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Cardiovascular Responses and Nitric Oxide Production in Cerebral Ischemic Rats

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We investigated that the role of nitric oxide (NO) on ischemic rats in brain and heart. Ischemia was induced by both common carotid arteries (CCA) occlusion for 24h following reperfusion. Then tissue samples were removed and measured NOx. In brain, NOx was increased by about 40% vs. normal and it was significantly inhibited by aminoguanidine, selective iNOS inhibitor. This result showed that NOx concentration was increased by iNOS. We investigated the role of Ca^{2+} during ischemia. Nimodipine, L-type calcium channel blocker, didn't inhibit the increases of NOx concentration during ischemia. It suggested that increased NOx was due to calcium-independent NOS. MK-801, which N-methyl-D-aspartate (NMDA) receptor antagonist, didn't significantly prevent the increases of NOx. In heart, ischemia caused NOx decrease and it is inconsistent with NOx increase in brain. Aminoguanidine and nimodipine didnt affect on NOx decrease. But MK-801 more lowered NOx concentration than those of ischemia control group. It seemed that Ca^{2+} influx in heart partially occurred via NMDA receptor and inhibited by NMDA receptor antagonist. The mean arterial pressure (MAP) in ischemic rats after 24h of CCA occlusion was decreased when compared to normal value, whereas the heart rates (HR) was not different between two groups. Aminoguanidine or MK801 had no effect on MAP or HR, but nimodipine reduced MAP. There was no difference the effects of aminoguanidine, nimodipine, or MK-801, on MAP and HR between normal rats and ischemic rats. In summary, ischemic model caused an increase of NOx concentration, suggesting that this may be produced via iNOS, which is calcium independent in brain. However in heart, ischemia decreased NOx concentration and NMDA receptor was partially involved. The basal MAP was decreased in ischemic rats but HR was not different from normal control, suggesting that increased NOx in brain of ischemic rat may result in the hypotension.

Key words: Nitric oxide, NO, *N*-methy-D-aspartate, NMDA, NO, Nitric oxide, NMDA, *N*-methyl-D-aspartate, NOS, Nitric oxide synthase, iNOS, Inducible nitric oxide synthase, eNOS, Endothelial nitric oxide synthase, nNOS, Neuronal nitric oxide synthase, CCA, Common carotid artery, MAP, Mean arterial pressure, HR, Heart rate, BP, Blood pressure

INTRODUCTION

There is increasing evidence that nitric oxide (NO) has a dual role in the pathophysiological mechanisms of cerebral ischemia. As a neurotoxin, NO may mediate the ischemic excitotoxic brain injury induced by glutamate release and N-methyl-D-aspartate (NMDA) receptor overactivation. In contrast, as a signaling molecule, NO may induce an increase in the blood perfusion of the ischemic penumbra in the early stages of cerebral ischemia (Iadecola, 1997).

In the brain, distinct isoforms of NOS are basally expressed; one isoform in the neurons and another isoform in the endothelial cells. These constitutive NOS isoforms of (cNOS) produce NO transiently during the agonist-induced increase in intracellular calcium (Murphy and Snyder, 1982). Another isoform of NOS is expressed in a wide variety of cells, most notable in the macrophages during inflammation. The inducible isoform of NOS (iNOS) is continuously active and produces NO independently of the intracellular calcium levels (Cho *et al.*, 1992).

The brain can be damaged during cerebral ischemia as a result of an amplifying cascade of neurochemical events beginning with formation of free radicals and the loss of cell energy through the lower aerobic production of ade-

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nosine triphosphate (ATP), and finishes with an overload of cytosolic calcium (del Pilar Fernandez *et al.*, 1998). The excessive influx of calcium into the neurons that follows hypoxia/ischemia plays a critical role in post-ischemic cell death (Siesjo and Bengtsson, 1989). Many neurochemicals produced during and after ischemia are changed in many biochemical pathways (Rami and Krieglstein, 1994).

NO production is increased during or after ischemia, sepsis and endotoxemia (Mulder *et al.*, 1994; Thiemermann *et al.*, 1993). However, there is little information is concerning the activity of nitric oxide synthase (NOS) available in permanent (Iadecola *et al.*, 1995; Yoshida *et al.*, 1995) or transient (Iadecola *et al.*, 1996) focal ischemia models. The increased NO levels produced by inducible NOS (iNOS) may be involved in the haemodynamic and functional derangements observed during sepsis or ischemia (Wang *et al.*, 1994).

