Bioactive 6-Nitronorepinephrine Identified in Mammalian Brain*

Norepinephrine (NE) (von Euler, U. S. (1972) in Catecholamines (Blaschko, H., and Muscholl, E., eds.) pp. 186–230, Springer-Verlag, Berlin) and nitric oxide (NO) function as neurotransmitters in the nervous system. We have shown that NE levels in the rat hypothalamic paraventricular nucleus (Shintani, F., Kato, T., Kinoshita, N., Kanba, S., Asai, M., and Nakaki, T. (1995) Proceedings of the Satellite Symposium, 4th IBRO World Congress on Neuroscience, Otsu, 1995) diminish in the presence of NO. This observation prompted us to explore the possibility of an interaction between NE and NO or NO-related molecules. In fact, nitration of NE has been shown to occur in vitro (d’Ischia, M., and Costantini, C. (1995) Bioorg. Med. Chem. 3, 923–927). We now report the identification of 6-nitronorepinephrine in the mammalian brain. Amounts of 6-nitronorepinephrine in the rat brain were attenuated by intraperitoneal administration of an inhibitor of nitric oxide synthase, N^0-nitro-O-arginine methyl ester (L-NAME). This was reversed by coadministration of l-arginine, suggesting that nitric oxide synthase participated in the formation of 6-nitronorepinephrine. Moreover, we found that 6-nitronorepinephrine inhibits the activity of catechol O-methyltransferase, as well as NE transport into rat synaptosomes. A rat brain microdialysis experiment showed that perfusion of 6-nitronorepinephrine into the rat paraventricular nucleus significantly elevated NE while decreasing 3-methoxy-4-hydroxyphenylglycol and that l-NAME administered intraperitoneally decreased NE and increased 3-methoxy-4-hydroxyphenylglycol. These observations suggest that 6-nitronorepinephrine generated in nuclei containing both adrenergic and nigral neurons inhibits NE inactivation. We propose that 6-nitronorepinephrine is a potential signal molecule linking the actions of NE and NO.

Norepinephrine (NE) is a catecholamine that functions as a neurotransmitter in the nervous system (1). Nitric oxide (NO), a gaseous radical, is also thought to function as a neurotransmitter (2, 3). We previously found that NE levels detected by microdialysis of the rat hypothalamic paraventricular nucleus (PVN) (4) were diminished by perfusion of a solution containing NO. Other investigators have suggested that NO or NO-related molecules may interact. In fact, nitration of NE at position 6 has been shown to occur in vitro (5, 6). However, there have been no reports to date showing that 6-nitronorepinephrine exists in the mammalian brain.

Therefore, we attempted to measure 6-nitronorepinephrine in the mammalian brain and then asked whether intraperitoneal administration of a nitric oxide synthase inhibitor to rats affects brain levels of this amine. Moreover, we examined the effects of this amine on the uptake of NE into synaptosomes, binding to adrenergic receptors, and the enzyme activities of catechol O-methyltransferase (COMT) and monoamine oxidase in vitro. Finally, we studied the effects of 6-nitronorepinephrine on NE release from PVN neurons in vivo.

**Experimental Procedures**

Identification of 6-Nitronorepinephrine—We purchased 100 porcine brains (150–200 g brain) from Tokyo Shibaura Zoki (Tokyo, Japan). Catechol derivatives were extracted from the brain tissues by a previously described method (7). The samples were injected into a high performance liquid chromatography (HPLC)-electrochemical detection (ECD) system according to a previously described method (8). A peak with a retention time identical to that of the 6-nitronorepinephrine synthesized in vitro was obtained. The fractions were pooled and subjected to the following analyses by UV spectrometry, mass spectrometry (MS), and NMR spectroscopy. Absorbance was measured at a wavelength between 190 and 500 nm using a UV photometer (Waters 990; Waters Japan). The mobile phase was a water solution that consisted of 5.5 mM formate and 6.7 mM ammonium formate (pH 3.7), and a strong cation exchange column (Whatman Japan, Tokyo, Japan) and a mobile phase of 0.09% trifluoroacetic acid (v/v in water) and 0.07% acetonitrile (v/v in water). Mass spectral analysis was carried out on a J MS-SX/SX102A (BBE configuration) tandem mass spectrometer (J EOL, Tokyo, Japan). Positive and negative ion fast atom bombardment MS, employing 3-nitro-benzyl alcohol as the matrix, produced [M + H]+ ions at m/z 215.0669 (calculated for C_{9}H_{11}N_{2}O_{5}, 215.0668) and [M − H]− ions at m/z 213. Collisionsally activated dissociation was performed with argon as the collision gas at a pressure sufficient to reduce the precursor ion signal by 80%. The accelerating voltage was 10 kV and the collision cell potential was set at 5 kV. The 1H NMR spectrum was recorded on a J EOL GX-400 NMR spectrometer (J EOL), using tetramethylsilane as an internal reference.

