J Neural Transm (1988) 71: 29–37

— Journal of — Neural Transmission

Stress increases endogenous benzodiazepine receptor ligand-monoamine oxidase inhibitory activity (tribulin) in rat tissues*

Inés Armando, Gloria Levin, and **Marta Barontini**

Centro de Investigaciones Endocrinológicas, Hospital de Niños, Buenos Aires, Argentina

Accepted April 28, 1987

Summary. The presence of both MAO and benzodiazepine (BZ) receptor binding inhibitory activities in rat tissues has been reported previously. The two activities were similarly and unevenly distributed in the tissues. This dual inhibitory activity has been termed tribulin. We report here the effect of 1 1/2 hrs cold restraint stress on tribulin activity in rat tissues together with biochemical evidence to support the concept of a physiological role of tribulin. Stress induced a significant increment of both activities in heart and kidney while no significant changes were observed in the other tissues studied. Hearts and kidneys from stressed rats also showed a significant decrease of MAO activity, a significant increase of dopamine content and a significant decrease of the binding of ^{3}H-Ro 5-4864 to peripheral BZ receptors. Scatchard analysis of the saturation curves carried out using ^{3}H-Ro 5-4864 (0.4–10 nM) showed significant Bmax decreases in both organs. No significant change in either of these inhibitory activities was observed in the other tissues studied. These data provide support for a role of tribulin in the biochemical response to stress.

Keywords: Tribulin, MAO, stress, benzodiazepine receptors.

Introduction

Normal human and rat urine contains a substance (s) which is able to inhibit competitively MAO activity and to act as a benzodiazepine (BZ) receptor ligand, inhibiting the binding of BZ to specific receptors (Clow et al., 1983).

Partial purification by high pressure liquid chromatography showed that both activities eluted from the column in the same fractions (Elsworth et al., 1984; Glover et al., 1985). The urinary output of these activities is raised in rats after cold-restraint stress (Glover et al., 1981; Glover et al., 1985) and in humans after exhausting muscular exercise (Armando et al., 1984). In conditions

* This study was supported by grant PID 3-078500 from the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

of raised output the two activities always showed a significant correlation, although neither correlated with creatinine concentration.

This dual inhibitory activity has been termed tribulin (Sandler, 1982) and cannot be accounted for by any of the major urinary constituents, the known putative BZ receptor ligands or MAO inhibitors (Clow et al., 1983; Glover et al. 1980). We have previously described that both MAO inhibitory activity and BZ receptor ligand material are present in rat tissues. The two activities are unevenly distributed in different tissues and the tissues content of these activities were found to be highly correlated (Armando et al., 1986). We report here the effect of cold-restraint stress in the tissue content of both activities. In order to determine whether tribulin could play a role in the biochemical response to stress we have also studied the activity of MAO, the binding of specific ligands to BZ receptors and the content of catecholamines in tissues from control and stressed animals.

Materials and methods

Experiments were carried out on 2 month old male Sprague-Dawley rats. Rats were purchased from ELEA Lab. Argentina and placed for at least 4 days in a quiet room with food and water "ad-libitum". Control rats were killed without any previous handling. Animals were stressed by placing them in restraint cages for 1 1/2 hrs in a cold room (4 °C). Rats were killed by cervical dislocation. Tissues were rapidly removed, placed on ice and stored frozen until assays were performed.

MAO and BZ binding inhibition assays

For each experiment tissues collected from 6 rats were pooled, weighed and homogenized (20–50%) with an Ultraturrax in cold 2N HCl. Homogenates were centrifuged (0–4 °C) for 10 min at 7000 rpm. A 50 µl aliquot of the supernatants was separated for measuring tissue content of catecholamines (CA) and the rest was transferred to glass tubes and extracted into 2 vol. redistilled ethyl acetate. After centrifugation the organic layer was carefully removed, divided into two aliquots and dried down under N_2. Blanks containing equal volumes of 2N HCl to those used for tissue homogenization were also extracted into ethyl acetate and carried through the whole procedure.