The generation of NO from L-arginine by NOS plays an essential role in regulating the cerebrovascular tone under both physiological and pathological conditions (Faraci, 1993; Iadecola *et al.*, 1994). An ischemic model by the occlusion of the carotid artery or/and middle cerebral artery affect the cardiovascular responses. Holtz et al. reported that an ischemic model by a carotid artery occlusion had a decreased blood pressure (BP) and produced iNOS expression during post-ischemic reper-fusion in the rat brain (Holtz *et al.*, 2001).

This study aimed to determine whether NO production can be affected in the brain or heart of ischemic rats, and if so, whether these cardiovascular responses can be affected. In addition, the effects of aminoguanidine (an iNOS inhibitor), MK-801 (a NMDA receptor antagonist), and nimodipine (a L-type Ca^{2+} channel blocker) on the NOx concentration were investigated to clarify the role of NO in cerebral ischemic rats.

MATERIALS AND METHODS

Drug

Nimodipine, Aminoguanidine, b-NADPH, FAD, nitrate reductase and other reagents were purchased from the Sigma Chemical Co. (St. Louis, MO). MK-801 was purchased from Tocris Cookson Ltd. (Bristol, UK) and pentobarbital sodium was obtained from Merck (Rahway, NJ).

Animals and Surgical Procedures

Male Sprague-Dawley rats (250-300 g body weight) were used throughout these studies. They were allowed free access to food and water and were anesthetized with either sodium pentobarbital (40 mg/kg, i.p.) or ketamine (50 mg/kg, i.v.). Their breathing was unaided. Ischemia was induced by a carotid artery occlusion (Cai *et al.*, 2002; Kobayashi *et al.*, 2000; Ohtani *et al.*, 2000). This consisted of a bilateral clamping of the carotids with ligatures of nylon thread for 24 h. The ligatures were then removed to allow recirculation. The rectal temperature was controlled and maintained at 36-37°C during the experiment. Animals were divided into 5 groups: (1) normal control rats, where neither the carotids were exposed nor occluded; (2) ischemia control rats, where the carotids were exposed and occluded; (3) rats receiving MK-801 (3.0 mg/kg, i.p.) after inducing cerebral ischemia; (4) rats receiving aminoguanidine (100 mg/kg, i.p.) after the inducing cerebral ischemia; (5) rats receiving nimodipine (0.3 mg/kg, i.p.) after cerebral ischemia. After inducing ischemia, the rats were sacrificed by decapitation and their brains were removed immediately. The tissues were stored at -70°C for several days without a loss of NOS activity.

NOx assay

The tissue NOx concentration was determined using the method reported by Tracey *et al.* (Tracey *et al.*, 1995). The tissue samples were weighted and homogenized (Vibra cell™, Sonics &Materials, USA) in 400 μl of deionized water and centrifuged at 20,000 g, for 10 min, at 4°C. Fifty microlitres of the supernatant were mixed with 20 μl of 0.31 M of a potassium phosphate buffer (pH 7.5), 10 μl of 0.86 mM β-NADPH, 10 μl of 0.11 mM FAD and 20 mu of nitrate reductase (2 units/ml). The samples were allowed to incubate for 1 h at room temperature in the dark. Afterward, 5 μl of 1 M $ZnSO_4$ were added to the samples in order to precipitate the proteins. The samples were centrifuged at 20,000 g, for 5min, at 4°C and the supernatants were removed. One hundred microlitres of Griess reagent (1:1 mixture of 1% sulphanilamide in 5% H_3PO_4 and 0.1% N-(1-naphytyl) ethylenediamine) were added to 50 μl of the supernatant and the mixture was then incubated for 10 min at room temperature (Grandati *et al.*, 1997). NO undergoes a series of reactions with several molecules that are present in biological fluids. The final products of NO *in vivo* are nitrite and nitrate. The sum of the nitrite and nitrate concentration can be an index of the total NO production. Nitrite, a stable NO oxidation product, was determined using the Griess reaction. First, nitrate was converted to nitrite utilizing nitrate reductase, and then the Griess reagents were added to convert the nitrite into a deep-purple azo compound. The absorbance of the azo chromophore was measured by a spectrophotometer using a plate reader (Takeda *et al.*, 1999) and the nitrite concentration was determined at 540 nm, which was converted to NOx levels by using a nitrate standard curve.

