REVIEW

The Actions of NO in the Central Nervous System and in Thymocytes[†]

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ABSTRACT—Nitrogen monoxide (NO) has been suggested to be involved in many physiological and pathological functions. In rat hippocampus, chemical NO donors stimulated noradrenaline release in the presence of thiols such as dithiothreitol and L-cysteine. *S*-Nitrosocysteine, which is proposed to be a stable and endogenous *S*-nitrosothiol molecule, stimulated noradrenaline release by itself. The effect of *S*-nitrosothiol on noradrenaline release was calmodulin-dependent and cyclic GMP-independent. *S*-Nitrosocysteine was incorporated into the slice via the L-type-like amino acid transporter. These findings suggest the physiological significance of *S*-nitrosocysteine on neurotransmitter release and propose the existence of a specific uptake system of *S*-nitrosothiols in neuronal tissues. In rat thymocytes, chemical NO donors inhibited DNA synthesis. Hydrocortisone treatment in vivo inhibited DNA synthesis via the expression of the inducible NO synthase protein, and the accumulation of NO and cyclic GMP. Although it is known that glucocorticoids regulate inducible NO synthase expression negatively in several types of cells in vitro, glucocorticoid treatment in vivo regulates the expression positively. In primary cultured rat glial cells, a combination of cytokines stimulated production of nitrite via expression of inducible NO synthase. In these cells, simultaneous addition of endothelin decreased inducible NO synthase expression induced by cytokines. On the other hand, pretreatment with endothelin for 24 hr enhanced the inducible NO synthase expression. Endothelin has two effects on inducible NO synthase expression, positive and negative, depending on treatment time. The actions of NO on the hippocampus and thymocytes and the regulation of inducible NO synthase expression in glial cells are discussed.

Keywords: *S*-Nitrosocysteine, Amino acid transporter, Hippocampus, Thymocyte, Glial cell

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[†]In many cases, the term NO is used for nitric oxide (NO radical, [•]NO). In this paper, however, NO means all types of nitrogen oxides including nitric oxide and other metabolites, because the active species of NO seem to be different in various cells and tissues and under different conditions.

Abbreviations used are (in alphabetical order): BCH, 2-aminobicyclo[2.2.1]heptane-2-carboxylate; CaM, calmodulin; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; cNOS, constitutive NO synthase; Con A, concanavalin A; DTT, dithiothreitol; GC, glucocorticoids; GSH, glutathione; GSSG, glutathione disulfide; HC, hydrocortisone; IL-1 β , interleukin-1 β ; iNOS, inducible NO synthase; LPS, lipopolysaccharide; NA, noradrenaline; NOC-18, 1-hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazene; SIN-1, 3-morpholinonydonimine HCl; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNC, *S*-nitrosocysteine; SNP, sodium nitroprusside; TNF- α , tumor necrosis factor- α ; W-7, N-(6-aminoheptyl)-5-chloro-1-naphthalenesulfonamide.

I. Introduction: NO as neurotransmitter and immunomodulator

NO is known to be a diffusible messenger that mediates cell-cell interactions in a variety of tissues. NO has been suggested to be involved in many physiological and pathological functions including smooth muscle relaxation, platelet inhibition, neurotransmission, immune regulation, enzyme regulation, cytotoxicity and brain damage (for reviews, see Refs. 1 and 2). NO includes many species with distinctive properties and reactivities: NO^+ (nitrosonium cation), nitric oxide (NO radical, $\cdot\text{NO}$), and NO^- (nitroxy anion). These NO species react rapidly with O_2 or H_2O_2 in cells and tissues, yielding higher oxides of nitrogen such as nitrogen dioxide free radicals ($\cdot\text{NO}_2$) and peroxynitrite (ONOO^-). The form in which NO is active in cells has been much debated. In this review, we summarize our recent reports (3–9) and propose that *S*-nitrosocysteine (SNC) is one of the physiological NO species to stimulate noradrenaline (NA) release in rat hippocampus and that SNC is incorporated into neurons via endogenous L-type-like amino acid transporter. In glial cells of rat brain, inducible NO synthase (iNOS) expression was stimulated by cytokines, and the iNOS expression was regulated by the neuropeptide endothelin. In rat thymocytes, iNOS expression was modulated by *in vivo* glucocorticoid treatment, and nitric oxide (NO radical)-cyclic GMP induced inhibition of DNA synthesis.

II. NA release from rat hippocampus by NO compounds and thiols

II-A. NA release induced by SNP and DTT (3)

The roles of NO as a neurotransmitter and/or neuro-modulator in the nervous system have been demonstrated in several types of cells. NO modulates secretion of growth hormone (10) and luteinizing hormone (11) in the rat pituitary gland. In the hypothalamus, NO plays a role in the release of luteinizing hormone-releasing hormone (12) and corticotropin-releasing factor (13). NO also stimulates glutamate release from hippocampal synaptosomes in a Ca^{2+} -independent manner (14). We examined the role of NO using NO donors on [^3H]NA release from prelabeled rat hippocampal slices.

