Inhibition by Nitric Oxide of the Uptake of $[^3H]$Serotonin into Rat Brain Synaptosomes

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Received May 26, 1997
Accepted July 2, 1997

ABSTRACT—$[^3H]$Serotonin (5-HT) uptake by synaptosomes of rat brain was dose-dependently inhibited by nitric oxide (NO) donors such as sodium nitroprusside (SNP), 3-(2-hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-propanamine, 3-morpholinosydnonimine and S-nitroso-L-cysteine (NO-CYS). The inhibitory effect was blocked by reduced hemoglobin. The effect was not mimicked by ferrocyanide and ferricyanide. 8-Bromoguanosine 3',5'-cyclic monophosphate (8-bromo cGMP) did not affect $[^3H]$5-HT uptake into rat cortical synaptosomes. The reduced activity of $[^3H]$5-HT uptake into the cortical synaptosomes pretreated with NO-CYS was partially reversed by washing the preparation after the treatment. Kinetic analysis showed that NO-CYS (100 μM) decreased the $V_{\text{max}}$ value without any change in the $K_m$ value. NO-CYS did not affect the specific binding of $[^3H]$paroxetine, a ligand that binds to the 5-HT transporter, in membranes. NO-CYS and SNP, like iodoacetic acid and sodium cyanide, decreased the ATP content in cortical synaptosomes, but the effect on ATP content was not related to that on $[^3H]$5-HT uptake. These findings suggest that NO inhibits reversibly $[^3H]$5-HT uptake into rat brain synaptosomes without affecting the recognition site of the 5-HT transporter in a cGMP-independent manner, and the observed effect is not due to its metabolic effect.

Keywords: Serotonin (5-HT), Uptake, Nitric oxide (NO), S-Nitroso-L-cysteine (NO-CYS), Synaptosome

Nitric oxide (NO) is a diffusible radical gas that may serve as a chemical messenger or cytotoxic agent within the nervous system (1). Previous neurochemical studies indicate that NO is capable of stimulating the release of glutamate or other neurotransmitters (2–8). We (9) also reported using a microdialysis technique that local application of sodium nitroprusside (SNP) and 8-bromoguanosine 3',5'-cyclic monophosphate (8-bromo cGMP) caused an increase in serotonin (5-HT) release from the striatum of freely moving rats. NO-induced neurotransmitter release may be mediated by its stimulatory action on cGMP formation, but the exact mechanism is not known. There is growing evidence that neurotransmitters can be released not only by exocytosis but also through the membrane carriers responsible for transmitter reuptake (10). The $Ca^{2+}$- or $Na^+$-dependence of the NO effect suggests that NO evoked-neurotransmitter release is mediated by two distinct systems, a $Ca^{2+}$-dependent exocytosis and the reverse process of a $Na^+$-dependent carrier-mediated transporter (3, 5, 7, 11, 12). In relation to the latter process, it was reported that NO donors inhibited the uptake of $[^3H]$dopamine (13, 14) and $[^3H]$5-HT (14) into rat striatal synaptosomes. In contrast, Launay et al. (15) and Miller and Hoffman (16) showed that NO or cGMP increased 5-HT uptake in human platelets and rat basophilic leukemia cells, respectively. The effect of NO on 5-HT uptake in the brain has not been studied in detail. In this paper, we examined the effects of NO donors on the uptake of $[^3H]$5-HT and the binding of the 5-HT transporter ligand $[^3H]$paroxetine in rat cortical synaptosomes.

MATERIALS AND METHODS

Animals and preparation of synaptosomes

Thirty male Wistar rats (Shimizu Lab. Supplies Co., Ltd., Kyoto), weighing 100–200 g, were maintained under controlled environmental conditions (22 ± 1 °C; 12–12 hr light-dark cycle, lights on at 08 hr 00 min food and water ad libitum). The rats were sacrificed and their...
Brains dissected out. The cerebral cortex was homogenized in 10 vol. of sucrose buffer (0.32 M sucrose, 2 mM Tris HCl, pH 7.4) with a Teflon glass homogenizer. The homogenate was centrifuged at 1,000 × g for 10 min and the resulting supernatant centrifuged at 17,000 × g for 20 min. The final pellet was resuspended in the original volume of homogenizing buffer and kept on ice. All experiments were performed according to the guiding principles for the care and use of laboratory animals approved by the Japanese Pharmacological Society.