Cardiovascular study (Measurement of BP and HR)

The rats were anesthetized with ketamine (50 mg/kg, i.p.) and a transverse incision was made in the ventral

neck area. Heparinized saline-filled polyethylene catheters were then inserted into both the left common carotid artery and the right jugular vein for to determine the arterial pressure and the extent of drug infusion, respectively. The blood pressure was measured by connecting the arterial catheter to a disposal transducer via a length of the polyethylene tubing attached to a swivel device. A second length of polyethylene tubing was attached to the venous catheter to facilitate drug infusion. Both the blood pressure and heart rate were measured simultaneously and recorded on a Grass 7E polygraph.

Statistical analysis

All values are expressed as a mean ± S.E.M. Statistical differences between groups were established using the Students *t*-test or the analysis of the variance (ANOVA). A P value < 0.05 was considered significant.

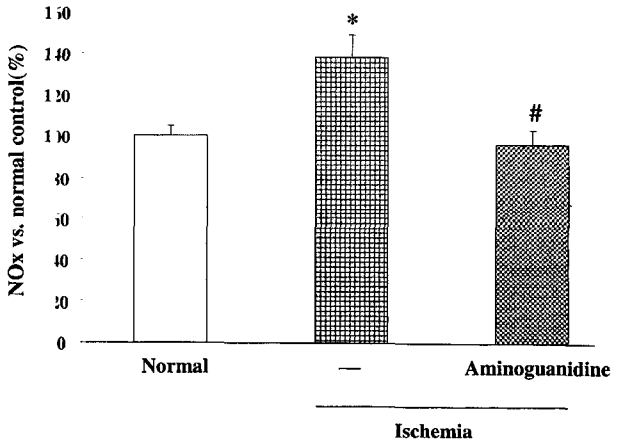

Fig. 1. The effects of aminoguanidine (100 mg/kg, i.p.) on the brain NOx levels in cerebral ischemic rats. The values are represented as a mean ± S.E.M.(n = 8) and are expressed as a percentage of the normal value. *P < 0.05 vs. the normal control, #P< 0.05 vs. the ischemia control.

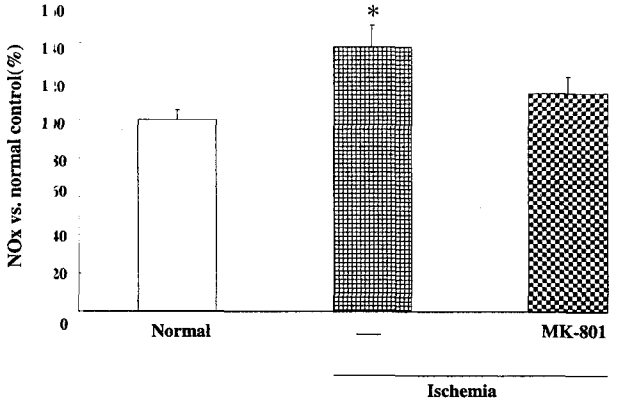

Fig. 2. The effects of MK-801 (3 mg/kg, i.p.) on the brain NOx levels in cerebral ischemic rats. The values are represented as a mean ± S.E.M (n = 8) and are expressed as a by percentage of the normal value. *P < 0.05 vs. the normal control.

RESULTS

The effects of aminoguanidine, nimodipine and MK-801 on ischemic rats in brain

The brain NOx levels in the normal control rats was 33.0 ± 2.0 nmol/mg wet weight. The post-ischemic changes in the brain NOx levels are shown in Fig. 1, 2, 3 and Table 1. After ischemia for 24 h, which was followed by reperfusion, the NOx levels were increased by 40% when compared to the normal (P < 0.05). However, aminoguanidine (100 mg/kg), significantly inhibited the increases