Sodium nitroprusside (SNP) stimulated [^3H]NA release from prelabeled rat hippocampal slices in the presence of 5 mM dithiothreitol (DTT) (Fig. 1A). The effect of SNP was dose-dependent, not observed in the absence of DTT, and had an ED_{50} value of 0.55 ± 0.23 mM ($n=4$). Figure 1B shows the dose-response curves of DTT with or without 1 mM SNP. DTT had no effect on [^3H]NA release by itself. Although [^3H]NA release stimulated by 20 mM KCl was dependent on extracellular CaCl_2 , the effect of SNP/DTT in the absence of CaCl_2 was the same as that in the presence of 1 mM CaCl_2 (Table 1). The addition of desipramine, an inhibitor of NA transporter, from 5 μM to 20 μM , did not inhibit the effect of SNP/DTT (data not shown). Thus, the

Fig. 1. Effects of SNP and DTT on [^3H]NA release from rat hippocampal slices. Increasing concentrations of SNP with (▲) or without (○) 5 mM DTT (Panel A) or increasing concentrations of DTT with (□) or without (●) 1 mM SNP (Panel B) in Tyrode's buffer were kept at 4°C for 1 h in assay tubes. The labeled hippocampal slices were added and incubated for 8 min at 37°C. Data are means $\pm$ S.D. of three determinations in a typical experiment and are representative of three similar experiments.

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Table 1. SNP stimulated [^3H]NA release in a Ca^{2+} -independent manner

Additions	[^3H]NA release (% of total)	
	0.2 mM EGTA	1 mM CaCl_2 and 0.2 mM EGTA
None	4.0 ± 1.6	6.5 ± 1.0
20 mM KCl	6.3 ± 1.4	37.3 ± 0.5
1 mM SNP and 5 mM DTT	32.6 ± 3.4	34.6 ± 3.8

KCl or SNP and DTT in Tyrode's buffer were kept at 4°C for 1 h in assay tubes. Then the labeled slices were added and incubated in the presence or absence of 1 mM CaCl_2 . 0.2 mM EGTA was further added to the assay mixture. Data are means $\pm$ S.D. of three determinations in a typical experiment and are representative of three independent experiments. (Reprinted with permission from Ref. 3)

release by SNP/DTT did not seem to be derived from the activation of the reversal transport system under our conditions.

Table 2 illustrates the effects of various NO donors on [^3H]NA release from rat hippocampal slices in the presence or absence of 5 mM DTT. 3-Morpholinysydnonimine HCl (SIN-1), which is known to yield peroxynitrite, had no effect on [^3H]NA release. NOC compounds are believed to be specific releasers of nitric oxide in acidic and neutral buffer such as phosphate buffer (pH 7.4) without co-factors (15). Neither 1-hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazene (NOC-18), NaNO_2 or NaNO_3 was effective in the presence or absence of DTT.

Nitric oxide and nitrovasodilators cause vasorelaxation by activating guanylate cyclase and increasing cyclic GMP levels in vascular smooth muscle. SNP stimulated cyclic GMP content in rat hippocampal slices (Table 3), with the maximal level at 1 min after addition, which was sustained for at least 10 min in the presence of inhibitors of

Table 2. Effects of NO compounds on [^3H]NA release from rat hippocampal slices

Additions	[^3H]NA release (% of total)	
	None	5 mM DTT
None	5.4 ± 0.1	6.4 ± 0.1
1 mM SNP	6.0 ± 0.2	39.2 ± 2.5
1 mM SIN-1	8.6 ± 1.0	9.4 ± 1.1
3 mM NaNO_2	6.6 ± 0.5	6.0 ± 0.4
3 mM NaNO_3	5.7 ± 0.1	6.3 ± 0.6
3 mM NOC-18	5.5 ± 0.2	5.8 ± 0.3

The labeled slices were incubated with various stimulants in the presence or absence of 5 mM DTT. Data are mean $\pm$ S.D. of three determinations in a typical experiment and are representative of three independent experiments.

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Table 3. Effects of NO compounds on cyclic GMP content in rat hippocampal slices

Additions	Cyclic GMP content (pmol/mg protein)	
	None	5 mM DTT
None	5.6 ± 0.6	4.9 ± 0.6
20 mM KCl	4.1 ± 1.5	5.2 ± 0.3
1 mM SNP	19.3 ± 1.3	9.4 ± 0.8
1 mM NaNO_2	10.4 ± 0.7	10.6 ± 1.0
1 mM NOC-18	12.8 ± 1.5	14.2 ± 2.7

The hippocampal slices were incubated from 1 min with or without 5 mM DTT. Data are means $\pm$ S.D. of three determinations in a typical experiment and are representative of three independent experiments. (Reprinted with permission from Ref. 3)

phosphodiesterase. The addition of 1 mM NaNO_2 or 1 mM NOC-18 also stimulated cyclic GMP content in the slices. The addition of 20 mM KCl had no effect on cyclic GMP content despite being a potent stimulator of NA release. Hemoglobin is known to bind and inactivate nitric oxide and to block the nitric oxide-induced increase in prostaglandin release from the hypothalamus (12). In this experiment, we used oxyhemoglobin. The addition of oxyhemoglobin inhibited cyclic GMP increases by SNP, NaNO_2 and NOC-18 almost completely. 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) reacts with nitric oxide in a stoichiometric manner in neutral solution. The addition of 1 mM carboxy-PTIO inhibited increases in cyclic GMP content by SNP, NaNO_2 and NOC-18. These findings suggest that the species of NO involved in the increase of cyclic GMP may be nitric oxide.

