Nitric oxide in focal cerebral ischemia, an experimental study

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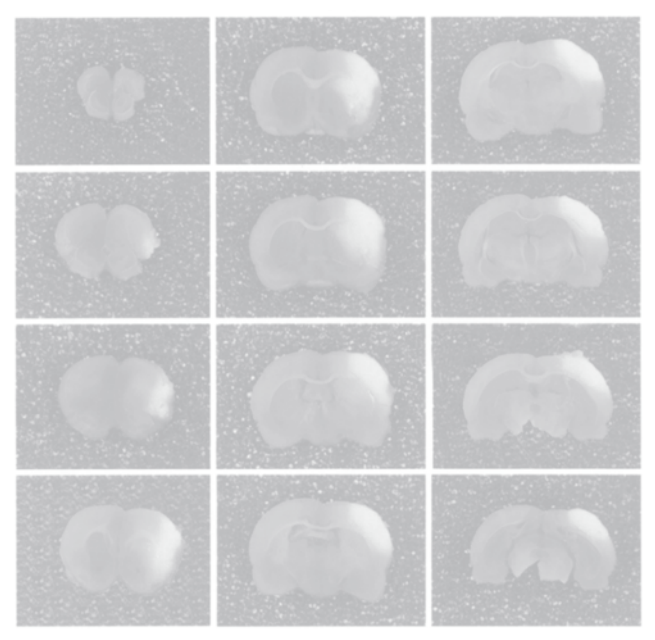
Chapter 6

Effects of the Nitric Oxide Donor 3-Morpholinosydnomine (SIN-1) in Focal Cerebral Ischemia Dependent on Intracellular pH.



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ABSTRACT

A nitric oxide (NO) donor that has been successfully used in the treatment of myocardial infarction, 3-morpholinosydnonimine (SIN-1), may be a potential neuroprotective agent. Production of NO in brain microsomes is dependent on the pH. The purpose of this study was to determine the efficacy of SIN-1 and its dependence on pH in vivo during periods of focal cerebral ischemia. At 0.1 or 1 mg/kg, SIN-1 was administered to 54 Wistar rats 30 minutes before a 2-hour period of focal cerebral ischemia under moderate hypo-, normo-, and hyperglycemic conditions. Measurements of brain intracellular pH (pHi); regional cortical blood flow, and the redox state of nicotinamide adenine dinucleotide were obtained in three additional animals to confirm the effects of the serum glucose manipulations. The animals were killed at 72 hours after the ischemic period to obtain infarction volumes. Administration of SIN-1 significantly reduced infarction in normoglycemic animals and, to a lesser extent, in hyperglycemic animals, indicating that SIN-1 was less effective under hyperglycemic conditions. At either dose SIN-1 had no significant effect on infarction volume in moderately hypoglycemic animals because moderate hypoglycemia in itself significantly ($p < 0.005$) reduced infarction volume. The NO donor SIN-1 may be a useful intraoperative cerebral protective agent. Furthermore, it is hypothesized that a mechanism that could explain the published discrepancies regarding the effects of NO donors in vivo may be affected by differences in ischemic brain acidosis.

INTRODUCTION

The role of NO in the development of ischemic brain damage is complex. Endothelial cells, which produce endothelium-derived relaxing factor NO, regulate the basal tone of cerebral vessels[37], platelet aggregation[36], neutrophil infiltration[7], and neuronal function[44]. The NO donor SIN-1 is currently being used in interventional cardiology to minimize myocardial infarction[17]. Treatment by NO donors in different animal studies has shown a trend toward neuroprotection[11,13,30,39,50-52]; however, isoenzyme- and concentration-dependent dual mechanisms have been proposed to explain both protective and detrimental effects of nonselective inhibitors of NOS at different dosages[15]. Hecker, et al.[22,23], demonstrated that the activities of endothelial and neuronal NOS enzymes[23] were dependent on pH. The "enzyme activity pH curve" was shown to be a narrow bell-shaped curve with an optimal enzyme activity at a pH of 7.6 for eNOS[22] and at a pH of 6.7 for nNOS[23]. Therefore, the variable effects of endogenous NO donors described in different published studies not only might be related to the ischemia model, dosage of the drug treatment, and timing, but also to the effects of intra- and periischemic pHi on NO activity. Supporting this concept is the observation that the nonselective NOS inhibitor, L-NAME, was not as efficacious in situations of severe focal cerebral ischemia compared with those of moderate ischemia[1].