Residues from one of the two dried down aliquots were taken up in 220 µl of 100 mM phosphate buffer pH 7.4 and the MAO inhibitory activity was tested as described by Glover et al. (1980). Briefly, 100 µl aliquots were incubated (30 min at 37 °C) with 20 µl of MAO preparation (1% w/v homogenate of rat liver) and 10 µl of ^{14}C-tyramine (Specific Activity 58.9 mCi/mmol, New England Nuclear) diluted with unlabelled tyramine to give a final concentration in the incubation mixture of 83 µM. The radioactive product formed was extracted into 3 ml of toluene: ethyl acetate (1:1) and after centrifugation the organic phase was transferred to vials for liquid scintilliation spectrometry.

Residues from the remaining aliquot were taken up in 220 µl of 25 mM phosphate buffer pH 7.1 and 100 µl aliquots were incorporated into an assay of ^{3}H-flunitrazepam binding to rat brain membranes, as described by Braestrup et al. (1980) to determine inhibition of BZ receptor binding. ^{3}H-flunitrazepam (^{3}H-FNZ) (Specific Activity 74.8 Ci/mmol, New England Nuclear) was used in a final concentration in the incubation mixture of 2 nM. The final concentration of rat brain membranes in the assay was 0.5% w/v. Both inhibitory activities were measured in duplicate for each sample. Controls for both assays

were carried out using 100 µl of buffer instead of tissue or blank extracts. Blanks inhibited both reactions by 10–15%.

Catecholamine determinations

Tissue contents of epinephrine (E), norepinephrine (NE) and dopamine (DA) were determined using a radioenzymatic method as modified in our laboratory (Armando et al., 1983).

Tissue activity of MAO

For determination of MAO activity tissues were homogenized in 5–10 vol. 100 mM phosphate buffer pH 7.4; centrifuged (0–4 °C) at 500 rpm for 10 min and 20 µl aliquots of the supernatant were incubated with 14C-tyramine (final concentration 16 µM). The assay procedure was similar to that used to test MAO inhibition.

BZ binding assays

Membranes for binding assays were prepared as follows: tissues were homogenized in 3–5 vol. 25 mM phosphate buffer pH 7.1, centrifuged (0–4 °C) at 2000 rpm for 10 min, pellets were discarded and supernatants recentrifuged at 12000 rpm for 30 min. The crude membrane pellets, including mitochondrias, were resuspended in the original volume of buffer and aliquots (containing 0.2–0.4 mg protein as determined by Lowry's method) were incubated with the radioactive ligands. Assays were carried-out as described by Braestrup et al. (1980). Non-specific binding was determined using 1 µM diazepam. ^{3}H-FNZ (final concentration 8 nM) was used for binding assays to cerebrum and cerebellum membranes. ^{3}H-FNZ (final concentration 20 nM) and ^{3}H-Ro 5-4864 (Specific Activity 76.5 Ci/mmol, New England Nuclear) (final concentration 1 nM) were used for binding assays to membranes from peripheral tissues.

Statistical analysis was performed using, Student's t-test for paired and unpaired data.

Results

Figure 1 shows the MAO and BZ receptor binding inhibitory activities found in tissues from control and cold-restraint stressed rats. Both inhibitory activities are expressed in units per g of wet tissue. A unit of each activity was arbitrarily defined as that producing 25% of inhibition in each test assay system (Armando et al., 1986). The MAO inhibitory activity found in hearts and kidneys from stressed rats was significantly higher than that found in those tissues from control rats. Although not significant, a decreased MAO inhibitory activity was found in cerebellum from stressed rats. BZ receptor binding inhibitory activity was also found to be significantly increased in both heart and kidney from stressed rats and decreased although not significantly, in cerebellum. In the other tissues studied the levels of the two activities were found to be similar in both control and stressed animals. The two inhibitory activities were found to be positively and significantly ($r=0.99$, $p<0.001$) correlated in tissues from both control and stressed rats.

Table 1 shows the content of DA, NE and E in tissues from control and stressed rats. The catecholamine content of heart atrium and ventricle were measured separately. Stress induced significant increases in DA content in heart (atrium and ventricle) and in kidney. A significant decrease in NE content was found in the atrium.

