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Nitric oxide-dependent blood-brain barrier permeability alteration in the rat brain*

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Abstract. The role of nitric oxide (NO), a well known vasodilator, in the regulation of blood-brain barrier (BBB) permeability is not clear. Therefore, the present study was planned to assess the role of NO-releasing compounds like sodium nitroprusside (SNP) and the active metabolite of molsidomine, SIN-1, as well as a precursor of NO, L-arginine, on this physiological barrier. The permeability was assessed by using several tracers. All three agents increased the permeability of BBB to the tracer. The increase in permeability caused by L-arginine was not blocked by N-nitro-L-arginine methyl ester (L-NAME). L-Arginine-treated brains did not show an elevation of nitrite content, thus ruling out the possibility of NO generation and its involvement in BBB permeability alteration. It is concluded that NO itself causes an increase in the permeability of BBB. However, arginine-induced opening is not NO mediated.
Key words. Blood-brain barrier; nitric oxide; arginine; sodium fluorescein; N-nitro L-arginine methyl ester.

The barrier between the circulatory system and the central nervous system is relatively impermeable to most ions and substances such as amino acids, small peptides and proteins. The BBB exists at the level of endothelial cells that make up brain capillaries[1]. Macrovascular endothelial cells are known to play a critical role in regulation of vascular tone through the secretion of factors like NO and endothelin[2]. Nitric oxide can be synthesized from L-arginine by at least two different nitric oxide synthase enzymes (NOS), recently characterized by molecular cloning[3,4]: one is calcium- and calmodulin-dependent and is constitutively expressed in few cells[5], the other is inducible by cytokines[6]. Both are potentially active in the endothelium[7]. The constitutive NOS-dependent NO synthesis activates soluble guanylate cyclase which results in elevated cyclic GMP (cGMP) levels that are responsible for vascular relaxation and regulation of blood pressure[8]. It has been demonstrated in vitro that elevation of cGMP levels by the NO-releasing compound SNP causes lowering of resistance of BBB endothelial cells[9].

Nitric oxide is known to be released from different cells after immunological activation. Such stimulation can be brought about in several ways, such as intraperitoneal injection of *E. coli* lipopolysaccharide, Bacillus Calmette Guerin, and treatment by gamma interferon or tumor necrosis factor[10–12]. There are also many nitrovasodilators that are used clinically in hypertensive emergencies, heart failure and for controlled hypotension during surgery. The effect of these agents on BBB permeability is not known. In the present study it was, therefore, planned to investigate the effect of some NO-releasing or -synthesizing compounds on BBB permeability. Potent NO-releasing compounds such as SNP, the active metabolite of molsidomine, SIN-1, and the NO precursor L-arginine were used to evaluate the effect of NO on BBB permeability.

Materials and methods

Male Sprague Dawley rats (150–200 g) were anaesthetized with urethane (1.25 g/kg, ip). The carotid artery was cannulated towards the brain with a twenty-seven gauge needle connected to polyethylene tubing through which NO-generating compounds were injected towards the brain. The jugular vein was cannulated with a similar needle and tubing towards the heart for delivering the dye. Control animals were sham operated and received normal saline instead of the test compounds through the carotid artery but received the same amount of fluorescein. The body temperature of animals was maintained at 37 °C by heating with an electric bulb. The integrity of the BBB was measured 30 min after intracarotid administration of SNP (1.0 and 10.0 mM, 0.2 ml/150 g, Sigma), SIN-1 (10.0 mM, 0.2 ml/150 g, gift from H. Bohn, Pharmacol. Div., Casella AG, Frankfurt/Main) and L-arginine (50, 100, 250, 400 and 1000 mg/kg, Sigma) by using the micromolecular tracer sodium fluorescein[13], a bioinert dye that normally does not cross the BBB (2%, 0.5 ml/100 g, iv, S.D. Fine-Chemical Ltd. Bombay, India) which was given immediately following the above mentioned agents. The intracarotid route was selected for administration of different compounds instead of iv in order to have the maximum concentration of NO at the site i.e.