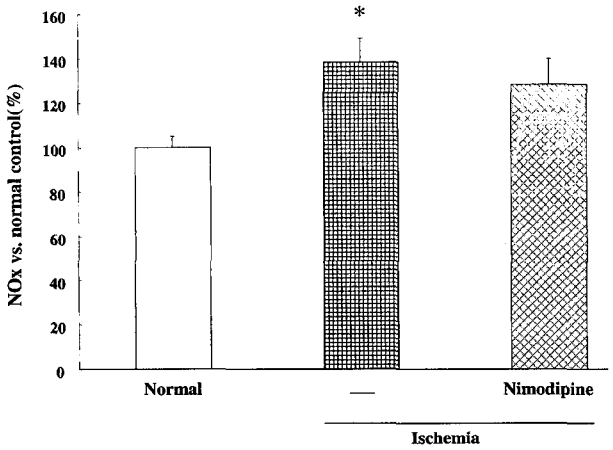

Fig. 3. The effects of nimodipine (0.3 mg/kg, i.p.) on the brain NOx levels in cerebral ischemic rats. The values are represented as a mean ± S.E.M.(n = 8) and are expressed as a percentage of the normal value. *P < 0.05 vs. the normal control.

Table 1. Nox (nmol/mg wet weight) levels in brain and herat

	Brain	Heart
Normal	33.0± 2.0	17.0± 1.0
ischemia	45.0± 4.0*	10.0± 2.0**

Values are mean ± S.E.M. (n = S). *p<0.05. **P<0.01 vs. normal values.

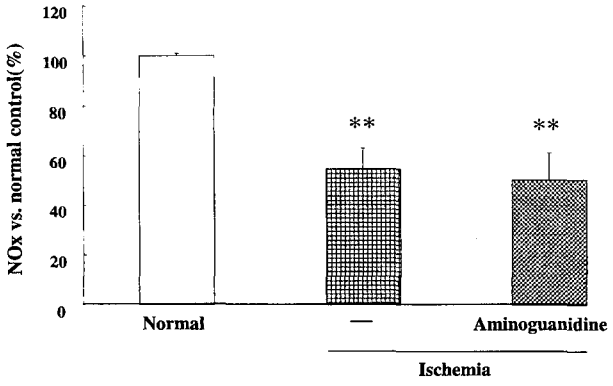

Fig. 4. The effects of aminoguanidine (100 mg/kg, i.p.) on the heart NOx levels in cerebral ischemic rats. The values are represented as a mean ± S.E.M (n=8) and are expressed as a percentage of the normal value. **P < 0.01, vs. the normal control.

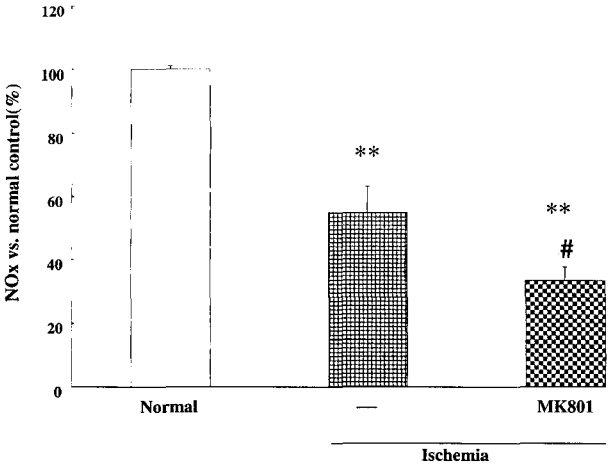

Fig. 5. The effects of MK-801 (3 mg/kg, i.p.) on the heart NOx levels in cerebral ischemic rats. The values are represented as a mean ± S.E.M. (n = 8) and are expressed as a percentage of the normal value. **P < 0.01 vs. the normal control, #P< 0.05 vs. ischemia value.

