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Short communication

Acute inactivation of tryptophan hydroxylase by amphetamine analogs involves the oxidation of sulfhydryl sites

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The activity of rat hippocampal tryptophan hydroxylase was reduced from 30-60% 3 h after the administration of a 10-15 mg/kg dose of either fenfluramine, methamphetamine or 3,4-methylenedioxyamphetamine (MDMA). Tryptophan hydroxylase inactivated by these drug treatments could be reconstituted by a prolonged anaerobic incubation in the presence of 5 mM dithiothreitol and 50 μ M Fe²⁺. Drug-inactivated enzyme obtained from rats killed 18 h after multiple doses of either D(+) or L(-)-MDMA could not be similarly restored. These observations suggest that the rapid decrease in central tryptophan hydroxylase activity induced by amphetamine analogs results from the reversible oxidation of a sulfhydryl site(s) within the enzyme molecule, whereas the irreversible decrease in enzymatic activity measured 18 h after multiple-dose MDMA treatment may reflect serotonergic toxicity.

Fenfluramine; Methamphetamine; 3,4-Methylenedioxyamphetamine (MDMA); Thiol/sulfhydryl; Tryptophan hydroxylase (TRH); (Inactivation)

1. Introduction

Several amphetamine analogs, including 3,4-methylenedioxyamphetamine (MDMA), methamphetamine and fenfluramine, induce a rapid decrease in the activity of rat central tryptophan hydroxylase (TPH) *in vivo*. This decrease occurs within 1 h of systemic drug administration (Bakhit and Gibb, 1981; Invernizzi et al., 1986; Stone et al., 1987) and has been associated with a decline in the enzyme V_{max} with no change in K_m for substrate or cofactor (Schmidt and Taylor, 1987). Amphetamines appear to have no direct inhibitory effect on TPH *in vitro* (Schmidt and Taylor, 1987). Multiple doses of MDMA, methamphetamine or fenfluramine can induce

neuronal degeneration of serotonin nerve terminals, as evidenced by a decrease in the number of central 5-hydroxytryptamine (5-HT) uptake sites and an irreversible loss of brain TPH activity and 5-hydroxyindole content (Battaglia et al., 1987; Ricaurte et al., 1980; Steranka and Sanders-Bush, 1979; Stone et al., 1987). Whereas the long-term loss of TPH activity can be attributed to a drug-induced reduction in the number of 5-HT nerve terminals, the mechanism by which amphetamine analogs induce the initial decline in enzymatic activity is not understood.

Due to the rapidity of the drug-induced decline in TPH activity, an inactivation of enzyme molecules resulting from a structural change may be a more likely explanation than an increase in enzyme catabolism or the onset of neuronal degeneration. Kuhn et al. (1980) described a similar rapid loss of TPH activity *in vitro* following exposure of the enzyme to molecular oxygen; TPH inactivated in this manner could be restored by an incubation

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under anaerobic conditions with dithiothreitol and Fe^{2+} . We therefore explored the possibility that a similar model of TPH oxidation might explain the acute decrease in enzymatic activity observed following a single dose of MDMA, methamphetamine or fenfluramine.

2. Materials and methods

2.1. Animals and drug administration

Male Sprague-Dawley rats weighing 180-250 g were housed in groups (3-4 per cage) in a temperature-controlled room (24°C) with a 12 h light/dark cycle. The animals were administered a single s.c. injection of either (\pm)-MDMA (10 mg/kg), (+)-methamphetamine (15 mg/kg), (\pm)-fenfluramine (10 mg/kg), or 0.9% saline vehicle, and killed 3 h later. Dosages of drugs are expressed in terms of the free base form. In a separate experiment, rats were administered 5 doses (6 h intervals) of either (+)- or (-)-MDMA (10 mg/kg per dose) and killed 18 h after the last administration. The brain was quickly removed from the skull and placed on a chilled glass plate. Hippocampi were removed, frozen on dry ice, and stored at -80°C until assayed.

2.2. Assay for TPH activity

Individual tissues were weighed and homogenized in 200 μl of 50 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES) buffer (pH 7.4) containing 0.2% (v/v) Triton X-100 and 5 mM dithiothreitol. Following centrifugation at 26000 $\times g$ for 15 min (4°C), duplicate 7.5 μl aliquots of the supernatant were removed and assayed for TPH activity by a $^{14}\text{CO}_2$ -trapping procedure, as described by Hotchkiss et al. (1979). The assay was run under V_{max} conditions with respect to the synthetic enzyme cofactor, 6-methyl-5,6,7,8-tetrahydrobiopterin (Sigma Chemical Co.).