Groups of four male Sprague-Dawley rats (Sankyo Laboratory, Tokyo, Japan) were given both 200 mg/kg of either N^0-nitro-arginine methyl ester (l-NAME) or N^0-nitro-arginine methyl ester (d-NAME) and 800 mg/kg of l- or d-arginine hydrochloride intraperitoneally at 60 min before decapitation under ether anesthesia. The drugs were dissolved in saline, and the solution was adjusted to pH 7.4 before use. The brain contents of 6-nitronorepinephrine were then extracted by the method described above. The peak areas of samples were compared with those of the standard 6-nitronorepinephrine determined for quan-
tification. All experiments conformed to the standards put forth in the Handbook for the Use of Animals in Neuroscience Research.

NE Uptake—Uptake of L-[3H]NE into synaptosomes was measured by a previously described method (9). In brief, male Sprague-Dawley rats were decapitated, followed by immediate dissection of the brain to remove the frontal cortex (~600 mg) according to the method of Glowinski and Iversen (10). The tissue was homogenized in 20 volumes of ice-cold 0.3M sucrose containing 11 mM glucose, pH 7.4, at 37°C (buffer 1) in a glass homogenizer with a Teflon pestle. The homogenate was centrifuged for 10 min at 1000 × g in a refrigerated centrifuge to obtain a crude nuclear fraction. The pellet was discarded and the supernatant was centrifuged for 20 min at 20,000 × g. The supernatant was discarded and the crude synaptosomal pellet was resuspended in 20 volumes of buffer 1. Preparation of the crude synaptosomal suspension was performed at 4°C. The transport of 8 nM L-[3H]NE (DuPont NEN) into cortical synaptosomes was measured in the presence of various concentrations of 6-nitronorepinephrine and related compounds to determine the rank order of potency for the blockade of monoamine uptake. Aliquots of the synaptosomal suspension (50 μl) were added to tubes containing 50 μl of the test drug and 350 μl of a modified Krebs-Ringer phosphate buffer (122 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl2, 1.2 mM MgSO4, and 15.8 mM Na2HPO4) containing 2.0 mM glucose, 0.2 mg/ml ascorbic acid, and 12.5 μM nialamide (buffer 2). After a 5-min preincubation at 37°C in a shaking water bath (80 oscillations/min), the uptake was initiated by the addition of 50 μl of the radiolabeled monoamine. This transport was stopped after 5 min by adding 4.5 ml of ice-cold 0.9% NaCl (buffer 3) and immediately filtering through Whatman GF/B glass fiber filters under a vacuum. The tubes were rinsed three times with 5 ml of buffer 3, and the rinses were then applied to the filters. Filters were removed and placed in plastic scintillation vials containing 4 ml of scintillator, and the radioactivity was measured after a minimum of 8 h by liquid scintillation spectrometry. Nonspecific transport was determined to be the amount of uptake in the presence of 10 μM imipramine for NE transport. Data are presented as the mean ± S.E. of at least three independent experiments.

Microdialysis—Surgery and the microdialysis experiments were performed according to the previously described methods (11). Male Sprague-Dawley rats (7–8 weeks of age) were used. A microdialysis probe was inserted into the left PVN (1.5 mm posterior to the bregma, 0.6 mm lateral to the midline, and 7.0 mm below the skull surface, according to the atlas of Paxinos and Watson (12)). Under nonanesthetized and freely moving conditions, Hanks’ solution (137 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl2, 0.8 mM MgSO4, 0.338 mM Na2HPO4, 0.4 mM KH2PO4, 5.6 mM glucose, 4.2 mM NaHCO3, pH 7.4) was perfused into the probe at a flow rate of 1.5 μl/min for 3 h to allow equilibration. The dialysate was collected at 20-min intervals into microtubes (Eicom) containing 70 μl of acetic acid/water solution (0.02 M). The microtubes were maintained at 4°C to prevent oxidation of catechol compounds.