In this study we tested the efficacy of SIN-1, an NO donor, as a neuroprotectant, as well as the hypothesis that the severity of ischemic brain acidosis influences the neuroprotective effects of the NO donor. The degree of brain acidosis was manipulated by altering serum glucose concentrations. A sydnonimine, SIN-1 is considered to be an endogenous endothelium-derived relaxing factor that is produced by the endothelium and is used to mimic the intravascular actions of NO[32]. Given the current technology, it is not possible to measure brain pH and NO simultaneously in vivo. We therefore conducted a comparative analysis of the literature to elucidate the possible effects of pHi on the production of NO.

MATERIALS AND METHODS

Following approval by the Institutional Animal Care and Use Committee, anesthesia was induced in 60 adult male Wistar rats, each weighing between 300 and 450 g. Anesthesia was achieved by administration of halothane in a mixture of O_2 and air, which was given through a face mask at a concentration of 2% during the surgical procedure and 1% during the occlusion period. Atropine was administered subcutaneously (0.08 mg/kg) preoperatively to reduce respiratory secretions. Core body temperature was maintained at 36.5 to 37.5°C by using a heating pad, and the temperature was monitored continu-

ously by using a rectal temperature probe. Polyethylene catheters (type PE-50) were inserted into the femoral artery to monitor arterial blood pressure and to facilitate arterial blood sampling ($PaCO_2$, PaO_2, pH, hematocrit, and serum glucose); similar catheters were inserted into a vein for administration of drugs. These animals' physiological parameters were recorded 30 and 60 minutes before experimental ischemia was initiated, during the 2- hour ischemic period, and 30 minutes after restoration of blood flow. Antibiotic medication (Durapen, 30,000 U) was administered intramuscularly before wound closure.

Model of Focal Cerebral Ischemia

A modification by Coert, et al. [14], of the technique described by Tamura, et al. [45], was used. A 2-cm ventral coronal incision was made in the neck to expose and place snares (No. 2 silk suture) around both CCAs. A 2-cm skin incision was made between the right outer canthus and the tragus of the right ear. The temporal muscle was deflected anteriorly and the zygomatic arch was partially removed. After retraction of the musculature, the mandibular nerve was identified and followed back to the foramen ovale. Using a high-speed drill (Hall Surgical, Largo, FL) a small 3- to 4-mm craniectomy was made just anterior and superior to the foramen ovale. The dura mater was then opened with a sharp needle and the MCA was freed from the arachnoid. The MCA was temporarily occluded for 2 hours at the point where it crosses with the olfactory tract, by using a No. 3 Sundt arteriovenous malformation microclip (Johnson and Johnson Professional, Inc., Raynham, MA). Both CCAs were simultaneously temporarily occluded using the snares. In three animals used for fluorescent imaging, an extra 4 x 5 mm craniectomy was made over the frontal and parietal area of the cortex. The dura was removed and the edges at the margins of the craniectomy were carefully cauterized; the cortex was then covered with plastic wrap (Saran Wrap) to prevent surface oxygenation and keep the brain moist.

Experimental Groups

The animals were randomly divided into nine groups in the following arrangement: 1) there were three control groups: moderate hypoglycemia, normoglycemia, and hyperglycemia (seven rats each); 2) three treatment groups in which SIN-1 (0.1 mg/kg) was administered intravenously to animals with moderate hypoglycemia (seven rats), normoglycemia (six rats), and hyperglycemia (six rats); and 3) three treatment groups in which SIN-1 (1 mg/kg) was administered intravenously to rats with moderate hypoglycemia, normoglycemia, and hyperglycemia (seven rats each). Three additional animals, one with moderate hypoglycemia, one with normoglycemia, and one with hyperglycemia were studied before, during, and after induction of focal cerebral ischemia for imaging of brain pHi, regional cortical blood flow, and the NADH redox state. Moderate hypoglycemia (1.7-4.4 mmol/l) was obtained using a single dose of Humilin-N U-100 (1 U/kg; Eli Lilly and Co., Indianapolis, IN), which was administered subcutaneously 1 hour before ischemia