2.3. In vitro enzyme reactivation

A 30 μl aliquot of the hippocampal supernatant

from each rat was transferred into a 6 \times 50 mm silanized glass tube, into which 5 μl of a 5 mM dithiothreitol solution containing ferrous ammonium sulfate was added; after dilution, the final concentration of Fe^{2+} was 50 μM . The tubes were placed in a vacuum desiccator (without desiccant) and incubated for 20-24 h under a nitrogen atmosphere at 25°C, essentially according to the method of Kuhn et al. (1980). Following the incubation, tubes were removed and placed in an ice-cooled water bath (4°C); duplicate 7.5 μl aliquots were removed from each tube and assayed for TPH activity as described above.

3. Results

Hippocampal TPH activity was dramatically decreased, to less than 50% of control, 3 h after the acute administration of either MDMA or methamphetamine and to less than 70% of control 3 h after fenfluramine (table 1, before incubation). The decline in TPH activity induced by these drugs was completely reversed by a prolonged anaerobic incubation of the crude TPH preparations in the presence of dithiothreitol and Fe^{2+} ; i.e. TPH activity from rats treated acutely with either MDMA, methamphetamine or fenfluramine did not differ significantly from similarly in-

TABLE 1

Hippocampal TPH activity, both before and after a 20-24 h anaerobic incubation in the presence of dithiothreitol and Fe^{2+} , from rats killed 3 h after a single injection of (\pm)-MDMA (10 mg/kg), (+)-methamphetamine (15 mg/kg) or (\pm)-fenfluramine (10 mg/kg). Results are the means \pm S.E.M.; the number of animals is indicated in parentheses.

Drug treatment	n	TPH activity (nmol/g tissue per h)	
		Before incubation	After incubation
Saline	7	65.0 \pm 4.9	77.7 \pm 4.0 ^b
MDMA	7	25.3 \pm 2.1 ^a	75.7 \pm 3.6 ^c
Saline	6	77.9 \pm 4.1	143.9 \pm 3.6 ^c
MA	6	33.7 \pm 1.9 ^a	129.1 \pm 6.4 ^c
Saline	6	62.0 \pm 2.9	78.8 \pm 6.5 ^b
FA	6	42.3 \pm 2.4 ^a	83.6 \pm 3.7 ^c

^a P < 0.001 vs. time-matched saline; ^b P < 0.05, ^c P < 0.001 vs. respective 'before incubation', by the two-tailed Student's t-test.

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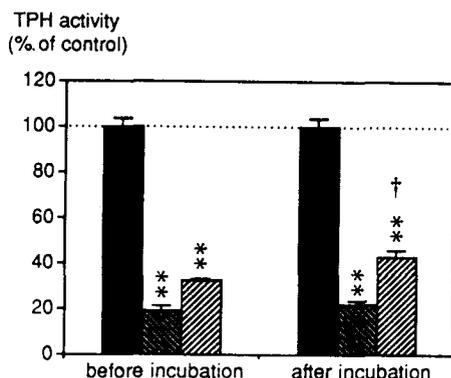


Fig. 1. Hippocampal TPH activity, both before and after a 20-24 h anaerobic incubation in the presence of dithiothreitol and Fe^{2+} , from rats administered five 10 mg/kg doses (6 h intervals) of (+)- or (-)-MDMA. Results are the means \pm S.E.M. (n = 6-7), expressed as a percent of time-matched control. Control TPH activity, before and after incubation, respectively, was 60.7 ± 2.3 and 123.5 ± 5.0 nmol/g tissue per h. **P < 0.001 vs. time-matched control, † denotes significant enzyme reactivation (i.e. P < 0.05 vs. respective 'before incubation' percentage). ■ Control; ▨ (+)-MDMA; ▩ (-)-MDMA.

cubated saline-treated controls (table 1, after incubation). Anaerobic incubation of control enzyme preparations (from saline-treated rats) under identical reducing conditions resulted in a 20-80% enzymatic activation (see table 1).

Figure 1 shows the effect of the anaerobic, reducing incubation on hippocampal TPH activity obtained from animals treated with 5 successive doses (6 h intervals) of either (+)- or (-)-MDMA. Treatment with (+)-MDMA, the more potent of the 2 isomers, reduced TPH activity to 18% of control, whereas treatment with (-)-MDMA reduced TPH activity to 32% of control. Anaerobic incubation in the presence of dithiothreitol and Fe^{2+} failed to reactivate the (+)-MDMA-inactivated enzyme (before and after incubation, respectively, TPH activity was 18 and 22% of respective time-matched control), and only partially restored the (-)-MDMA-induced loss of TPH activity (from 32 to 43% of time-matched control, before and after incubation, respectively). Incubation of control TPH preparations under identical conditions resulted in an approximate doubling of enzymatic activity (see fig. 1, legend).