To examine the effects of 6-nitronorepinephrine, Hanks’ solution containing 100 μM 6-nitronorepinephrine was perfused into the microdialysis probe without stopping the aforementioned sampling; perfusion was continued for up to 60 min. To examine the effects of a nitric oxide synthase inhibitor on NE and 3-methoxy-4-hydroxyphenylglycol (MHPG) levels in PVN dialysates, 200 mg/kg L-NAME combined with
L-NE hydrochloride (Sigma), 0.14 ml of several concentrations of 6-nitronorepinephrine was injected into the HPLC system setup as described above. Peak areas of samples were compared with those of the standard 6-nitronorepinephrine determined for quantification. Ordinate, amounts of the amine in 1 g of brain tissue. Vertical bars, S.E. of four independent experiments.* significantly different (p < 0.05) from other columns.

800 mg/kg d-arginine hydrochloride or the same amount of L-NAME combined with equivalent doses of L-arginine hydrochloride were administered intraperitoneally. Each dialysis sample was immediately injected into an HPLC system to allow measurement of the levels of NE and MHPG by a previously described method (8). All drugs were freshly prepared and adjusted to pH 7.4 on the day of use. The rats were given free access to food and water throughout the microdialysis procedure.

HPLC Analysis—The HPLC-ECD system was set up based on a previously described method (8) with minor modifications. In brief, the chromatographic system consisted of a dual plunger pump (EP-10, Eicom), an electrochemical detector with a carbon-graphite electrode (Coulochem II, ESA), an autosampling injector (231–401, Gilson Medical Electronics Inc.) with a 100-μl loop, and an ODS column. We used a CA-SODS column (150 × 4.6 mm, inner diameter; Eicom); the mobile phase consisted of 95% 0.1 M phosphate buffer solution (0.0862 M Na₂HPO₄·2H₂O and 0.0138 M NaH₂PO₄·2H₂O; pH 6.0), 2% methanol, 200 mg/liter sodium 1-octanolsulfonate, and 50 mg/liter EDTA-2Na. The buffer was sonicated to degas before use. Separation was achieved at 25°C using a flow rate of 1 ml/min. An electrochemical detector with a carbon-graphite working electrode was set at 400 mV for the guard cell, at 50 mV for the oxidation electrode, and at -200 to -300 mV for the reduction electrode. Data were recorded on an integrator (Chromatograph 12, J ASCO, Tokyo, Japan), and peak heights/area of dialysis samples were compared with those of standards determined each day for quantitation. The detection limit was 0.5 pg/100 μl.

Binding Assays—Several concentrations of 6-nitronorepinephrine were incubated in a receptor binding preparation containing [¹H]prazosin (1.10 TBq/mmol, DuPont NEN) (13), [¹H]yohimbine (2.59 TBq/mmol, DuPont NEN) (13), or [¹H]CGP12177 (1.11 TBq/mmol, DuPont NEN) (13), the α₁, α₂, and nonselective β-adrenergic receptor ligands, respectively.

Enzyme Assay—COMT activity was determined by measuring the amount of normetanephrine formed from NE (15). The incubation mixture consisted of the following components in a total volume of 0.5 ml: 0.1 ml of 0.5 M sodium phosphate buffer, pH 7.8, 0.05 ml of 100 mM MgCl₂, 0.1 ml of 10 mM S-adenosylmethionine (Sigma), 0.01 ml of 15 mM L-NE hydrochloride (Sigma), 0.14 ml of several concentrations of 6-nitronorepinephrine, and 20 units of COMT (Sigma) dissolved in 0.1 ml of the buffer containing 10 mM dithiothreitol. The mixture was incubated for 60 min. The reaction was stopped by the addition of 50 μl of 4 M perchloric acid. Protein was removed by centrifugation. The supernatant was injected into the HPLC system set up as described above. Peak areas were compared with those of the standard normetanephrine (Sigma) determined for quantification. Monoamine oxidase activity was determined with serotonin or L-NE serving as a substrate based on a previously described method (16) with minor modifications. Levels of monoamines were determined by using the HPLC system.

The 6-nitronorepinephrine used in these experiments was synthesized in vitro from L-NE and NO. After 10 mg of L-NE hydrochloride had been dissolved in 100 ml of distilled water, 200-300 ml of NO gas were bubbled in a gas-tight glass tube at 37°C at atmospheric pressure for 2 min. The reaction products were separated and purified with the HPLC system described above. The structure was confirmed using both MS and NMR spectroscopy (data not shown). The purity of 6-nitronorepinephrine exceeded 95%.

RESULTS AND DISCUSSION

Catechol-containing molecules were extracted from porcine brain tissue and separated by HPLC with ECD (Fig. 1A). The presence of 6-nitronorepinephrine was then detected in porcine brain using a UV detection method (Fig. 1B), MS (Fig. 1C), and NMR spectroscopy (Fig. 1D). The concentration of this catecholamine in pig brain was 75 ± 7 pg/g. The average rat brain concentration was 78 ± 8 pg/g (data not shown). The 6-nitronorepinephrine content of the whole brain constitutes about 0.01% of total NE.