was induced. Hyperglycemia (16.6-27.8 mmol/l) was achieved by an intravenous infusion of 0.5 g/ml dextrose (50% concentration) at 6 g/kg/hr over a 20-minute period, followed by 1.2 g/kg/hr for maintenance. Fresh solutions of SIN-1 were administered intravenously at 0.1 or 1 mg/kg, dissolved in saline (1 ml/kg) as a bolus injection, 30 minutes before MCA and bilateral CCA occlusion.

In Vivo Fluorescent Instrumentation

To determine changes in brain pHi in the cortex, fluorescent imaging was used before, during, and after focal cerebral ischemia. As has been described previously, pHi as well as regional cortical blood flow, and the NADH redox state, can be measured in vivo by using umbelliferone, a fluorescent indicator[3]. A PE-10 catheter was placed in the right external CA, with the tip at the carotid bifurcation for retrograde injection of umbelliferone. A video-fluorescent microscope was focused on the parietal cortex to measure brain pHi, regional cortical blood flow, and the NADH redox state. The umbelliferone solution (0.2 g in 200 ml of 5% glucose) was injected into the external CA catheter at 30- to 60-minute intervals before, during, and after MCA and bilateral CCA occlusion. The pH indicator, umbelliferone, has two fluorophors, anionic and isobestic, which are excited at 370 and 340 nm, respectively, and have a common emission wavelength of 450 nm. The fluorescence of the anion varies directly with the pH, whereas the isobestic fluorescence varies with the drug concentration. Brain pHi can then be calculated from the 340/370–nm ratio. The NADH fluorescent images excited at 370 nm are acquired before umbelliferone is injected to correct for background fluorescence and for analysis of mitochondrial function[46]. The scale factor for the percentage of change in NADH fluorescence from baseline is set so that 100% represents the level of NADH fluorescence in the healthy brain, whereas an increase to 300% represents brain death. The images obtained from the 340-nm excitation were processed to compute the regional cortical blood flow by using the 1-minute initial slope index with a partition coefficient of unity for umbelliferone[3]. All images of pHi, regional cortical blood flow, and NADH redox states are stored on tape for analysis. Regional cortical blood flow as measured using umbelliferone is defined as those areas that are relatively avascular and primarily contain arterioles and capillary beds[3]. The imaging system allows the measurement of regional cortical blood flow by allowing the investigator to outline cortical areas of interest, which are devoid of major surface conducting vessels.

Histopathological Study

Seventy-two hours after flow restoration, the animals were again placed in a state of anesthesia, induced by pentobarbital, and then intracardially perfused using a warm (37°C) 2% TTC solution. The animals' brains were quickly removed and immersed in the TTC solution for 15 minutes to enhance staining, after which the brains were placed in a 10%

buffered formaldehyde solution for 5 days. Eleven serial coronal sections were cut from each brain at 1-mm intervals and photographed. Total cortical infarction volume was calculated by integrating the infarct areas of all 11 sections (area of infarction in square millimeters x thickness of section). The total infarction volume was multiplied by the ratio between total left hemisphere volume and total right hemisphere volume to correct for cerebral edema[19].

Statistical Analysis

Analysis of variance was followed by the Fisher post hoc test for multiple comparisons to test the statistical significance of differences between groups. The Student unpaired t-test was used to compare measurements between different time points within a group. A probability value lower than 0.05 was considered significant. Data are presented as the means ± standard errors of the means for all groups, with the exception of data obtained from the video images (brain pHi and regional cortical blood flow), which are presented as the means ± standard deviations. All analysis was conducted using STATVIEW statistical software (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

In Vivo Fluorescence Imaging of Brain pHi, regional cortical blood flow, and the NADH Redox State

