Since DS binds specifically to a single reactive cysteine residue (Cys-302), we decided to investigate the role of the diethylamino groups in directing the binding of ALDH inhibitors to the enzyme active site. In an attempt to mimic disulfiram and substrate binding, several non-thiol-containing inhibitors incorporating dialkylamino and carbonyl substituent groups were screened as potential inhibitors by direct spectrofluorometric enzyme assay. 4-(Diethylamino) benzaldehyde (DEAB) was identified as a potent, partial competitive inhibitor of the cytosolic (but not mitochondrial) isozyme of mouse ALDH. In addition, DEAB was shown to confer sensitivity to L1210/CPA cells using *in vitro* cell survival assays.

Materials and methods

NAD, aprotinin, leupeptin, and disulfiram were purchased from the Sigma Chemical Co. (St. Louis, MO). 4-(Diethylamino)benzaldehyde (DEAB), 4-(dimethylamino)benzaldehyde (DMAB), 4-(diethylamino)benzoic acid (DEABA), 4-(diethylamino)salicylaldehyde (DEAS), and *N*,*N*-diethylaniline (DEA) were obtained from the Aldrich Chemical Co. (Milwaukee, WI), and propionaldehyde and benzaldehyde were from the Kodak Chemical Co. (Rochester, NY). 4-Bis(2-chloroethyl)aminobenzaldehyde (BAM) was synthesized as previously described [18].

Female BD2F1 mice, weighing 20–25 g, were used for i.p. passage of L1210/CPA cells. Ascites tumor cells (5×10^8 cells/mouse) were collected on day 8 post-injection and cooled in phosphate-buffered saline (PBS) at 4°. Cells were washed three times in cold PBS and resuspended in freeze/thaw buffer (0.1 M sodium phosphate, pH 7.4, 1 mM EDTA, 5 mM 2-mercaptoethanol, 200 units/ml aprotinin, and 50 μg/ml leupeptin) with 200 μl/5×10^7 cells. The cells were subjected to three cycles of freezing/thawing in a solid CO_2/methanol bath and centrifuged for 10 min at 5000 *g*. The lipid layer was discarded, and the supernatant fraction was removed and centrifuged for 45 min at 48,000 *g*. The supernatant fraction was collected (protein concentration approximately 10 mg/ml) and used for ALDH assays. The WEHI-3 cell line, a mouse myelomonocytic leukemia line (gift from James Ihle, Frederick Cancer Research Center, Frederick, MD) was grown in RPMI 1640 medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The 1C1C7 cell line, a mouse hepatoma line (gift of Mary DeLong, Department of Pharmacology, Johns Hopkins School of Medicine, Baltimore, MD), was maintained in