**[3H]5-HT uptake and [3H]paroxetine binding**

[3H]5-HT uptake into the synaptosomes was measured as reported previously (17). A 50-μl aliquot of the crude synaptosome suspension was added to tubes containing 400 μl of incubation buffer (120 mM NaCl, 5 mM KCl, 1.2 mM MgCl₂, 2.5 mM CaCl₂, 10 mM glucose, 100 μM pargyline, 1 mM ascorbic acid and 20 mM Tris HCl, pH 7.4) aerated with 95% O₂ and 5% CO₂. The tubes were preincubated at 37°C for 15 min in the presence or absence of drugs; then 50 μl of [3H]5-HT (939.8 GBq/mmol) in concentrations ranging from 40 to 100 nM was added to each tube, followed by a 3-min incubation. Blank tubes representing nonspecific uptake were incubated at 37°C in the presence of 10 μM fluoxetine. Incubation was terminated by the addition of 5 ml of ice-cold incubation buffer and subsequent filtration through Whatman GF/B filters. The filters were washed twice with 5 ml of ice-cold incubation buffer and dried, and the retained radioactivity was determined by liquid scintillation spectrometry. Net uptake was defined as the difference between the total and the nonspecific uptake. The maximum uptake rate and affinity constant were calculated from Scatchard analysis of saturation binding data. Whatman GF/B filters. The filters were washed 3 times with 5 ml of ice-cold buffer and dried, and the retained radioactivity determined by liquid scintillation spectrometry. The maximum number of binding sites and equilibrium dissociation constant were calculated from Scatchard analysis of saturation binding data.

**Assay of ATP content**

Measurement of intrasynaptosomal ATP levels was carried out as described previously (20). In brief, the synaptosomal suspension was centrifuged at 16,000 × g for 5 min, and the resulting pellet was resuspended in 0.5 M perchloric acid. The suspension was centrifuged at 16,000 × g for 5 min, and the supernatant obtained was neutralized by 3 M KOH/1.5 M triethanolamine. ATP in the extract was measured by the luciferase reaction, and the bioluminescence was detected with a Packard liquid scintillation counter (21).

**Statistics**

Statistical analyses were conducted by one-way ANOVA followed by Fisher’s least significant difference (LSD) test, using statistical data analysis software (SPSS, Chicago, IL, USA). P values of 0.05 or less were considered statistically significant.

**Chemicals**

[3H]5-HT creatinine sulfate and [3H]paroxetine were purchased from NEN (Boston, MA, USA). SNP, iodoacetic acid (IAA) and sodium cyanide (NaCN) were from Wako Chemicals (Osaka). 3-Morpholinosydnonimine (SIN-1), 3-(2-hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-propanamine (NOC-7) and N-ethyl-2-(1-ethyl-2-hydroxy-2-nitrosohydrazino)ethanamine (NOC-12) were from Dojindo Lab. (Kumamoto). S-Nitroso-L-cysteine (NO-CYS) was prepared according to the previously reported procedure (22). Luciferase and luciferin were from Sigma Chemical Co. (St. Louis, MO, USA). Pargyline HCl was from Nacalai Tesque (Kyoto). Fluoxetine was a gift from Eli Lilly (Indianapolis, IN, USA).

**RESULTS**

Various NO donors such as NOC-7, NOC-12, SNP, SIN-1 and NO-CYS caused a dose-dependent inhibition of the uptake of [3H]5-HT into the cortical synaptosomes (Fig. 1). NOC-7 and NO-CYS were more potent than SNP, NOC-12 and SIN-1 in inhibiting the uptake. NOC-12 at 0.1 mM increased rather than inhibited the uptake. K₅Fe(CN)₆ and K₅Fe(CN)₉ at 1 mM were without effect on [3H]5-HT uptake (data not shown). The inhibitory effect of NO-CYS at 1 mM was attenuated by hemoglobin at 100 μM (Fig. 2). A similar antagonism by hemoglobin was observed in the effect of SNP (data not shown). NO-
CYS at 100–300 μM decreased the uptake of [3H]5-HT into the synaptosomes, and the reduced activity was increased by subsequent washing of the synaptosomes (Fig. 3). The effect of washing depended on the NO-CYS concentration used for treatment. In contrast to NO donors, the cGMP agonist 8-bromo cGMP at 1 pM–1 mM did not affect the uptake of [3H]5-HT into the synaptosomes (Fig. 4).

