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Mechanism of Stress-Induced Subsensitivity to Norepinephrine

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STONE, E. A. *Mechanism of stress-induced subsensitivity to norepinephrine.* PHARMAC. BIOCHEM. BEHAV. **14**(5) 719–723, 1981.—Chronic footshock stress in rats produces a persistent reduction in the sensitivity of the norepinephrine (NE)-cAMP generating system in the cerebral cortex, an effect similar to that reported after chronic antidepressant treatment. The present studies show that footshock-induced subsensitivity is not related to changes in either beta or alpha-1 adrenergic receptors, phosphodiesterase or total adenylate cyclase activity. The stress does induce a small, selective decrease in binding at high affinity alpha-2 receptor sites but this change does not appear to explain the decreased responsiveness to NE. These data and related findings by others using restraint stress indicate that the mechanism of subsensitivity after chronic stress resembles in part that seen after antidepressants but may also involve additional phenomena which may not occur after the latter agents.

Stress Adaptation Subsensitivity Norepinephrine Brain Adrenergic receptors Phosphodiesterase Adenylate cyclase

ANIMALS exposed to prolonged or repeated stress undergo a group of complex changes known as adaptation which serve to reduce the deleterious effects of stress [22]. Recent studies in our laboratory suggest that a reduction in the sensitivity of brain NE receptors may be one of the biochemical changes underlying adaptation [24,25]. Using the stress of electric footshock, we have found that if rats are subjected to 9 daily exposures to this treatment and killed 24 hrs afterwards, the animals show a decrease in the sensitivity of NE receptors in the cerebral cortex. The measure of receptor sensitivity employed in these studies was the ability of NE to stimulate the formation of cAMP in the brain slice preparation. Stress reduced this measure by approximately 40%. We have suggested that subsensitivity to NE may play a role in adaptation to stress because the two processes have a similar time course, i.e., subsensitivity does not occur after acute footshock when rats are most disturbed by the stress but only after chronic footshock when the animals show clear evidence of resistance to its adverse effects [26]. We have also suggested that subsensitivity may represent a link between antidepressant therapy and adaptation to stress since most antidepressant agents are known to reduce central sensitivity to NE and are presumed to increase resistance to emotional stress [26,28].

The biochemical basis of stress-induced subsensitivity to NE is not presently known. Previous pharmacological research has shown that a reduced cAMP response to NE can result from changes in a number of factors including alterations in adrenergic receptors [10], elevations in phosphodiesterase (PDE) activity [16] and reductions in the degree of activation of adenylate cyclase [8]. In the present study we have examined the effect of chronic footshock on the latter variables in an attempt to clarify the mechanism of this change.

METHOD

Experimental Design

Male Sprague Dawley rats (Charles River Inc., 150–200 g) were used. Animals were housed 2 to a cage and maintained on a 12 hr light-dark cycle (lights on at 0800 hrs) with food and water freely available. Footshock was applied as described previously [24] by placing the rat in a 15×24×24 cm chamber containing a grid floor connected to a constant current shock source with a shock scrambler. The rats received one hr of footshock per day on 9 consecutive days. Shocks of one sec duration were given on a variable interval schedule of 20 sec. The intensity of the shock was 1.5 mA for days 1–3, 2.5 mA for days 4–6 and 3.5 mA for days 7–9. This gradual increase in shock intensity was used to minimize the risk of an acute lethal effect of the stress since the latter has been reported on occasion when naive rats are subjected to high levels of footshock [34]. Control rats were handled but not shocked. Twenty-four hrs after the last session the animals were decapitated and the cerebral cortices dissected free. For studies of ligand binding the tissues were frozen on dry ice and stored in liquid N_2 until assay. For assays of cAMP formation in brain slices and adenylate cyclase activity in homogenates the tissues were used immediately after sacrifice. Each of the variables under consideration was examined in a separate experiment using an independent group of stressed and control rats. All statistical evaluations were based on planned comparisons between 2 groups (*t*-test, two tailed). Since there were 2 comparisons per exper-

iment the Type 1 error rate per comparison was set at $\alpha=0.025$ to maintain an experimentwise error rate of $\alpha=0.05$ [11].