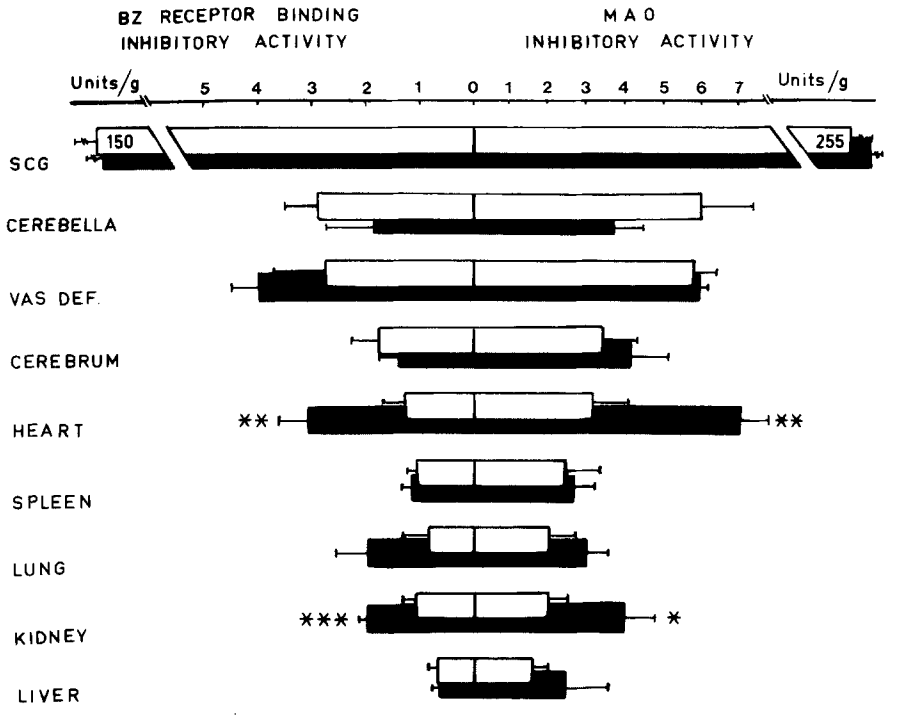

Fig. 1. Effect of 1 1/2 hrs of cold-restraint stress on benzodiazepine receptor ligand activity and MAO inhibitory activity in rat tissue extracts. Tissues from 6 rats were pooled for each experiment homogenized in acid and extracted into ethyl acetate. The dried down residues were taken up in buffer and incorporated into the MAO and benzodiazepine receptor assays using a final concentration of 83 μM ^{14}C-tyramine and 2 nM ^{3}H-flunitrazepam respectively. Each bar represents the mean ± SEM of 5 experiments. □ Control; ■ stress. * $p<0.05$ vs control; ** $p<0.0025$ vs control, *** $p<0.00125$ vs control

Table 2 shows in control and stressed rats the activity of MAO found in tissue homogenates and the binding of specific BZ receptor ligands to crude membrane preparations. The activity of the enzyme in heart and kidney from stressed rats was significantly lower than in controls whereas it remained unchanged in the other tissues studied.

The binding of the peripheral BZ receptor ligand ^{3}H-Ro 5-4864 (1 nM) was found to be significantly decreased in heart and kidney from stressed rats. Furthermore, in these tissues the binding of ^{3}H-FNZ (20 nM) a central and peripheral BZ receptor ligand was also found to be significantly decreased. The binding of ^{3}H-FNZ (8 nM) to membranes from cerebellum and cerebrum was found to be similar in control and stressed rats.

In heart and kidney from control and stressed rats saturation curves were carried out using ^{3}H-Ro 5-4864 (0.4 to 10 nM). The results of the Scatchard analysis of the curves are shown in Table 3. The stressed hearts and kidneys showed significant decreases in the number of receptors (B_{max}) when compared to controls. No changes were observed in the Kd.