Next, we tested whether nitric oxide synthase is involved in the formation of 6-nitronorepinephrine in the brain. 6-Nitronorepinephrine levels, as measured by HPLC-ECD in rat brains minus the olfactory bulbs, fell following intraperitoneal injection of L-NAME (200 mg/kg), but not D-NAME (Fig. 2). This decrease was reversed by coadministration of 800 mg/kg of L-arginine hydrochloride, but not d-isomer drug (Fig. 2). These results suggest that nitric oxide synthase is involved in the formation of 6-nitronorepinephrine. Ischiropoulos et al. (18)
showed that peroxynitrite and Cu$^{2+}$ mediate nitration of the electrophilic center of the phenyl moiety of tyrosine. Although the nitration of NE may be mediated through peroxynitrite, the mechanisms governing nitration of 6-nitronorepinephrine in vivo remain to be determined.

We then examined whether 6-nitronorepinephrine exerts biological effects. Subsequent to identifying 6-nitronorepinephrine in the brain, we became aware of reports describing the in vitro synthesis of 6-nitronorepinephrine (5, 6). The authors of these reports did not, however, explore any potential biological actions of this compound. We found that at up to 100 $\mu$M this catecholamine did not displace the binding of [3H]prazosin, [3H]yohimbine, or [3H]CGP12177, the $\alpha_1$, $\alpha_2$, and nonselective $\beta$-adrenergic receptor ligands, respectively (data not shown). Likewise, the activity of monoamine oxidase was unaffected (data not shown). However, transport of 8 nM [3H]NE into rat brain synaptosomes prepared from frontal and occipital cortices was inhibited by 6-nitronorepinephrine at concentrations higher than 1 $\mu$M (Fig. 3A). The concentration of 6-nitronorepinephrine at 50% maximal transport (IC$_{50}$) was 31 $\mu$M, approximately 6 times less potent than the antidepressant bupropion (IC$_{50}$, 5 $\mu$M (9)). 6-Nitronorepinephrine inhibited the activity of COMT with an IC$_{50}$ of 7.5 $\mu$M (Fig. 3B), suggesting that this catecholamine may act as a COMT inhibitor. 3,4-Dihydroxy-5-nitrobenzene derivatives, such as entacapone, have been synthesized as selective COMT inhibitors (19). 6-Nitronorepinephrine is, however, much less potent than entacapone (IC$_{50}$, 10 nM (20, 21)). Since substitutions at position 1 of the phenyl moiety determine the potency of COMT inhibitors (20), it is possible that 6-nitrocatechols with side chains similar to those of entacapone would be more potent COMT inhibitors than 6-nitronorepinephrine.

To examine the effects of 6-nitronorepinephrine on central noradrenergic neurotransmission in vivo, we conducted a microdialysis experiment to determine the contents of NE and MHPG in the rat PVN, where both nitric oxide synthase-containing neurons and noradrenergic neurons exist (3). Significant elevations of NE and decreases in MHPG were observed during the perfusion of 6-nitronorepinephrine, and both were dose-dependent (Fig. 4). The inhibition by 6-nitronorepinephrine of both COMT activities and the reuptake of NE into presynaptic neurons may account for the above results.

In addition, intraperitoneal administration of L-NAME to rats resulted in decreased NE as well as increased MHPG in PVN dialysates (Fig. 5). These changes were reversed by coadministration of L-arginine (Fig. 5). Two explanations are possible for the results of L-NAME. (a) NO' by itself facilitates neurotransmitter release (22–24) and NO' inhibits catecholamine reuptake to synaptosomes (25); and (b) the inhibition of nitric oxide synthase activity might diminish the amounts of endogenous 6-nitronorepinephrine in the PVN, which inhibited NE reuptake and COMT activities in vivo.

Nitric oxide synthase-containing and NE-containing neurons coexist in both the locus coeruleus (26) and the PVN (3), but the two are apparently never found in a single neuron (26). This suggests that 6-nitronorepinephrine may be synthesized via an interaction between NE and NO' released from different neurons. Therefore, it is still an open question whether endogenous...
concentrations of 6-nitronorepinephrine inhibits catecholamine reuptake and COMT activities.

Our results suggest that NO\(^{z}\) reacts with NE in vivo, and that the resultant 6-nitronorepinephrine has distinct biological activities that modulate synaptic transmissions.

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