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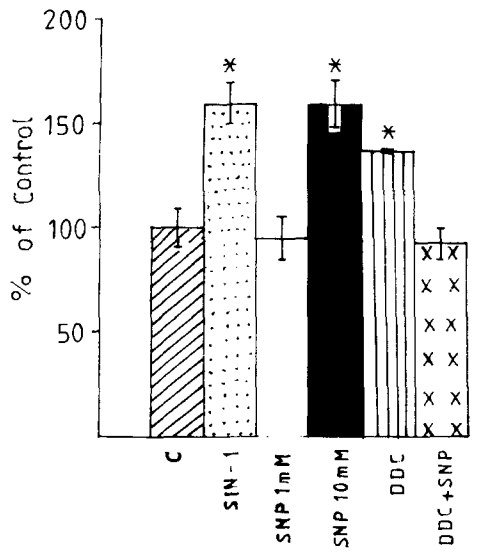

Figure 1. Effect of SIN-1 (10 mM), SNP (1 and 10 mM) and DDC (400 mg/kg) + SNP (10 mM) on blood-brain permeability. C = Control, *p < 0.001. (n = 5)

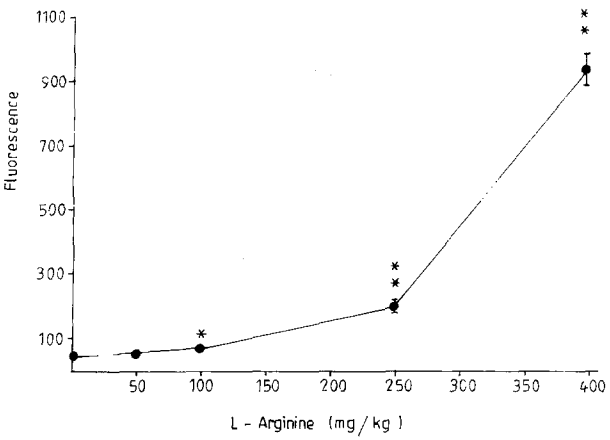

Figure 2. Effect of different doses (50, 100, 250 and 400 mg/kg) of L-arginine on blood-brain permeability. *p < 0.01, **p < 0.001. (n = 5)

at BBB. Sodium fluorescein in brain was measured after perfusing it with chilled saline (0.9%) for 2 min through the left ventricle of the heart, while the right auricle was cut open. The brain was then removed and washed twice with triple distilled water and a 10% homogenate was made in the water. To 2 ml of homogenate, 0.1 ml of 12% perchloric acid (PCA) was added and the mixture was kept at 4 °C for 1 h. After 1 h, samples were centrifuged at 4000 rpm for 20 min and supernatants were used for fluorescein estimation. To a tube containing 1.5 ml borate buffer (0.2 M, pH 10.0) and 0.1 ml 1 N NaOH, 1.5 ml supernatant was added and the fluorescence was read at 490 (Ex) and 522 (Em) nm using a Schimadzu spectrophotofluorometer.

To show that the opening mediated by these agents is not specific for sodium fluorescein only, in one experiment with L-arginine three tracers (^{14}C sucrose, sodium fluorescein and Evans blue) were used. ^{14}C Sucrose (BARC, Bombay), 2.2 μCi/100 g, was given through the carotid artery 10 min after L-arginine injection, the rats were sacrificed five seconds later and radioactivity in the brain of control and L-arginine-treated rats was counted after digesting the tissue with Protosol (tissue solubilizer, 0.5 N, Research Product International). In the case of Evans blue, a macromolecular tracer, only a qualitative assessment was done in which 2% solution of dye at a dose of 4 ml/kg was given intravenously immediately after L-arginine, the brain was removed 25 min later after perfusion, and staining of brain was assessed qualitatively using a standard procedure of grading[14] (0 = no staining, + = mild staining, ++ = moderate staining and +++ = heavy staining).