Table 4. Effects of nitric oxide scavengers on SNP-stimulated [^3H]NA release from rat hippocampal slices

Additions	[^3H]NA release (%)	
	None	1 mM SNP and 5 mM DTT
None	100%	100%
Oxyhemoglobin	95 ± 5	63.1 ± 9.1^a
Carboxy-PTIO	94 ± 4	65.0 ± 0.6^a

The labeled slices were incubated with indicated agents and 5 mM DTT. The concentrations of NO antagonists were $500 \mu\text{g/ml}$ of oxyhemoglobin and 1 mM carboxy-PTIO. Data are normalized as percentages of non-stimulated release or net increase by SNP and DTT. The absolute [^3H]NA release (% of total incorporated NA) were 10.5 ± 2.1 , and 38.5 ± 4.6 (mean $\pm$ S.E.M. of three independent experiments), in non-stimulated and SNP-stimulated slices, respectively. Data are means $\pm$ S.E.M. of three independent experiments. Data were analyzed by Student's *t*-test. $P < 0.01$ was considered to be significant. $^aP < 0.01$ vs without NO antagonist.

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The effects of oxyhemoglobin and carboxy-PTIO on [3 H]NA release by SNP in the presence of DTT were examined (Table 4). The effect of SNP was significantly inhibited by the addition of oxyhemoglobin or carboxy-PTIO, although the maximal inhibition did not exceed 40% even in the presence of 1 mg/ml of oxyhemoglobin and 1 mM carboxy-PTIO. The inhibitory effect of scavengers of nitric oxide on NA release does not seem to be due to the inhibition of increases in cyclic GMP content, because SNP-stimulated NA release in the presence of DTT seemed to be independent of cyclic GMP as described above.

II-B. NA release by SNAP and L-cysteine in vitro and in vivo (4)

The addition of another NO donor, *S*-nitroso-*N*-acetylpenicillamine (SNAP), stimulated [3 H]NA release in the presence of 5 mM DTT, although SNAP had no effect by itself. These findings showed the role of DTT as a reducing agent or a thiol compound. Next, we investigated other reducing agents. Figure 2 shows the effect of L-cysteine on [3 H]NA release. The simultaneous addition of L-cysteine with 1 mM D-SNAP stimulated [3 H]NA release, although L-cysteine had no effect by itself. Addition of 1 mM SNP also stimulated [3 H]NA release to the

Fig. 2. NO donor-stimulated [3 H]NA release from rat hippocampal slices in the presence of L-cysteine. The labeled hippocampal slices were incubated with 5 mM D-SNAP (■), 5 mM SNP (▲), or vehicle (○) in the presence of increasing concentrations of L-cysteine. Data are means $\pm$ S.D. of three determinations in a typical experiment and are representative of 3 similar experiments. (Reprinted with permission from Ref. 4, Copyright 1996 by Wiley-Liss, Inc.)

equivalent of about 50% of the effect of D-SNAP in the presence of 5 mM L-cysteine. The effect of L-cysteine was much higher than that of the D-isomer, although SNAP did not show stereospecificity.

SNAP stimulated endogenous NA release in the presence of L-cysteine from rat hippocampus in vivo. With the addition of 1 μ M desipramine to the perfusion fluid, low levels of NA were detectable in all animals (82.2 ± 4.8 fmol/30 min; range from 45.0 to 139.5 fmol/30 min, $n=12$). This value was similar to that reported recently by Kuczenski et al. (16). Addition of 1 mM D-SNAP or 0.5 mM L-cysteine had no effect on NA release over a period of 3 hr (Fig. 3). However, co-addition of D-SNAP and L-cysteine stimulated NA release 30 min immediately following injection, and the NA level peaked to about twofold at 60 min. The levels were $147 \pm 16.0\%$ at 30 min and $209.7 \pm 28.6\%$ at 60 min ($n=6$). The levels were $141.6 \pm 21.8\%$ at 90 min after injection, which were not significantly compared with the non-stimulated or pre-incubation levels. These findings showed that co-addition of D-SNAP and L-cysteine stimulates NA release from the hippocampus not only in vitro but also in vivo, and the necessity of thiol agent for effects of SNP and SNAP to stimulate NA release from the hippocampus.

The effect of glutathione (GSH), another physiological thiol agent, was examined. Addition of 0.5 mM GSH,

Fig. 3. SNAP-stimulated endogenous NA from rat hippocampus. One mM D-SNAP (Δ), 0.5 mM L-cysteine ($\square$), and both drugs ($\bullet$) were dissolved in modified Ringer's solution and applied for 180 min. Data are means $\pm$ S.E.M. expressed as percentages of basal release (perfusion with Ringer's solution). Basal NA release was 82.2 ± 4.8 fmol/30 min ($n=9$). Asterisks indicate significant effects compared to the mean of basal release before each stimulation (paired t -test, $P < 0.01$).