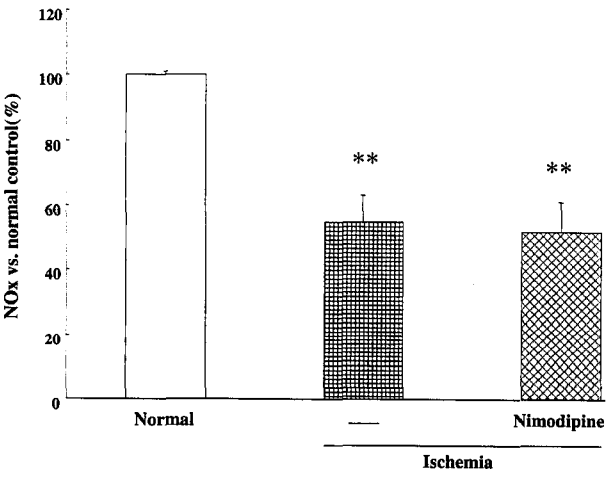

Fig. 6. The effects of nimodipine (0.3 mg/kg, i.p.) on the heart NOx levels in cerebral ischemic rats. The values are represented as a mean ± S.E.M. (n = 8) and are expressed as a percentage of the normal. **P < 0.01 vs. the ischemia control.

in the NOx levels (Fig. 1, P < 0.05). MK-801, had no significant effect on the NOx levels (Fig. 2). In addition, nimodipine, did not affect the NOx levels (Fig. 3).

The effects of aminoguanidine, nimodipine and MK-801 on global ischemic rats in heart

The heart NOx level in the normal control rats was 17.0 ± 1.0 nmol/mg wet weight. The post-ischemic changes in the heart NOx levels are shown in Fig. 4, 5, 6 and Table 1. After ischemia for 24 h, which was followed by reperfusion, the NOx levels were reduced by 45% when compared with the normal (P < 0.01). Aminoguanidine, and nimodipine had no effects on the NOx levels (Fig. 4, 6). However in the MK-801 treatment group, the NOx levels were decreased by 35% when compared with the ische-

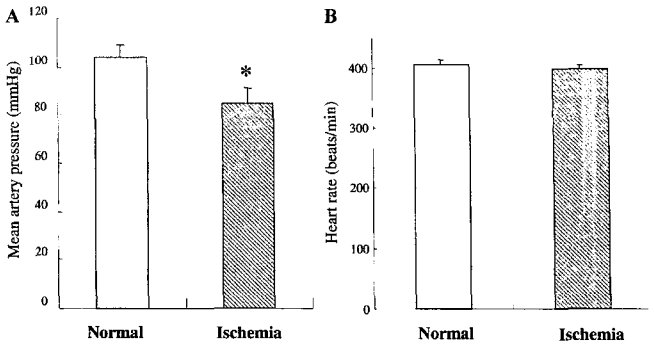

Fig. 7. Basal levels of blood pressure and heart rate before and after ischemia. Ischemia in the rats were performed by occluding the CCA (common carotid artery) for 24 hours with subsequent reperfusion for 1hour. The normal mean artery pressure or heart rate was 103.5 ± 5.4 mmHg, 403.5 ± 9.7 beats/min, respectively. In ischemia, the mean artery pressure was lower than the normal controls, but the heart rate was not different from the normal controls. *P < 0.05 versus the normal value.

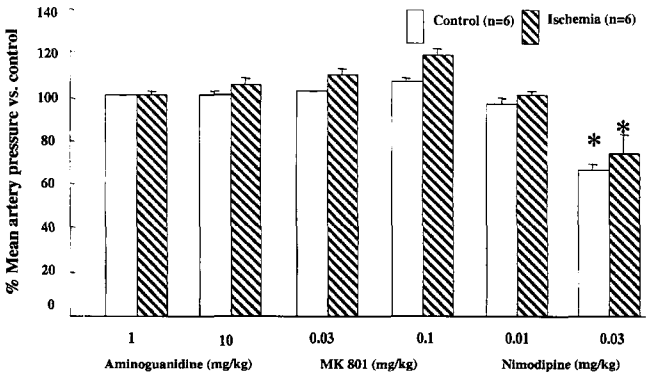

Fig. 8. Effect of aminoguanidine, MK-801, and nimodipine on the Blood Pressure before and after ischemia. Aminoguanidine (iNOS inhibitor), MK801 (NMDA-receptor antagonist), nimodipine (L-type Ca^{2+}-channel blocker) were injected (i.v.). The ratio expresses the mean arterial pressure of post-treatment divided by the mean arterial pressure of the pre-treatment groups. *P < 0.05 vs. the control.