The following agents were administered to various groups:

1. Diethyl dithiocarbamate (400 mg/kg, ip, DDC, Sigma) was administered 2 h prior to SNP treatment.
2. Animals were treated with indomethacin (3 mg/kg, ip, Merck Sharp and Dohme, USA) 1 h prior to L-arginine.
3. Methylene blue (10 and 50 mg/kg, iv, Himedia Lab. Bombay, India) was injected 30 min prior to L-arginine.
4. Atropine (10 mg/kg, ip, Sigma, USA) was administered 30 min prior to L-arginine.
5. L-NAME (10 mg/kg, iv, Sigma, USA) was given 10 min prior to L-arginine.
6. In L-arginine-treated animals arterial blood pressure (BP) and heart rate (HR) was recorded.

For all the above mentioned experiments the dose of L-arginine was 250 mg/kg. Particular doses and route of administration for the above mentioned agents were selected from the published literature.

Another basic amino acid, L-lysine (250 mg/kg, intracarotid route), was also studied for its effects on BP and BBB permeability.

Constitutive nitric oxide synthase (NOS) assay was done in brain microvessels using two methods: 1) nitrite estimation[15] and 2) the conversion of oxyhemoglobin to methemoglobin[16].

Every effort was made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques, if available. Ethical principles and guidelines for scientific experiments on animals provided by the Swiss Academy of Medical Sciences were followed.

The significance of difference between the two groups was evaluated by using Student's t-test. p value <0.05 was considered to be significant.

Table 1. Effect of L-arginine (250 mg/kg, intracarotid) on blood-brain permeability as assessed by Evans blue dye (80 mg/kg, i.v.) method.

Group (n)	Score/No. of animals			
	0	+	++	+++
Control (5)	3	2	0	0
L-arginine (6)	1	2	1	2

Results

SNP and SIN-1 (10 mM) increased the permeability of BBB for sodium fluorescein significantly. However, a lower dose of SNP (1 mM) was ineffective (fig. 1). In control rat brain the value of sodium fluorescein was 350 ± 21 pg/g tissue. Methylene blue, given to inhibit guanylate cyclase, itself produced a 61% increase in BBB permeability. DDC, a known inhibitor of superoxide dismutase, completely blocked the SNP-mediated opening of BBB (fig. 1).

L-Arginine was ineffective at 50 mg/kg but 100, 250 and 400 mg/kg doses produced marked increases in the permeability of BBB to all three tracers. The increases were found to be dose-dependent (fig. 2, table 1). In the experiments where ^{14}C sucrose, a micromolecular tracer, was used to study the effect of L-arginine, control values were $63,743 \pm 6826$ cpm/g, which increased to $104,471 \pm 6406$ cpm/g following L-arginine treatment. A very high dose of 1 g/kg was found to be toxic as all the animals in this group died within a few minutes. In order to work out the pathway of L-arginine action, the effect of L-NAME pretreatment was studied. L-NAME

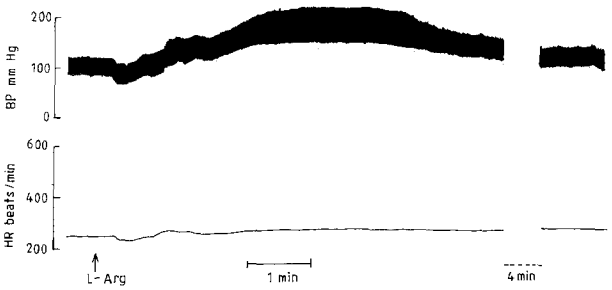

Figure 4. Changes in the blood pressure (BP) and heart rate (HR) following L-arginine (250 mg/kg) administration into the carotid artery.

itself had no effect on the permeability, and it could not block the changes induced by L-arginine (fig. 3).