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which had no effect by itself, did not stimulate [3 H]NA release from the slices in the presence of 1 mM D-SNAP. Additionally, D-SNAP/L-cysteine-stimulated NA release was not modified by GSH. The reduced form of GSH, glutathione disulfide (GSSG), showed no effect in the presence or absence of SNAP. Addition of 1 mM S-nitroso-GSH had no effect on NA release in the presence or absence of 0.5 mM L-cysteine in our experimental conditions.

Ca^{2+} is a critical signal for neurotransmitter release and a ubiquitous secretagogue. To verify the dependency on extracellular Ca^{2+} , SNAP/L-cysteine-induced release was assayed in the presence or absence of extracellular CaCl_2 . [3 H]NA release stimulated by 20 mM KCl was dependent on extracellular CaCl_2 . The effect of SNAP/L-cysteine in the absence of CaCl_2 was the same as that in the presence of 2 mM CaCl_2 . A stimulatory effect of SNAP/L-cysteine was observed in slices pretreated with 50 μM BAPTA-AM, a cell-permeable chelator of Ca^{2+} , in the absence of CaCl_2 . Table 5 shows the effects of calmodulin (CaM) antagonists on SNAP/L-cysteine-stimulated [3 H]NA release. Treatment of slices with *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) for 10 min inhibited the effect of SNAP/L-cysteine in a dose-dependent manner. Treatment with *N*-(6-aminohexyl)-1-naphthalenesulfonamide (W-5), which was considered as a control for W-7 because W-5 had many similar effects to W-7 without inhibition of CaM, did not inhibit the SNAP/L-cysteine-stimulated [3 H]NA release. Another CaM antagonist, trifluoperazine, also inhibited the effect of SNAP/L-cys-

teine. Therefore, we concluded that SNAP/L-cysteine was able to induce NA release from hippocampal slices by a mechanism independent of a rise in intracellular Ca^{2+} , and it stimulated the release in a CaM-dependent manner.

III. SNC as a physiological NO molecule

III-A. NA release by SNC (5)

As described above, NO donors such as SNP and SNAP stimulated NA release from the rat hippocampus in vitro and in vivo in the presence of thiol compounds such as DTT and L-cysteine. NO donors had no effect on NA release by themselves without thiol compounds, although both of them stimulated cyclic GMP accumulation alone via nitric oxide (NO radical) in rat hippocampal slices. These results suggest that nitrosothiol compounds, not nitric oxide, are the active species that stimulate NA release. Several reports have showed that SNC acts as a NO donor in various tissues (17–20). Although there is not direct evidence showing that SNC is an endogenous molecule, Myers et al. (21) showed that endothelium-derived relaxing factor is much more likely to be SNC than nitric oxide. Recently, Gow et al. (22) reported that under physiological conditions, nitric oxide reacts directly with thiols to form a S-nitrosothiol such as SNC. Next, we investigated the effects of SNC.

The addition of SNC stimulated [3 H]NA release from the prelabeled slices in a dose-dependent manner (Fig. 4). The effect of 0.5 mM SNC on [3 H]NA release in the ab-

Table 5. Inhibition of D-SNAP/L-cysteine-stimulated [3 H]NA release by CaM antagonists

Addition	[3 H]NA release (% of total)		
	None	1 mM D-SNAP and 0.5 mM L-cysteine	20 mM KCl
Experiment I			
None	6.3 $\pm$ 1.5	36.6 $\pm$ 5.0	48.6 $\pm$ 2.6
W-7 (5 μM)	5.8 $\pm$ 1.0	23.2 $\pm$ 3.4	46.7 $\pm$ 0.5
(10 μM)	4.1 $\pm$ 1.3	13.0 $\pm$ 2.1	33.0 $\pm$ 2.6
(20 μM)	6.0 $\pm$ 0.9	11.4 $\pm$ 1.2	29.9 $\pm$ 3.6
Experiment II			
None	10.6 $\pm$ 2.2	30.9 $\pm$ 3.0	30.7 $\pm$ 1.2
5 μM trifluoperazine	9.7 $\pm$ 0.6	18.8 $\pm$ 1.3	22.1 $\pm$ 3.2
20 μM W-5	11.4 $\pm$ 1.6	26.5 $\pm$ 2.8	31.9 $\pm$ 0.3

The labeled slices were preincubated with indicated concentrations of CaM antagonists for 10 min. The washed slices were incubated with vehicle, 1 mM D-SNAP and 0.5 mM L-cysteine, or 20 mM KCl in the presence of the same concentration of CaM antagonists. Data are mean $\pm$ S.D. of three determinations in a typical experiment and are representative of 3–4 independent experiments. (Reprinted with permission from Ref. 4, Copyright 1996 by Wiley-Liss, Inc.)