Kinetic analysis showed that NO-CYS at 100 μM decreased the maximal uptake without changing the affinity for 5-HT (Fig. 5). V_{max} values (pmol/mg protein /3 min, means ± S.E.M. of 3 experiments) were 4.40 ± 0.82
control) and 2.63±0.33 (NO-CYS, P<0.001), and 
$K_m$ values (nM, means±S.E.M. of 3 experiments) were 
25.4±9.5 (control) and 24.9±6.2 (NO-CYS). Figure 6 
shows the Scatchard analysis of $[^3H]$paroxetine binding 
in the membranes. NO-CYS at 100 pM did not change 
the binding parameters (means ±S.E.M. of 3 experiments): 
$B_{max}$ values (pmol/mg protein) were 1.85 ± 0.22 (control) 
and 2.07±0.18 (NO-CYS), and $K_d$ values (nM) were 
3.52±0.73 (control) and 4.00±0.66 (NO-CYS).

The effect of SNP and NO-CYS on the synaptosomal 
ATP content was examined (Fig. 7). At 100 pM, SNP, 
IAA and NaCN, but not NO-CYS, decreased the ATP 
content, whereas only the NO donors SNP and NO-CYS 
decreased the uptake of $[^3H]$5-HT into the synaptosomes. 
At 1 mM, all these drugs decreased the ATP content, 
whereas IAA did not affect the uptake of $[^3H]$5-HT.

**DISCUSSION**

Previous studies suggest that NO evoked-neurotransmitter release is mediated by two distinct systems, a 
$Ca^{2+}$-dependent exocytosis and the reverse 5-HT trans 
port mechanism (3, 5, 7, 11, 12). Pogun et al. (14) showed 
that SNP inhibits the uptake of $[^3H]$5-HT into the striatal

![Fig. 5. Lineweaver-Burke plots of $[^3H]$5-HT uptake. The crude synaptosomes were treated with (closed) or without (open) 100 μM NO-CYS for 15 min and incubated with $[^3H]$5-HT at the indicated concentrations for 3 min. Results are means of three experiments.](image)

![Fig. 6. Scatchard plots of $[^3H]$paroxetine binding. The crude synaptosomes were treated with (closed) or without (open) 100 μM NO-CYS for 15 min and incubated with $[^3H]$paroxetine at the different concentrations at 22°C for 60 min. Results are means of three experiments.](image)

![Fig. 7. Effects of NO donors and metabolic inhibitors on $[^3H]$5-HT uptake and the synaptosomal ATP content. A: The crude synaptosomes were treated with the indicated drugs at 100 μM (open) and 1 mM (hatched) for 15 min and incubated to allow $[^3H]$5-HT uptake. B: The crude synaptosomes were treated with the indicated drugs at 100 μM (open) and 1 mM (hatched) for 15 min and centrifuged. ATP was extracted from the resulting pellet and measured. Results are means ±S.E.M. of 3–5 determinations. *P<0.05, **P<0.01, compared with the corresponding value without NO donors and metabolic inhibitors.](image)
Inhibition of 5-HT Uptake by NO