4. Discussion

Results from the present investigation suggest that amphetamine analogs decrease the activity of brain TPH by inducing oxidation of protein thiol groups. This conclusion is based on the demonstrated ability of dithiothreitol and reduced iron to reactivate acutely drug-inactivated TPH. Both the formation of intra- or inter-protein-S-S-protein crosslinks and/or mixed protein-thiol disulfides, e.g. between TPH and soluble cellular thiols such as glutathione (GSH), could contribute to the loss of TPH activity by critically altering enzyme conformation. The importance of free enzyme sulfhydryl groups in maintaining functional TPH has been previously demonstrated (Kuhn et al., 1980).

Enzymatic reactivation appeared to require a prolonged exposure (20-24 h at $25^{\circ}C$) to dithiothreitol and Fe^{2+} , since no or only partial enzyme restoration occurred within 1-4 h of incubation (data not shown). The reason for such a lengthy incubation period is unknown, but might reflect a relative inaccessibility of the oxidatively altered enzyme site to soluble thiols. Moreover, an anaerobic environment was essential for TPH reactivation to occur, as similar incubations under room air ($25^{\circ}C$) resulted in a complete loss of enzymatic activity (data not shown). The anaerobic atmosphere may act to stabilize TPH during the prolonged incubation (see Kuhn et al., 1980), thus allowing the dithiothreitol-mediated rereduction of sulfhydryl groups to occur.

The activation of control enzyme in the presence of dithiothreitol and Fe^{2+} (table 1) suggests either that a percentage of hippocampal TPH in vivo is maintained in a 'reserve' inactive state, or that active enzyme was partially inactivated prior to assay by manipulation and 'ageing' (see Kuhn et al., 1980).

TPH activity depressed 18 h after repeated drug administration could not be restored (or only slightly; see fig. 1) suggesting a change in the nature of the enzyme inactivation had occurred by this time. Following an identical multiple-dosing regimen of racemic MDMA, rat brain TPH activity remained significantly depressed for up to 110 days, with only partial enzyme recovery occurring

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between 18 h and 110 days after treatment (hippocampal TPH activity recovered from an initial 17% of control 18 h after treatment to 40% of control 110 days after treatment; Stone et al., 1987); this enzyme recovery was hypothesized to reflect regenerative 5-HT axonal sprouting and regrowth into and around MDMA-damaged terminal areas. In contrast, the recovery of TPH activity following a single dose of MDMA (10 mg/kg) was nearly complete by 2 weeks (Stone et al., 1987). These observations, in conjunction with the present demonstration of the change from reversible to irreversible TPH inactivation 3 h after a single dose of MDMA or 18 h after multiple doses, respectively, suggest that the *in vitro* reactivation capacity of central TPH following amphetamine analog administration may reflect the extent of toxic serotonergic damage. Thus, whereas the initial MDMA-induced TPH decline represents a reversible drug effect, the deficit remaining after prolonged drug exposure reflects irreversible enzyme damage, possibly indicative of the loss of 5-HT nerve terminal viability.

Brain TPH acutely inactivated by three different amphetamine analogs could be significantly restored *in vitro* (see table 1). These results suggest that a similar mechanism may underly the TPH inactivation induced by all amphetamine compounds. Since MDMA itself does not directly inhibit TPH (Schmidt and Taylor, 1987), metabolic activation of the parent compound, or some other intermediary process, must precede the induction of amphetamine-induced toxicity *in vivo*. The nature of the enzyme damage induced by these compounds suggests that oxidative stress (for review, see Kappus and Sies, 1981) may play a major role in the induction of serotonergic neurotoxicity. Quinone species formed via oxidative metabolism of either the parent amphetamine compound or of drug-liberated catecholamines represent potential mediators of oxidative stress, by virtue of their ability to undergo one electron oxidation-reduction reactions. Superoxide anion, hydrogen peroxide, and other reactive oxygen species formed via intracellular redox cycling might inactivate TPH directly, or indirectly by elevating cellular GSSG levels (since detoxification of H_2O_2 by GSH peroxidase involves the

oxidation of GSH to GSSG). Elevated levels of cellular GSSG have been associated with increased mixed disulfide formation.

In summary, TPH inactivation occurring within the first several hours of acute amphetamine analog administration was a reversible process associated with the oxidation of key enzyme sulfhydryl groups. In contrast, the TPH deficit measured 18 h after repeated drug exposure was irreversible, and most likely reflects the drug-induced loss of 5-HT nerve terminals.

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