Fig. 4. SNC-stimulated [3 H]NA release from rat hippocampal slices in the presence and absence of NaCl. The labeled hippocampal slices were incubated for 8 min at 37°C with the indicated concentrations of SNC in Na^+ -containing (○) or Na^+ -free, choline-containing (●) Tyrode's buffer. Data are the means $\pm$ SE of three independent experiments. (Reprinted with permission from Ref. 5)

sence of extracellular CaCl_2 was the same as that in the presence of 1 mM CaCl_2 . Next, we studied the effect of SNC on endogenous NA release from the rat hippocampus by in vivo microdialysis. Addition of 0.5 mM SNC to the perfusate stimulated NA release for 30 min immediately following the injection, and the NA level peaked at $465 \pm 33\%$ ($n=6$). The effect of SNC declined after 60 min, but the NA level was $211 \pm 30\%$ ($n=6$), which was still significantly elevated as shown by two-way ANOVA.

III-B. Involvement of L-type-like amino acid transporters in uptake of SNC (5)

As described above, SNAP/DTT and SNAP/L-cysteine stimulated NA release. One of the putative products is the *S*-nitrosothiols such as *S*-nitroso-DTT and SNC. Furthermore, SNC stimulated NA release by itself without co-factors. The *S*-nitrosothiols such as SNC seemed to be bioactive NO molecules in the hippocampus. In a series of experiments, we found that the addition of other amino acids to the assay mixture inhibited SNAP/L-cysteine-stimulated [^3H]NA release. The addition of L-leucine inhibited the SNAP/L-cysteine-stimulated [^3H]NA release and shifted the dose-response curve of L-cysteine to the right. The inhibitory effect of L-leucine was dose-dependent and was not complete at 3 mM in the normal Na^+ -containing buffer. These findings suggest the possibility that L-leucine inhibited the uptake of the putative *S*-nitrosothiol compound produced by SNAP and L-cysteine. Next, we investigated the effect of SNC on NA release, focusing on the transport system of SNC.

The uptake of amino acid across mammalian cytoplasmic membranes has been categorized in several ways, e.g., according to substrate specificity and Na^+ dependency. First, we investigated the effect of extracellular Na^+ on SNC-stimulated NA release from prelabeled hippocampal slices. Addition of SNC stimulated NA release in a Na^+ -free, 0.14 M choline chloride-containing buffer in a dose-dependent manner, although the maximal response was lower, and the dose-response curve was shifted slightly to the right in the Na^+ -free buffer, compared with the response in the Na^+ -containing buffer.

Next, we investigated the effects of various amino acids on 0.5 mM SNC-stimulated [^3H]NA release in Na^+ -containing and Na^+ -free, choline-containing buffers (Table 6). Addition of 3 mM L-leucine inhibited SNC-stimulated NA release by 30% in the Na^+ -containing buffer, as L-leucine inhibited the effect of SNAP/L-cysteine. Addition of L-methionine significantly inhibited the effect of SNC, and L-phenylalanine and L-tyrosine had an inhibitory effect on SNC-stimulated [^3H]NA release in the Na^+ -containing buffer. The L-type amino acid transporter system is Na^+ -independent and shows a preference for the transport of branched-chain and aromatic amino acids, such

Table 6. Effects of various amino acid on SNC-stimulated [^3H]NA release

Additions	[^3H]NA release (% of increase)	
	134 mM NaCl	134 mM choline chloride
0.5 mM SNC	100	100
Branched-chain and aromatic amino acids		
+L-Ala	111.7 $\pm$ 12.3	37.3 $\pm$ 1.1
+L-Leu	70.9 $\pm$ 5.8	12.0 $\pm$ 3.0
+L-Met	75.9 $\pm$ 6.9	32.1 $\pm$ 7.0
+L-Phe	48.5	10.2 $\pm$ 4.4
Hydrophilic amino acids		
+L-Asn	117.3	92.0
+L-Gln	95.8	87.7
+L-Ser	101.0	57.9
+L-Thr	102.4	58.0 $\pm$ 3.2
+L-Tyr	65.9	8.5 $\pm$ 3.4
Basic amino acids		
+L-Arg	102.2	63.7
+L-His	72.8	109.5
+L-Lys	114.4	97.5
Acidic amino acid		
+L-Asp	107.5	70.1

The labeled slices were incubated with 0.5 mM SNC and the indicated amino acids (3 mM) in Na^+ -containing or Na^+ -free, choline-containing Tyrode's buffer. Data are standardized to the percent-age of SNC-stimulated release without amino acids and represent the means $\pm$ SE of three or four independent experiments. In some experiments, data are the means of two independent experiments and the SD between each experiment was within $\pm 10\%$.

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as L-leucine, L-phenylalanine and L-tyrosine (23). In the Na^+ -free, choline-containing buffer, addition of large neutral or aromatic amino acids, such as L-leucine, L-alanine, L-methionine, and L-phenylalanine, inhibited SNC-stimulated [^3H]NA release significantly. L-Tyrosine also inhibited the release. The effects of the other amino acids tested were limited, although L-serine and L-threonine inhibited the release by 40%. Although the addition of D-leucine also inhibited the release in a dose-dependent manner, the effect of D-leucine was much less than the effect of L-leucine, and D-leucine had no inhibitory effect at a concentration of 0.3 mM.