[^{3}H]DHA Binding

Specific [^{3}H]dihydroalprenolol (DHA) binding in cortical membranes was determined according to Bylund and Snyder [2]. Saturation binding assays were conducted separately for each animal of each group. Tissues were homogenized in 30 volumes of 50 mM Tris buffer (pH 8.0) and centrifuged at 49,000 G for 15 min. This procedure was repeated once and the pellet resuspended in fresh buffer to give a final concentration of 20 mg original wet wt/ml. Incubation of the membrane preparation (10 mg) with [^{3}H]DHA (0.125–4.0 nM) was carried out in a final volume of 1 ml in triplicate for 25 min at 23°C. Incubations were terminated by rapid filtration over Whatman GF/B filters. The filters were rinsed with 16 ml of buffer, air-dried and counted in 10 ml of a toluene cocktail at 46% efficiency. Specific [^{3}H]DHA binding was defined as the excess over blanks containing 1 μM dl-propranolol. Specific binding accounted for 55–83% of total binding. In competitive binding experiments membranes were incubated with 1 nM [^{3}H]DHA in the presence of varying concentrations (10^{-9} to 10^{-4} M) of isoproterenol (ISO). The latter experiments were conducted both in the absence and presence of 5 mM $MgCl_2$. Linear regression by the method of least squares was used to calculate (a) the binding constants of [^{3}H]DHA from Scatchard plots, (b) the IC_{50} values of ISO from log-probit plots, and (c) the Hill coefficients from Hill plots.

[^{3}H]WB-4101 Binding

Specific [^{3}H]WB-4101 binding was determined by the procedure of U'Prichard *et al.* [31]. Membranes (10 mg) were prepared as above in Tris buffer (pH 7.7) and incubated for 25 min at 23°C with [^{3}H]WB-4101 at 0.125 to 2.0 nM in a final volume of 1 ml. Filtration, counting and analysis were as above. Specific [^{3}H]WB-4101 binding was defined as the excess over blanks containing 100 μM 1-NE and ranged from 45–77% of total binding.

[^{3}H]Clonidine Binding

[^{3}H]Clonidine was repurified on silica gel G and stored at 0.05 mCi/ml 70% (v/v) ethanol at −20°C to reduce degradation. Specific high and low affinity clonidine binding were determined by the method of U'Prichard *et al.* [30]. Membranes (20 mg), prepared as above in Tris buffer (pH 7.7) were given an initial preincubation for 30 min at 23°C. Total and nonspecific binding were determined by incubating the membranes with 1 nM [^{3}H]clonidine in the absence or presence of 10 μM 1-NE in a final volume of 2 ml at 23°C followed by filtration at 30 min. Residual binding was determined by adding 1-NE (10 μM final concentration) to a second set of total binding incubates and filtering these at 2 min after NE addition. Specific high affinity [^{3}H]clonidine binding was defined as the difference between residual and nonspecific binding. Specific low affinity binding was defined as the difference between total and residual binding. Under the present conditions, high and low affinity binding each accounted for 43% of total binding.

cAMP Formation in Brain Slices

Cortical slices (0.26×0.26×1.0 mm) were prepared with a McIlwain chopper and incubated in Krebs Ringer bicarbonate buffer (pH 7.4) by methods described previously [24,25]. After 40 min of preincubation the medium was changed and the slices incubated for an additional 7 min in the presence or absence of 100 μM 4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone (ZK-62711), a potent phosphodiesterase inhibitor [21]. NE (0 or 100 μM) was next added and the slices were incubated a further 15 min. The reaction was stopped by immersion of the tubes in a boiling water bath. After centrifugation, cAMP was assayed in aliquots of the supernatant by the method of Brown *et al.* [4]. The pellet was solubilized in a 1 N NaOH and assayed for protein according to Lowry *et al.* [13] using bovine albumin as the standard.