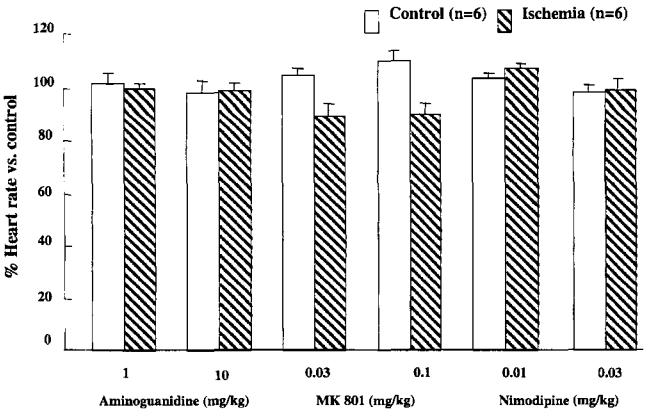

Fig. 9. Effect of aminoguanidine, MK-801, and nimodipine on the Heart Rate before and after ischemia. Aminoguanidine (iNOS inhibitor), MK801 (NMDA-receptor antagonist), nimodipine (L-type Ca^{2+}-channel blocker) were injected (i.v.). The ratio of the HR is expressed as the heart rate of the post-treatment group divided by the heart rate of the pre-treatment groups.

mia control (Fig. 5, $P < 0.05$).

There was no significant change in the plasma NOx levels in either the rats subjected to cerebral ischemia or the drug-treated rats. In this study, the plasma NOx levels did not reflect the brain enzymatic activity, possibly because the changes are too slight to induce these effects in the plasma (data not shown).

The effect of aminoguanidine, nimodipine and MK-801 in ischemic rats on BP and HR

The basal MAP and HR in the anesthetized normal rats used in this study were 103.5 ± 5.4 (mmHg), 403.5 ± 9.7 (beats/min), respectively. The MAP in the ischemic rats after 24 h of CCA occlusion was lower (85 ± 5.7 mmHg; $P < 0.05$) compared to the normal, whereas the HR was no different (397.4 ± 5.2 beats/min) (Fig. 7). Neither aminoguanidine nor MK801 affected the MAP, but nimodipine reduced MAP (Fig. 8). Neither aminoguani-dine, MK-801, nor nimodipine affected the changes in the HR (Fig. 9). However, there was no difference in either the MAP or HR between the normal and schemic rats, which were treated either aminoguanidine, MK-801, or nimodi pine.

DISCUSSION

There is a growing body of evidence that NO participates in the mechanism of cerebral ischemia. Distinct families of NOS have been identified, one constitutive and the other inducible. The constitutive isoforms are Ca^{2+}/calmodulin-dependent and release NO for short periods in response to physiological stimuli. The inducible isoforms are induced by endotoxins and cytokines by a calcium-independent mechanism, and once expressed, release NO for long periods. The genes for these enzymes have been encoded: the constitutive neuronal (nNOS) and endothelial (eNOS) isoform, and the inducible (iNOS) isoform (Grandati *et al.*, 1997).

Large fluxes of NO synthesized by iNOS have been implicated in the cytotoxicity in many cell systems (Morris and Billiar, 1994). After cerebral ischemia, there is a marked inflammatory reaction involving the area of the infarction (Garcia *et al.*, 1994; Shichijo *et al.*, 1991). Therefore, it is conceivable that iNOS induction occurs after cerebral ischemia. Indeed, after focal cerebral ischemia, there is the expression of substantial calcium-independent NOS enzymatic activity in the affected cerebral cortex, suggesting that iNOS is induced in the post-schemic brain.