2-Aminobicyclo[2.2.1]heptane-2-carboxylate (BCH) is known to be a specific inhibitor of L-type transporters (24). Addition of 1 mM BCH inhibited SNC- and SNAP/L-Cys-stimulated [^3H]NA release significantly from labeled slices in the Na^+ -free, choline-containing buffer. The inhibitory effect of BCH was dose-dependent and was much less in SNC- and SNAP/L-Cys-stimulated release in the Na^+ -containing buffer. These findings suggest that SNC is incorporated in at least two systems: one

Table 7. Effects of SNC, BCH, and amino acids on L-[³H]Leu uptake in rat hippocampal slices

Addition	L-[³ H]Leu uptake (fmol/assay tube)
none	467 ± 18
SNC	293 ± 15 ^a
BCH	217 ± 14 ^a
L-Phe	167 ± 13 ^a
L-Lys	405 ± 23
L-Cys	331 ± 26

The hippocampal slices were incubated with L-[³H]Leu in the Na⁺-free, choline-containing buffer containing 0.5 mM SNC, BCH, and the indicated amino acids. Data are the means ± SE of three independent experiments. Statistical significance was determined by an unpaired *t* test. ^aSignificantly different compared with no addition.

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is the Na⁺-independent and BCH-sensitive transporter, and the other, a transporter for SNC that is BCH-insensitive and works in the presence of extracellular NaCl.

Next, we investigated the effects of 0.5 mM SNC, BCH and amino acids on the uptake of L-[³H]leucine into hippocampal slices in Na⁺-free buffer (Table 7). Addition of 0.5 mM SNC and BCH inhibited L-[³H]leucine uptake by 35% and 50%, respectively. The inhibitory effects of SNC and BCH were dose-dependent. L-Phenylalanine (0.5 mM) also inhibited the uptake by >60%. This inhibitory effect of L-phenylalanine is comparable to that against [³H]NA release. Addition of L-lysine, which had no inhibitory effect on SNC-stimulated [³H]NA release, did not inhibit L-[³H]leucine uptake. Addition of 0.5 mM L-cysteine inhibited the uptake significantly, although the effect of L-cysteine was less than those of SNC and BCH.

A possible intracellular regulatory mechanism induced by NO in the brain is the S-nitrosylation of protein thiols. It has been proposed that SNC induced the S-nitrosylation of a cysteine in a redox modulatory site of the N-methyl-D-aspartate receptor-channel complex (18, 19, 25). In addition, NAD linkage (26), NADH attachment (27) and thiol-associated ADP-ribosylation (28) by NO donors to several proteins have been reported. Further studies on whether SNC modulates the activities of CaM or its kinase via covalent modification, are in progress in our laboratory. It is important to find the target protein(s) of SNC and the mechanism by which NO compounds/thiols and SNC regulate the release. Another important point to be determined is to show the physiological role of amino acid transporter on uptake of SNC in vivo. Our results showed that SNC is a physiological NO compound and that SNC is incorporated into cells via a specific type of amino acid transporter

IV. Effects of NO on rat thymocytes

IV-A. Enhancement of iNOS expression by in vivo glucocorticoid treatment (6, 7)

NO has been suggested to be involved in many physiological and pathological functions not only in the CNS but also in the immune system. NO is implicated in several vascular phenomena during inflammation and is involved in the effector function of lymphocytes (29–32). Glucocorticoids (GC) are hormones naturally released during the course of an immune response and are also widely used as immunosuppressive and antiinflammatory agents in the treatment of many diseases. One of the anti-inflammatory effects of GC is due to their ability to inhibit the expression of cytokine-induced iNOS. GC in vitro inhibited lipopolysaccharide/interferon-induced iNOS expression (30) and activation in macrophages (33). In thymocytes, addition of GC in vitro induced apoptotic death within 2–3 hr with endogenous endonuclease activation (34). In this study, we investigated the effect of in vivo treatment with GC on NO production, induction of iNOS protein and DNA synthesis on rat thymocytes.

Male Sprague-Dawley rats (5-week-old) were obtained from NRC Haruna Corp. (Gunma). They were fed commercial feed ad libitum for 1 week under the SPF condition. Animal experiments were carried out in accordance with the NIH Guide for Care and Use of Laboratory Animals and approved by the animal care and use committee of Hokkaido University. Six-week-old male Dawley rats were s.c. treated with hydrocortisone (HC) at 100 mg/kg (100 mg/ml, suspended in 50% ethanol/50% saline). Control rats were s.c. injected with vehicle (50% ethanol/50% saline). Forty-eight hours after the injection, the thymuses from control and HC-treated rats were removed. The contamination of antigen presenting cells such as dendritic cells and macrophages was under 2%, as measured by morphological observation and by the reactivity with anti-rat macrophage antibody (Inter-cell Tec., Inc., Hopewell, NJ, USA). The purity of thymocytes was over 95%.