TABLE 1

EFFECT OF CHRONIC FOOTSHOCK ON SPECIFIC BINDING OF [^{3}H]DIHYDROALPRENOLOL (DHA) AND [^{3}H]WB-4101 IN RAT CORTEX

Treatment	B_{max} (pmol/g)	K_D (nM)
	[^{3}H]DHA	
Control (10)	11.2 ± 1.2	0.92 ± 0.11
Footshock (10)	11.8 ± 0.6	0.96 ± 0.12
	[^{3}H]WB-4101	
Control (10)	10.4 ± 0.5	0.40 ± 0.06
Footshock (10)	10.2 ± 0.9	0.36 ± 0.04

Values are means ± SEM of number of rats (n) in parentheses.

TABLE 2

EFFECT OF CHRONIC FOOTSHOCK ON HIGH AND LOW AFFINITY [^{3}H]CLONIDINE BINDING IN RAT CORTEX

Treatment	High Affinity	Low Affinity
	pmol/g tissue	
Control (8)	1.16 ± 0.05	1.14 ± 0.03
Footshock (8)	0.99 ± 0.04*	1.14 ± 0.04

Values are means ± SEM of number of rats (n) in parentheses.
*Differs from control at $p<0.02$.

Adenylate Cyclase Activity in Homogenates

Basal and fluoride-stimulated adenylate cyclase activity in cortical homogenates were assayed by the method of Krishna *et al.* [12] as modified by Skolnick *et al.* [23].

RESULTS

Footshock had no effect on the density or affinity of cortical beta or alpha-1 adrenergic receptors as measured by [^{3}H]DHA and [^{3}H]WB-4101 binding, respectively (Table 1). The stress also did not affect the ability of ISO to displace [^{3}H]DHA from beta receptors. IC_{50} values of ISO in 4 control and 4 stressed rats were (means±SEM) $3.84\pm0.57\times10^{-8}$ M and $3.90\pm0.63\times10^{-8}$ M, respectively. Hill coefficients derived from these data were 0.82±0.04 for controls and 0.80±0.04 for footshocked rats and were not significantly different. The presence of $MgCl_2$ (5 mM) did not appreciably

TABLE 3

EFFECT OF CHRONIC FOOTSHOCK ON NE-STIMULATED FORMATION OF cAMP IN CORTICAL SLICES IN PRESENCE AND ABSENCE OF PHOSPHODIESTERASE (PDE) INHIBITOR

Treatment	Basal Level	NE 100 μM	Increment due to NE
	pmol cAMP/mg protein		
	PDE Inhibitor Absent		
Control (7)	11.8 ± 1.0	50.1 ± 4.7	38.4 ± 3.9
Footshock (7)	12.1 ± 0.8	37.4 ± 2.5	25.3 ± 1.9
p-diff			<0.01
	PDE Inhibitor Present (ZK-62711 100 μM)		
Control (7)	38.9 ± 4.0	205.7 ± 17.7	166.9 ± 14.4
Footshock (7)	48.9 ± 3.9	176.0 ± 8.6	127.0 ± 5.4
p-diff			<0.025

Increments were computed for each rat's cortex by subtraction of the basal from the NE-stimulated levels. Differences in increment values were used for statistical tests. Values are means ± SEM for number of rats (n) in parentheses.