Table 1. Endogenous catecholamines in tissues from control and cold-restrained rats

		Dopamine ng/g	Norepinephrine ng/g	Epinephrine ng/g
Kidney	Control	1.6±0.4	83±18	5.2±1.8
	Stress	2.5±0.4*	79±7	4.8±1.4
Cerebellum	Control	5.2±1.0	144±25	3.7±1.5
	Stress	9.2±2.7	157±30	3.0±0.6
Ventricle	Control	6.9±1.1	217±39	9.7±1.7
	Stress	13.5±1.7*	223±25	22.5±8.9
Atrium	Control	11.6±3.9	789±160	19.2±3.7
	Stress	27.5±5.8**	637±126**	37.0±11.1
Vas deferens	Control	60.0±8.9	4545±543	37.9±9.8
	Stress	74.8±17.2	4289±268	28.6±2.6
Cerebrum	Control	382.0±57.8	259±24	23.1±3.9
	Stress	353.0±17.0	197±22	20.6±2.4

Results are mean ± SEM of 4 experiments
* $p<0.02$ vs control; ** $p<0.05$ vs control

Discussion

Our results show that cold-restraint stress induced an increase in the MAO and BZ receptor binding inhibitory activities in heart and kidney. In both tissues together with the increment in this dual inhibitory activity we have also found a decrease in MAO activity, an increase in DA content and a decrease in the binding of peripheral benzodiazepine receptor ligands.

We have previously described the presence of both activities in rat tissues and showed that they are similarly and unevenly distributed. The distribution of the two activities found in control tissues from Sprague-Dawley rats is similar to that we have reported before in Wistar rats (Armando et al., 1986). The only difference between these two strains of rats seems to be higher values of both activities in the cerebellum from Sprague-Dawley rats. Although still short of proof, in urine the two inhibitory activities have been ascribed to only one molecule since partial purification by high pressure liquid chromatography demonstrated that both eluted in the same column fractions (Clow et al., 1983). Similar activities from rat brain and human plasma extract at the same pH, are of low molecular weight and behave in a similar way on high pressure liquid chromatography to tribulin from human urine (Glover et al., 1984). Is not unlikely then that the activities in the tissues could also be due to tribulin.

The release of an endogenous reversible MAO inhibitor has been postulated by several authors to explain the brain levels of CA and serotonin metabolites produced during different stressful conditions (Welch and Welch, 1970) and the decrease of MAO activity in rat liver and brain after environmental stress (Maura and Vaccari, 1975). Such an inhibitor would be of obvious physiological

Table 2. Effect of stress on the tissues MAO activity and on the binding of benzodiazepine receptor ligands to tissue membranes

		MAO activity 16 μM ^{14}CTyramine pmol product/mg tissue/h	Binding of ^{3}H-Ro 5-4864 1 nM pmol/gr prot	Binding of ^{3}H-FNZ 20 nM pmol/gr prot	Binding of ^{3}H-FNZ 8 nM pmol/gr prot
Cerebrum	Control	396 ± 19(6)	—	—	790 ± 106 (6)
	Stress	419 ± 30 (6)	—	—	756 ± 69 (6)
Cerebellum	Control	429 ± 17 (6)	—	—	375 ± 63 (6)
	Stress	437 ± 9 (6)	—	—	260 ± 27 (6)
Heart	Control	1651 ± 130 (6)	753 ± 152 (6)	888 ± 164 (5)	—
	Stress	1211 ± 122 (6)*	324 ± 33 (6)*	494 ± 129 (5)*	—
Kidney	Control	522 ± 29 (12)	565 ± 100 (6)	206 ± 31 (6)	—
	Stress	445 ± 27 (12)*	320 ± 35 (6)*	85 ± 23 (6)**	—
Spleen	Control	179 ± 17 (6)	478 ± 79 (6)	—	—
	Stress	174 ± 10 (6)	632 ± 151 (6)	—	—
Vas deferens	Control	488 ± 14 (3)	668 ± 441 (3)	—	—
	Stress	474 ± 41 (3)	680 ± 15 (3)	—	—
Lung	Control	505 ± 13 (6)	443 ± 113 (6)	—	—
	Stress	477 ± 13 (6)	349 ± 67 (6)	—	—
Liver	Control	1310 ± 67 (4)	—	—	—
	Stress	1347 ± 223 (4)	—	—	—

Results are $\bar{X}$ ± SEM
In parenthesis number of experiments
*$p < 0.05$ vs control; ** $p < 0.01$ vs control

Table 3. Scatchard analysis of ^{3}H-Ro 5-4864 saturation curves (0.4–10 nM) in heart and kidney membranes from control and stressed rats