First, we investigated the dose-dependency on concanavalin A (Con A) of the DNA synthesis of rat thymocytes. With 10% fetal calf serum, the addition of Con A stimulated [³H]thymidine incorporation in a dose-dependent manner up to 5 µg/ml, and the maximal response was obtained with 5 µg/ml of Con A. Next, we compared [³H]thymidine incorporation in thymocytes from normal rats and that from HC-treated rats. HC treatment (100 mg/kg, s.c.) significantly caused a decrease in thymus size, as previously reported (6). HC treatment inhibited the DNA synthesis induced by Con A (5 and 10 µg/ml) significantly.

To investigate the role of NO in the proliferation of

Fig. 5. Rats were s.c. injected with HC (100 mg/kg body weight) or vehicle 48 h before thymocyte isolation. Thymocytes (5×10^6 cells/ml/well) were incubated with the indicated concentrations of Con A for 72 h. Nitrite levels in the culture medium were determined. Data are the means $\pm$ S.E. of 3 independent experiments. $n=3$ in normal and HC-treated rats. * $P<0.05$ and ** $P<0.01$ vs vehicle. (Reprinted with permission from Ref. 7)

thymocytes, nitrite accumulation and the amounts of iNOS proteins were determined. Basal nitrite accumulation without Con A in the control thymocytes was very limited. Addition of Con A to the control thymocytes increased nitrite accumulation in the culture medium slightly, but not significantly (Fig. 5). On the other hand, basal nitrite accumulation in thymocytes from HC-treated rats (HC-treated thymocytes) was significantly higher than that in the control thymocytes. Addition of Con A significantly enhanced nitrite accumulation in the HC-treated thymocytes in a dose-dependent manner. Figure 6 shows the immunoblot analysis of the iNOS protein in rat thymocytes. The iNOS protein was not detected in either the control (lane 1) or HC-treated thymocytes (lane 3) without Con A stimulation. Addition of Con A (5 μ g/ml) to the culture medium increased the expression of the iNOS protein expression in the control thymocytes (lane 2). The Con A-induced increase in iNOS protein expression was much higher in the HC-treated thymocytes (lane 4). The induction of the iNOS protein by Con A in thymocytes was significantly enhanced by treatment with HC in vivo, 14.2 $\pm$ 0.7-fold compared to the expression in control thymocytes ($P<0.01$, $n=4$). The constitutive NO synthase (eNOS) protein was present both in the control and the HC-treated thymocytes without Con A stimula-

Fig. 6. Rats were s.c. injected with HC (100 mg/kg body weight) or vehicle 48 h before thymocyte isolation. Thymocytes (5×10^6 cells/ml/well) were incubated with Con A (5 μ g/ml) for 72 h. The same amount of proteins from control and HC-treated thymocytes were analyzed with anti-iNOS antibodies. The data shown represent a typical experiment and are representative of 3 independent experiments. (Reprinted with permission from Ref. 7)

tion, and the amounts were almost the same.

IV-B. Inhibition of DNA synthesis of rat thymocytes by NO-cyclic GMP system (6, 7)

Effects of chemical NO donors and the cyclic GMP analog dibutyryl cyclic GMP on [3 H]thymidine incorporation in the control thymocytes was investigated (Table 8). Addition of 50 μ M SNP inhibited Con A-stimulated [3 H]thymidine incorporation remarkably. Another NO donor, NaNO₂ (50 μ M), also inhibited DNA synthesis. NO donors such as SNP release nitric oxide (NO radical) and stimulate soluble guanylyl cyclase. In rat thymocytes, addition of 50 μ M SNP and 50 μ M NaNO₂ stimulated cyclic GMP accumulation, from 0.6 ± 0.1 to 15.3 ± 0.5 and 2.3 ± 0.2 pmol/ 10^6 cells, respectively. Addition of 1 mM dibutyryl cyclic GMP also inhibited Con A-stimulated [3 H]thymidine incorporation significantly.

These findings suggest that NO and cyclic GMP inhibited DNA synthesis in rat thymocytes and that HC treatment in vivo inhibited DNA synthesis via the expression of the iNOS protein and the accumulation of NO. Hochman and Cudkowicz (35) reported that the adminis-

Table 8. Effects of NO donors and a cyclic GMP analog on Con A-stimulated DNA synthesis in rat thymocytes

Addition	[³ H]Thymidine incorporation (%)
None	100
50 μ M SNP	29.5 $\pm$ 5.0 ^a
50 μ M NaNO ₂	73.1 $\pm$ 7.9 ^b
1 mM dibutyl cyclic GMP	67.1 $\pm$ 5.7 ^b

Rat thymocytes (10⁶ cells/200 μ l/well) were cultured with the indicated NO donors and cyclic GMP analog for 54 h. To this medium 2 μ g/ml of Con A and 10% FCS were also added. And then [³H]-thymidine (1 μ Ci/well, 2.5 μ M) were added to wells and the cells were further incubated for 18 h. Data are expressed as percentages of the control value (none). Data are means $\pm$ S.E. of 3–5 independent experiments. ^aP < 0.001, ^bP < 0.05 vs none.