Careful analysis of the molecular mechanism of the underlying increases in the $[Ca^{2+}]i$ indicates that there are several routes by which Ca^{2+} can enter the cytosol. Ca^{2+} enters a cell through a variety of voltage-sensitive calcium channels (VSCC), via the receptor-operated calcium channels (ROCCs) and via the non-selective cation channels (NSCC) (Callewaert *et al.*, 1988; Mayer and Miller, 1990; Tsien *et al.*, 1991). Several drugs to reduce the deleterious transfer of Ca^{2+} ions across the cell membranes have been developed. An important group of these calcium antagonists are dihydropyridines. The prominent derivative is nimodipine, which acts preferentially on the cerebrovascular system and exhibits_neuro- and psychopharmacological action (Murphy and Snyder, 1982). Nimodipine has been extensively studied in cerebral ischemia because of its lipid solubility and preferential action on the cerebral vessels. Nimodipine blocks the L-type of voltage-sensitive calcium channels present throughout the central nervous system (Rami and Krieglstein, 1994). In this study, nimodipine did not affect the NOx levels and showed that the increase in NOx was due to calcium-independent NOS, which is iNOS. An increase in the cytosolic calcium concentration activates constitutive nitric oxide synthase (cNOS), a Ca^{2+}/calmodulin-dependent flavoenzyme. The increase in cNOS activity results in a burst of NO production, which activates brain guanylate cyclase and leads to the formation of cyclic guanosine monophosphate (cGMP) in the brain (East and Garthwaite, 1991; Kader *et al.*, 1993).

NOS induction might participate in the inflammatory response after cerebral ischemia. This study has shown that a treatment with aminoguanidine, a relatively selective inhibitor of iNOS, has a neuroprotective effect in cerebral ischemia. Aminoguanidine inhibited the increase in NOx levels in the brain after ischemic induction (Iadecola *et al.*, 1996). The calcium-independent activity that was observed in this study could be related to an inducible isoform of NOS. However, this needs to be confirmed by western blot experiments.

Recent work has shown that treatment alone with the NMDA receptor antagonists (MK-801) has a neuroprotective effect in animal models of brain damage (Buchan *et al.*, 1992; Green *et al.*, 1995). This study showed that a post-treatment with MK-801 did not significantly reduce the increase in the NOx levels in the rat brain homogenates but reduced by cerebral ischemia. This result caused by MK-801 may be explained by its property of being a noncompetitive NMDA receptor antagonist, which inhibits the inward Ca^{2+} flux through the calcium channel, thereby partially diminishing intracellular calcium concentrations. NO is an endogenously synthesized effector molecule that acts as a neurotransmitter with novel properties in both the central and peripheral nervous system. It has been suggested that there is transient expression of the NMDA receptor in a developing rat heart. Seeber *et al.* reported that NMDA receptor mRNA and its protein were detected in the heart tissue of rats from embryonic day 14 until postnatal day 21 but this receptor disappeared

10 weeks after birth. However, its function still remains elusive (Seeber *et al.*, 2000). It is also suggested that NO and the NMDA receptors may interact in central cardiovascular regulation (Lo *et al.*, 1997). In this study, MK-801, NMDA receptor inhibitor, decreased the NOx levels in the heart of rats but the mechanism still remains elusive.

Ischemia/reperfusion is associated with the systolic and diastolic dysfunction and a decrease in endothelium-dependent vasodilation (Brunner, 1997). The basal release of nitric oxide from the rat hearts was diminished after ischemia and reperfusion (Maulik *et al.*, 1995), and the pathophysiological consequences due to impaired nitric oxide release were mitigated or abolished by providing exogenous nitric oxide donors (Pabla *et al.*, 1995).

Holtz *et al.* showed that the mean arterial pressure (MAP) was significantly lower than that determined before ischemia (Holtz *et al.*, 2001). They suggested that this reduction in MAP during ischemia was a stress reaction in lightly anesthetized rodents, which is consistent with our results. In our study, MAP was significantly lower than the normal control (103.5 ± 5.4 for the normal rats and 85 ± 5.7 mmHg for the ischemic rats). However, the HR was not different from the normal control. Salom *et al* showed that SNP-infused rats did not show significant hypotension when the MAP values during ischemia were compared with the baseline values (Salom *et al.*, 2000). In this study, both aminoguanidine and MK801 had no effect on BP, but nimodipine reduced the BP. Neither aminoguanidine, MK-801, nor nimodipine affected the changes in the HR. There was no difference in the BP or HR between the normal rats and ischemic rats, which were treated with either aminoguanidine, MK-801, or nimodipine.

In summary, the ischemic model caused an increase in the NOx concentration, suggesting that this may be produced via iNOS, which is calcium independent in the brain. However in the heart, ischemia decreased the NOx concentration, and the NMDA receptors were partly involved. The basal BP was lower in the ischemic rats but HR was no different from the normal control, suggesting that increased NOx levels in the brain of ischemic rats may result in the hypotension observed.

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