TABLE 4

EFFECT OF CHRONIC FOOTSHOCK ON ADENYLATE CYCLASE ACTIVITY IN RAT CORTICAL HOMOGENATES

Treatment	Basal Level	NaF 5 mM
	nmol/mg protein/min	
Control (8)	0.422 ± 0.016	0.666 ± 0.027
Footshock (8)	0.408 ± 0.014	0.655 ± 0.030

Values are means ± SEM of number of rats (n) in parentheses.

affect the latter results except for producing a slight elevation in the IC_{50} values for both groups (data not shown). There was a small (15%) but significant reduction in high affinity [^{3}H]clonidine binding in cortices from stressed rats (Table 2). Low affinity clonidine binding did not differ between the two groups.

In confirmation of previous findings, cortical slices from stressed rats showed a significant reduction of cAMP accumulation in response to NE (Table 3). This reduction was significant both in the absence and presence of the phosphodiesterase inhibitor, ZK-62711, despite the fact that the inhibitor produced a 4-fold increase in cAMP levels. No differences in basal or fluoride-activated adenylate cyclase activity in cortical homogenates was found between stressed and control rats (Table 4).

DISCUSSION

Previous pharmacological research has shown that subsensitivity of the NE-cAMP response in the CNS is most frequently accompanied by a reduced density of beta adrenergic receptors. A decreased number of beta receptors has been found after most desensitizing agents including antidepressant drugs [1,20], electroconvulsive shock [3] and beta adrenergic agonists [15]. Unlike the latter agents, footshock stress has no apparent effect on these receptors. The stress was not found to alter either the affinity or density of beta receptors as measured by DHA binding. Similar findings have been obtained by others using a shorter period of this stress [3]. Footshock also did not have any effect on the ability of a beta agonist, ISO, to displace DHA from beta receptors. The latter result argues against the possibility that the stress may have induced subsensitivity by causing a selective decrease in the proportion of beta receptors with high affinity for agonists [33].

It should be noted however that under certain conditions stressful procedures can affect central beta receptors. U'Prichard & Kvetnansky [32] reported that if rats were killed immediately after the last of 14 daily exposures to restraint stress there was a significant reduction in beta receptor density in the cerebral cortex. This change disappeared however if the animals were sacrificed 24 hrs after the end of the stress. These results are therefore in agreement with the present finding that at 24 hrs after chronic footshock there is no decrease in beta receptor density despite a significant reduction of the NE-cAMP response. Whether footshock like restraint is capable of producing a transient decrease in beta receptors is a matter for further study.

Other factors suspected of playing roles in subsensitivity of catecholamine-cAMP systems are an elevated phosphodiesterase activity [15,16] and a partial refractoriness of adenylate cyclase towards activation [7,8]. The latter change, whose mechanism is still unclear, is observed as a reduced activation of adenylate cyclase by fluoride ion, guanine nucleotides and catecholamines. Footshock stress however was not found to produce either of these effects. Blockade of phosphodiesterase with ZK-62711, a potent and selective enzyme inhibitor, did not alter the subsensitive cAMP response to NE in cortical slices from stressed rats. Assays of basal and fluoride-stimulated adenylate cyclase activity in cortical homogenates did not reveal any evidence of a stress-induced refractoriness of the enzyme towards activation. The latter result however does not completely rule out the presence of an intracellular inhibitory factor as this factor would be considerably diluted by the homogenization procedure [27].

The possibility of a change in alpha receptor binding was examined since there is evidence (a) that part of the action of NE on adenylate cyclase in the rat cortex is modualted by a receptor that has some of the characteristics of an alpha adrenergic receptor [5,17], and (b) that subsensitivity to NE has been reported to be accompanied by alterations in binding to certain subtypes of alpha receptors (cited below). Footshock was not found to affect either the density or affinity of binding sites for WB-4101 indicating no change in alpha-1 receptors. The stress did however produce a significant decrease in high affinity clonidine binding without altering low affinity binding. This suggests that footshock selectively lowers binding to high affinity alpha-2 receptors. Owing to technical limitations it was not feasible to perform saturation binding studies of the low and high affinity sites. It is not known therefore whether the reduced binding represents a change in the density or affinity of these receptors.