(Reprinted in permission from Ref. 7)

tration of GC to mice caused a reduction in splenic natural killer cell activity. Direct suppressive effects of GC on T cell- (36) and peripheral leukocyte- (37) mediated cytotoxic activities have also been reported. Thus, in general, GC inhibit the mitogen-induced proliferation of T cells. Regarding DNA synthesis, our present results showing the loss of proliferative activity in thymocytes from HC-treated rats concurred with previous reports.

It has been shown that GC inhibits the expression of iNOS in macrophages (30) and glial cells (38). GC also inhibited iNOS expression in endothelial cells directly and probably indirectly via the inhibition of cytokine production (31). On the other hand, our results showed the stimulatory effect of HC on iNOS expression. The mechanism(s) underlying the effect of in vivo treatment with HC has to be determined. The stimulatory effect of HC may be derived from in vivo treatment with HC, because expression of iNOS is modulated by various cytokines and neurotransmitters. GC is used as an immunosuppressant in the allergic state. However, care should be taken when using GC in vivo, because GC had a stimulatory effect on iNOS expression in thymocytes.

V. Regulation of iNOS expression by endothelin in primary cultured rat glial cells (8, 9)

NO is synthesized by at least two major forms of NOS, a constitutive Ca²⁺-dependent NOS (cNOS) and an inducible, Ca²⁺-independent NOS (iNOS), which are expressed after stimulation with bacterial lipopolysaccharide (LPS) and/or inflammatory cytokines, such as tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β). LPS and/or cytokines are able to induce iNOS in glial cell cultures, and NO release from glial cells appears to be involved in neuronal cell death (39, 40). There is increasing evidence that iNOS is induced after focal and global

ischemia primarily in reactive astrocytes (41) and that NO release from glial cells appears to be involved in neuronal cell death (39, 40, 42). In glial cells, increased expression of endothelin has been observed following focal and global ischemia (43). It has been demonstrated that endothelins as well as endothelin receptors are present in the central nervous system and that endothelin acts as a neurotransmitter or neuromodulator (43–45). In addition, endothelin can be liberated from astrocytes during differentiation and proliferation (46, 47). Proliferation of astrocytes is observed exclusively after traumatic injury and after brain damage or brain ischemia. However, the effect of endothelin on iNOS expression in the nervous system has not been studied extensively. We examined the effects of endothelins on iNOS expression in glial cell cultures prepared from neonatal rat brains.

In primary cultured rat glial cells, a combination of cytokines such as TNF- α and IL-1 β stimulates production of nitrite via expression of iNOS. In these cells, simultaneous addition of endothelin decreased iNOS expression and nitrite accumulation induced by TNF- α /IL-1 β . The inhibitory effect of endothelin on TNF- α /IL-1 β -stimulated iNOS expression appears to be mediated by ET_B receptors, because a) both endothelin-1 and -3 inhibited the effects of TNF- α /IL-1 β on iNOS expression and nitrite accumulation, b) a selective ET_B-receptor agonist, Suc-[Glu⁹,Ala^{11,15}]-endothelin-1 (8–21) (IRL 1620), decreased the effects of TNF- α /IL-1 β , and c) a selective ET_B antagonist, N-cis-2,6-dimethylpiperidino-carbonyl-L- γ -methyleucyl-D-1-methoxycarbonyltryptophanyl-D-norleucine (BQ-788), abolished the inhibitory effects of endothelins and IRL 1620. Cyclic AMP-elevating agents (dibutyl cyclic AMP and forskolin) inhibited TNF- α /IL-1 β -induced iNOS expression and nitrite accumulation. Incubation of glial cells with LPS caused an increase in iNOS expression. Simultaneous addition of endothelin-3 decreased the effects of LPS in iNOS expression. These findings suggest that endothelins can decrease TNF- α /IL-1 β - and LPS-induced iNOS expression via ET_B receptors and that cyclic AMP may be involved in this process.

In contrast, pretreatment with endothelin for 24 hr enhanced LPS-induced nitrite accumulation and iNOS expression. Pretreatment with endothelin-1, -3 and the selective ET_B agonist IRL 1620 caused the same stimulatory effect with similar potencies, suggesting that the synergism was mediated via the ET_B receptor. A protein kinase C inhibitor, calphostin C, suppressed endothelin-3-enhanced iNOS expression. Pretreatment with phorbol 12-myristate 13-acetate also enhanced LPS-induced iNOS expression, although the phorbol had no effect on iNOS expression by itself. Our data clearly demonstrated that protein kinase C is involved in the

regulation of endothelin-enhanced iNOS expression.

The predominant mechanism underlying the induction of iNOS is transcriptional regulation. The iNOS gene is known to be activated by nuclear factor- κ B, nuclear factor-interleukin-6, interferon- γ -response element, and a palindromic TNF response element-like site (48). Recently, a novel LPS-response element, LRE_{AA}, containing critical adenosine residues was identified in the iNOS promoter (49). The two effects of endothelin, a stimulatory effect by simultaneous addition and an inhibitory effect by pretreatment for 24 hr, on nuclear factors have to be determined.

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