		B_{max} pmol/gr prot	Kd nM
Heart	Control	1403 ± 302	0.76 ± 0.30
	Stress	398 ± 89*	0.80 ± 0.08
Kidney	Control	482 ± 86	0.88 ± 0.17
	Stress	161 ± 98*	1.01 ± 0.60

Results are $\bar{X}$ ± SEM of 4 experiments
* $p < 0.01$ vs control

benefit to conserve CA levels during periods of maximal release. In vitro, tribulin inhibits rat liver MAO A and MAO B equally and kinetic analysis of the effect on tyramine shows the inhibition to be competitive (Elsworth et al., 1984). If tribulin plays a role in the in vivo regulation of MAO activity, its increased presence should cause a decrease in the activity of the enzyme in the tissue. Our present data, in heart and kidney, showed not only an increase in the MAO inhibitory activity after stress but also a decrease in the activity of MAO in homogenates of these tissues. Although a kinetic analysis of the inhibition of tyramine oxidation was not performed in these experiments, the decrease in MAO activity was found using a low concentration of the substrate. This is compatible with the presence of a competitive reversible inhibitor. If reversible with MAO then the dilution in the homogenate will underestimate the effects of the inhibitor in vivo.

On the other hand, an increase in DA content was observed in heart and kidney from stressed rats. Stress-induced increases in DA content have been reported in hamster hearts (Sole et al., 1977) and rat kidney (Snider and Kuchel, 1983). These increases have been explained assuming that under condition of increased sympathetic activity there is a shift in the rate-limiting step for NE synthesis from the hydroxylation of tyrosine to the hydroxylation of DA (Snider and Kuchel, 1983). However, it can also be speculated that these findings are related to a decrease in MAO activity since studies on neurotransmitter turnover have shown that inhibition of MAO activity results in tissue accumulation of DA while changes in NE concentration are usually small (Sharman, 1981).

Tribulin has been shown to inhibit selectively and competitively the binding of ^{3}H-clonazepam to the central type of BZ receptors in rat brain as well as to inhibit the binding of ^{3}H-Ro 5-4864 to the peripheral type of receptors in rat kidney (Elsworth et al., 1984). Stress induced in heart and kidney an increase in the BZ binding inhibitory activity. Crude membranes (including mitochondria) from these tissues showed a decrease in the binding of both ^{3}H-Ro 5-4864 and ^{3}H-FNZ. The Scatchard analysis of the saturation curves obtained with ^{3}H-Ro 5-4864 demonstrated a decrease in the number of receptors in both

tissues. These data are compatible with the presence of a non-competitive reversible inhibitor.

BZ receptors are capable of being altered by external stimuli (Braestrup and Nielsen, 1983). In this respect it has been reported that stress induced a decrease in the brain number of specific BZ binding sites (Medina et al., 1983). It has also been suggested that stress induces in brain cortex the release of a substance possessing similar characteristics to β-carbolines (Biggio et al., 1984). We have not found after stress significant changes in either the inhibitory potency of cerebrum homogenates or the binding of ^{3}H-FNZ to cerebrum membranes. However, since the changes reported in the number of receptors seem to be restricted to certain brain areas it is possible that a significant change could not be observed in these homogenates.

Peripheral BZ receptors have been found in many tissues including both kidney and heart (Arnholt et al., 1984). The regulation of kidney receptors by factors involved in the control of blood pressure has been suggested (Regan et al., 1981) as well as a role of peripheral receptors in the effect of BZ on the secretion of hormones from some endocrine organs (De Souza et al., 1985). Our data suggest that tribulin might be involved in the regulation of these receptors although the determination of the physiological significance of this finding must await the identification of the precise function of the peripheral BZ receptors.

The present data not only support the concept of a participation of tribulin in the complex mechanisms triggered by stress but also provide evidence of an in vivo role of these activities.