The decrease in alpha-2 adrenergic receptor binding after footshock is unlikely to be a factor in stress-induced subsensitivity as there is evidence that these receptors act to inhibit

adenylate cyclase in a variety of peripheral tissues [6, 7, 9] and possibly also in the CNS [19]. In support of this, an increased density of alpha-2 receptors in the rat cortex has been found following desensitization of the NE-adenylate cyclase system produced by some antidepressant drugs [18] and beta adrenergic agonists [14] and also following the application of restraint stress [29,32]. On the basis of these findings the decrease in alpha-2 receptor binding after footshock would be expected to enhance not diminish the adenylate cyclase response to NE. The reason why footshock produces a decrease in these receptors in contrast to the increase seen after other desensitizing procedures is not known. Several factors may have contributed to this difference. First, the increase in alpha-2 receptors after antidepressants and beta agonists appears to be linked in some manner to the decrease in beta receptors produced by these treatments. Since footshock does not produce a decrease in beta receptors it is not surprising that the stress also does not lead to an increase in alpha-2 receptor binding. Second, with regard to the effect of restraint, it is important to note that the greatest increase in alpha-2 binding found with this stress occurs after acute treatment. Chronic restraint produces much less of an effect suggesting that there is a decline in these receptor sites with prolonged exposure to stress. The difference between footshock and restraint may therefore be a matter of degree with footshock producing a more marked or rapid reduction in alpha-2 binding. Third, the lack of agreement in the above studies may have resulted in part form a difference in methodology. Total clonidine binding was examined in the studies of beta agonist treatment and restraint stress whereas high and low affinity clonidine binding were assayed separately in the present experiment with footshock and in the foregoing one with antidepressants. Owing to this difference in methodology therefore the results obtained with footshock are not strictly comparable to those found after restraint and beta agonists.

In conclusion, the persistent desensitizing action of chronic footshock on the cortical NE-adenylate cyclase system is not readily explained by changes in either beta or alpha adrenergic receptors, PDE or adenylate cyclase activation. Footshock therefore does not appear to produce prolonged changes in several of the factors that are most frequently associated with pharmacologically-induced subsensitivity. The mechanism by which the stress acts to induce this effect thus remains to be identified. Nevertheless the present findings do provide further information on the relationship between adaptation to stress and antidepressant therapy. As noted above, we have previously proposed that antidepressant therapy is a form of adaptation to stress [26]. According to this view the various antidepressant agents are presumed to exert their clinical effects by mimicking the desensitizing action of chronic stress at central adrenergic receptors and in this manner causing an increased resistance to emotional stress. This hypothesis would be considerably strengthened if antidepressants and stress were found to induce the same molecular form of subsensitivity. A comparison of these treatments based on studies of restraint, footshock and antidepressant drugs however reveals similarities as well as apparent differences. On the one hand, restraint stress resembles most antidepressant agents in that it produces a decrease in the number of beta adrenergic receptors and an increase in the density of alpha-2 receptors in the brain. On the other hand, footshock stress appears to differ from the antidepressants in that it does not lead to a fall in beta receptor density or to an increase in alpha-2 receptor binding. Whether these differing effects of restraint and footshock are the result of different forms of stressful stimulation or are due to the different post-stress assay intervals used in the above studies remains to be clarified. Nevertheless the above findings may be interpreted with regard to mechanism in one of two general ways. First, they can be taken to mean that the subsensitivity produced by stress differs from that seen after antidepressants because the former treatment compared to the latter evokes a broader range of neurochemical phenomena some of which are independent of changes in adrenergic receptors. Alternatively, it is possible that stress and antidepressants produce similar forms of subsensitivity, and that the assumption that antidepressants are acting solely via adrenergic receptors to achieve this effect is an incorrect one, i.e., the antidepressants, in addition to their effects on receptors, may also be producing a non-receptor alteration similar to that caused by footshock. Further research will be required to determine which of these interpretations is correct.

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