In this paper, we confirmed the inhibitory effect of SNP on the uptake of \(^{3}H\)5-HT in rat cortical synaptosomes and further studied the mechanism for inhibition. NO donors such as NO-CYS, NOC-7, SNP, SIN-1 and NOC-12 inhibited the uptake of \(^{3}H\)5-HT into the synaptosomes. Furthermore, reduced hemoglobin, which binds NO, attenuated the inhibitory effect of NO-CYS, but \(K_{\text{Fe(CN)}_{6}}\) and \(K_{\text{Fe(CN)}_{6}}\), compounds similar to SNP in chemical structure but which do not release NO, were without effect on uptake. These findings indicate that the uptake of \(^{3}H\)5-HT, like \(^{3}H\)dopamine (13, 14), is lowered by NO. The partial protection by hemoglobin may be explained by the high concentration of NO-CYS compared with hemoglobin. Alternatively, NO-CYS at the high concentration is considered to have an NO-unrelated effect on 5-HT uptake, although the details are not known. Kinetic study showed that NO-CYS caused a decrease in \(V_{\text{max}}\) without a significant change in \(K_{m}\) value. In addition, the binding experiment of \(^{3}H\)-paroxetine, a ligand for 5-HT transporter, indicates that NO-CYS does not affect the 5-HT transporter. That is, NO does not interact directly with the paroxetine recognition site. The similar observation was reported in the NO-CYS-induced decrease in \(^{3}H\)dopamine uptake: NO-CYS decreased the capacity of the dopamine transporter without having a significant effect on the affinity, and it had no effect on \(^{3}H\)mazindol binding (13).

The potencies of the NO donors used here were in this order: NO-CYS, NOC-7 > SNP, SIN-1 > NOC-12. The difference in potency among NO donors may be due to that in NO production, since NO liberation is more rapid in NOC-7 than in NOC-12 (23). NO can occur in free radical form (NO\(^{+}\)), as a nitrosoyl cation (NO\(^{+}\)), or as a nitroxy radical (NO\(^{-}\)), and NO\(^{+}\) can react with superoxide to form peroxyxynitrite, a highly toxic compound. Ohkuma et al. (24 - 26) reported that NO donor-evoked release of neurotransmitters might be due to the formation of peroxyxynitrite. In contrast, the present finding that the effects of NO-CYS and NOC-7 are more potent than that of SIN-1, a peroxyxynitrite donor (27), suggests that the inhibition of 5-HT uptake by NO donors may be due to NO rather than peroxyxynitrite, although it remains to be determined if superoxide is involved in the NO effect on 5-HT uptake.

The decreased activity of 5-HT uptake by NO-CYS treatment was partially reversed by washing the synaptosomes. The reversibility suggests that NO may be a physiological regulator of 5-HT uptake. One target molecule of NO is the soluble guanylyl cyclase, and the 5-HT transporter has multiple consensus sites for various protein kinases (16). NO-induced cGMP would activate cGMP-dependent protein kinase which would then phosphorylate either the 5-HT transporter directly or some other closely associated protein. In this relation, the increase in 5-HT transport by cGMP was shown in human platelets (15) and rat basophelic leukemia cells (16). Furthermore, Pogun et al. (14) reported in their preliminary experiments that dibutyryl cGMP inhibited \(^{3}H\)dopamine uptake. However, the present study showed that 8-bromo-cGMP did not affect \(^{3}H\)5-HT uptake. Then, it is unlikely that NO-induced inhibition of 5-HT transport in the brain is due to its action on the guanylate cyclase/cGMP system. This finding also suggests that the reverse 5-HT transport mechanism is not involved in cGMP-induced facilitation of in vivo 5-HT release. The autoribosylation of glyceraldehyde-3-phosphate dehydrogenase, which leads to a loss of its glycolytic enzyme activity, may be an alternative mechanism for the NO-induced decrease in 5-HT uptake (28). However, the present study showed that 5-HT uptake and cellular ATP content were not coordinately altered by metabolic inhibitors and NO donors. Thus, it is unlikely that the NO-induced decrease in 5-HT uptake is due to a general metabolic mechanism of NO. Further studies are required to clarify biochemical interactions responsible for the NO-induced decrease in 5-HT uptake.

In conclusion, the present study shows that NO inhibits 5-HT uptake into the synaptosomes through a cGMP-independent mechanism. This suggests that NO-induced 5-HT release is in part mediated by the reverse 5-HT transport mechanism and supports the hypothesis that NO is involved in neurotransmission of 5-HT neurons.

REFERENCES

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