References

Anholt RR, Murphy MM, Mack GE, Snyder SH (1984) Peripheral-type benzodiazepine receptors in the central nervous system: localization to olfactory nerves. J Neurosci 4:593–603

Armando I, Levin G, Barontini M (1983) Evaluation of sympathetic nervous system and adrenomedullary activity in normal children. J Auton Nerv Syst 8: 57–63

ArmandoI, Barontini M, Levin G, Simsolo R, Glover V, Sandler M (1984) Exercise increases endogenous urinary monoamine oxidase benzodiazepine receptor ligand inhibitory activity in normal children. J Auton Nerv Syst 11: 95–100

Armando I, Glover V, Sandler M (1986) Distribution of endogenous benzodiazepine receptor ligand-monoamine oxidase inhibitory activity (Tribulin) in tissue. Life Sci 38: 2063–2067

Biggio G, Concas A, Serra M, Corda MG, Nurchi V, Crisponi C, Gessa GL (1984) Stress and β-carbolines decrease the density of low affinity GABA binding sites: an effect reversed by diazepam. Brain Res 305: 13–18

Braestrup C, Nielsen N, Olsen CE (1980) Urinary and brain β-carboline-3-carboxylates as potent inhibitors of brain benzodiazepine receptors. Proc Natl Acad Sci USA 77: 2288–2292

Braestrup C, Nielsen M (1983) Biochemical studies of CNS receptors, lst edn. Plenum Press, New York, p 285–384

Clow A, Glover V, Armando I, Sandler M (1983) New endogenous benzodiazepine receptor

ligand in human urine: identity with endogenous MAO inhibitor? Life Sci 33: 735–741

De Souza EB, Anholt RR, Murphy MM, Snyder SH, Kuhar MJ (1985) Peripheral type benzodiazepine receptors in endocrine organs: autoradiographic localization in rat pituitary, adrenal and testis. Endocrinology 116: 567–573

Elsworth JD, Clow A, Glover V, Sandler M (1984) Characterization of an endogenous inhibitor of monoamine oxidase and benzodiazepine receptor binding. J Pharm Pharmacol 36 [Suppl] 1 W: 59 W

Glover V, Reveley MA, Sandler M (1980) A monoamine oxidase inhibitor in human urine. Biochem Pharmacol 29: 467–470

Glover V, Bhattacharya SK, Sandler M, File S (1981) Benzodiazepines reduce stress-augmented increase in rat urine monoamine oxidase inhibitor. Nature 292: 347–349

Glover V, Segarajasinghe CE, Clow A, Elsworth JD, Sandler M (1984) Endogenous monoamine oxidase inhibitors in rat brain and human plasma. J Pharm Pharmacol 36 [Suppl] 1 W: 12 W

Glover V, Clow A, Elsworth JD, Armando I, Sandler M (1985) Stress: the role of catecholamines and other neurotransmitters. Gordon and Breech, New York, p 457–465

Maura G, Vaccari A (1975) Relationships between age of submission to environmental stress and monoamine oxidase activity in rats. Experientia 31: 191–193

Medina JH, Novas ML, Wolfman CN, Levi de Stein M, De Robertis E (1983) Benzodiazepine receptors in rat cerebral cortex and hippocampus undergo rapid and reversible changes after acute stress. Neuroscience 9: 331–335

Regan JW, Yamamura HI, Yamada S, Roeske WR (1981) High affinity renal [^{3}H]-flunitrazepam binding: characterization, localization and alteration in hypertension. Life Sci 28: 991–997

Sandler M (1982) The emergence of tribulin. Trends Pharmacol Sci 3: 471–472

Sharman DF (1981) Central neurotransmitter turnover. University Park Press, Baltimore, p 20–58

Snider SR, Kuchel O (1983) Dopamine: an important neurohormone of the sympathoadrenal system. Significance of increased peripheral dopamine release for the human stress response and hypertension. Endocrine Rev 4: 291–309

Sole MJ, Kamble AB, Hussain N (1977) A possible change in the rate-limiting step for cardiac norepinephrine synthesis in the cardiomyopathic syrian hamster. Circ Res 41: 814–817

Welch BL, Welch AS (1970) Amphetamines and related compounds. Raven Press, New York, p 415–445

Authors' address: Dr. Inés Armando, Centro de Investigaciones Endocrinológicas, Hospital de Niños, Gallo 1330, (1425) Buenos Aires, Argentina.

Received January 8, 1987