L-Arginine infusion into the carotid artery produced immediate transient hypotension followed by a sustained hypertension and increase in the heart rate (fig. 4). Indomethacin pretreatment partially blocked the effect of L-arginine (fig. 5); indomethacin itself had some effect on BBB permeability (fig. 5).

Atropine pretreatment had no effect on L-arginine-induced blood-brain permeability (fig. 6). No detectable activity of constitutive NOS could be obtained using any of the described methods. The nitrite content of brain tissue in L-arginine-treated animals was not significantly different from that of control animals. L-Lysine at a dose of 250 mg/kg was found to have no effect on BBB permeability or on BP. Finally, the sodium fluorescein concentration in blood was not affected in the animals treated with L-arginine, SNP or

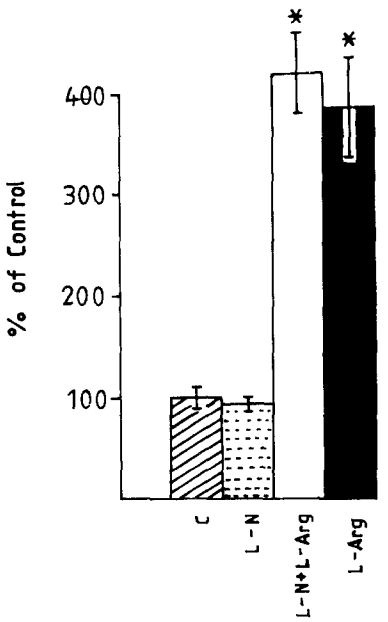

Figure 3. Blood-brain barrier permeability alterations in L-NAME (L-N, 10 mg/kg) + L-arginine (250 mg/kg)-treated rats. C = Control, *$p < 0.001$. (n = 5)

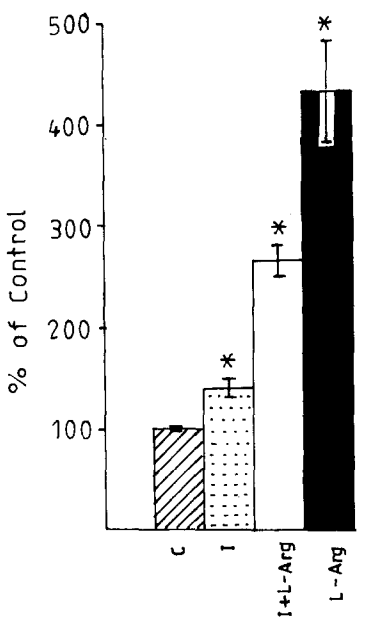

Figure 5. Effect of indomethacin (I, 3 mg/kg) pretreatment on L-arginine-induced BBB permeability. C = Control, *$p < 0.001$. (n = 5)

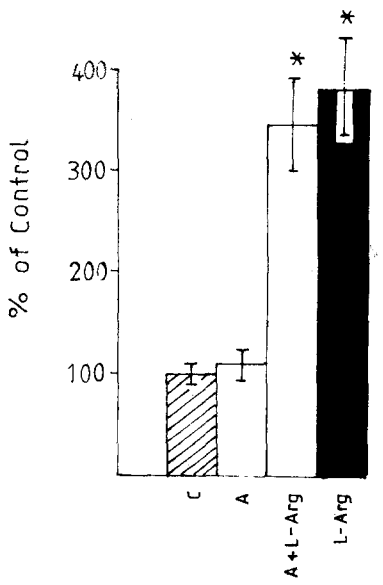

Figure 6. Effect of atropine (A, 10 mg/kg) pretreatment on L-arginine-induced alterations in BBB permeability. C = Control, *$p < 0.001$. ($n = 5$)

SIN-1, as compared to untreated controls (data not shown), showing these compounds had no effect on serum fluorescein levels.

Discussion

In the present investigation, the effect of some spontaneous nitric oxide-generating compounds, like SNP and SIN-1, on BBB permeability has been studied in rats. Both these compounds increased the permeability of BBB to sodium fluorescein, a micromolecular tracer. The sodium fluorescein detected in control brains enters through the circumventricular organs (CVOs) in brain tissue and not by crossing the BBB[17]. Further, the dye levels in CVOs of treated and control rats were not significantly different, showing that the higher brain uptake observed in the treated group did not result from an increased dye inflow through these organs. SNP has been shown to lower the resistance of endothelial cells in vitro, thereby increasing the permeability of these cells. This effect of SNP has been found to be mediated via cGMP[9]. The involvement of cGMP in the SNP effect has been demonstrated in the present study in rats by treating them with methylene blue which opened the BBB to the tracer, possibly owing to its free radical-generating property[18]. Free radicals have been found to alter the permeability of the BBB.

The effect of SIN-1 could be either due to NO or synergy between NO and free radicals, since this compound also generates superoxide radicals along with NO. L-Arginine, a precursor of NO via NOS, was used for evaluating the effect of NO on BBB permeability, since vascular endothelial cells are known to contain both constitutive as well as inducible NOS[19]. L-Arginine, at higher doses, produced a more than four fold increase in BBB permeability. To make sure that the effect was mediated by NO the animals were treated with L-NAME, an inhibitor of NOS, before administration of L-arginine. Interestingly, this did not block the effect of L-arginine. Nitrite, a degradation product of NO in the brain, was also measured in brain tissue in order to check whether it is formed from pathways involving lipoxygenase, cyclooxygenase and cytochrome P450, which are known to be involved in production of endothelial-derived relaxing factor in arteries[20]. The nitrite content in normal and L-arginine-treated rats was comparable. This suggested that L-arginine-mediated opening is not NO-dependent but involves some other mechanism. A recent report by Durieu-Trautmenn et al.[21] supports our observation that BBB endothelial cells do not possess constitutive NOS, a special feature of a special tissue, and this could be the reason why L-arginine is not effective at lower concentrations. L-Arginine administered through the intracarotid route produced a sharp but transient decline in blood pressure followed by a sustained increase. Arginine-induced hypertension could be a cause of altered permeability. Thomas et al.[22], reported that L-arginine-induced hypotension could in part be mediated by a cyclooxygenase product since indomethacin blocks the L-arginine-elicited increase in glomerular filtration and renal vascular dilation[23]. In our study indomethacin also partially blocked the response. The partial blockade by indomethacin could be due to the inhibition of the initial hypotensive response thereby minimizing the abrupt increase in blood pressure and thereafter decreasing the extent of increase in permeability. It is known that a more abrupt rise in blood pressure can increase the permeability of BBB to a greater extent[24]. Cernadas et al.[25] have suggested that the L-arginine-induced effect is cholinergic mediated, but the inability of atropine to block the effect of L-arginine in the present study ruled out this possibility. Another possible mechanism of L-arginine-induced increased permeability could be the action of polyamines. An increased concentration of polyamines is known to alter the BBB permeability[26]. Arginine gives rise to ornithine, a precursor of polyamines. The increased level of L-arginine may lead to increased polyamine synthesis, which in turn could lead to increased permeability. However, this possibility remains to be examined.

Being aware of the fact that NO can interact with superoxide radicals[27], an experiment was planned to show whether such interaction plays any part in altering the permeability of BBB or not. Rats were pretreated with DDC, an inhibitor of superoxide dismutase (SOD)[28], and then given SNP. DDC pretreatment blocked the effect of SNP completely. This could be due to the fact that in DDC-treated rats SOD is totally

inhibited and more superoxide radicals are available to scavenge NO, thereby decreasing the NO available at the site to make the BBB leakier. DDC itself produced some increase in BBB permeability. This may be because suppression of SOD allows the available superoxide radical to alter the permeability of BBB.

The findings of this study provide evidence that i) elevated NO levels can increase the permeability of BBB, and ii) L-arginine-evoked alterations in BBB permeability are not NO-mediated but related to its hypertensive effects.

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