Brain pHi, the NADH redox state, and regional cortical blood flow were measured in three typical animals (Fig.1 for colour version see backcover). Baseline values measured before initiation of ischemia in the normoglycemic animal (serum glucose 10.7 mmol/L, $PaCO_2$ 45.1 mm Hg, pHa 7.329, and MABP 86 mm Hg) were as follows: brain pHi 7.01 ± 0.03, the NADH redox state 100%, and regional cortical blood flow 66 ± 15.5 ml/100 g/min. After 2 hours of ischemia the brain pHi had declined to 6.58 ± 0.07, the NADH redox state had increased by 44% of baseline, and the regional cortical blood flow had significantly declined to 23.7 ± 13.4 ml/100 g/min. Thirty minutes after restoration of blood flow, the brain pHi was 6.69 ± 0.03, the NADH redox state had decreased to near baseline levels, and the regional cortical blood flow had increased to 85.7 ± 13.8 ml/100 g/min. In the moderately hypoglycemic animal (serum glucose 5.2 mmol/L, PaCO2 43 mm Hg, pHa 7.397, and MABP 86 mm Hg) the baseline brain pHi, which had been 7.01 ± 0.08, had decreased to 6.79 ± 0.06 after 2 hours of ischemia, the NADH redox state had increased by 14% of baseline, and the regional cortical blood flow had declined from 79.8 ± 14.4 to 32.8 ± 10.3 ml/100 g/min. Thirty minutes after restoration of blood flow, the brain pHi was 6.89 ± 0.05, the NADH redox state had decreased by 17%, and the regional cortical blood flow had increased to 89.1 ± 20.1 ml/100 g/min. Baseline values in the hyperglycemic animal (serum glucose 19 mmol/L, $PaCO_2$ 48 mm Hg, pHa 7.436, and MABP 89 mm

Hg) before initiation of ischemia were the following: brain pHi 7.01 ± 0.07, the NADH redox state 100%, and regional cortical blood flow 75.3 ± 24.4 ml/100 g/min. After 2 hours of ischemia, the brain pHi had decreased to 6.12 ± 0.05, the NADH redox state had increased by 75% of baseline, and the regional cortical blood flow declined to 16.4 ± 10.7 ml/100 g/min. Thirty minutes after restoration of blood flow, the brain pHi was 6.45 ± 0.1, NADH redox state had decreased by 60%, and the regional cortical blood flow had increased to 46.4 ± 17.4 ml/100 g/min. The difference in brain pHi during periods of focal cerebral ischemia between the normoglycemic and hyperglycaemic groups was significant ($p < 0.005$).

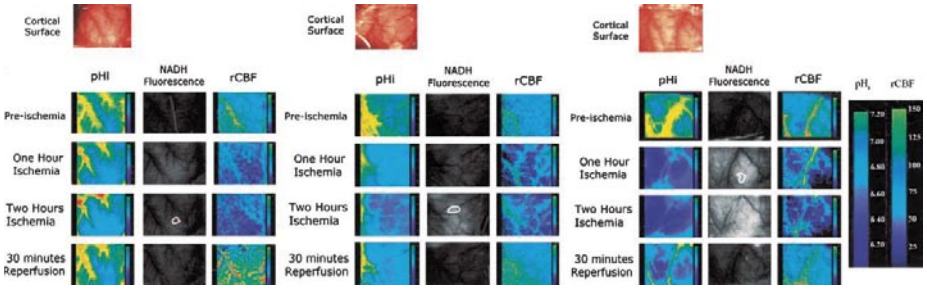

Figure. 1. Still pictures from videotapes of brain pHi, NADH redox state, and regional cortical blood flow (rCBF) in three typical animals during the following periods: moderate hypoglycemia (serum glucose 5.2 mmol/L; left), normoglycemia (serum glucose 10.7 mmol/L; center), and hyperglycemia (serum glucose 19 mmol/L; right). Calibration bars for pHi and regional cortical blood flow are placed at the far right of the three sets of images. The regions of interest (approximately 14,000 µm2) for a comparison of brain pHi, the NADH redox state, and regional cortical blood flow in these experiments are outlined in white. Each video frame originally was approximately 0.5 x 0.5 cm. (For the colour version see the backcover.)

Physiological Parameters

Treatment with SIN-1 did result in a temporary reduction in MABP directly after intravenous injection, but MABP recovered during the 30 minutes between injection of SIN-1 and occlusion of the MCA and CCAs. Weight loss was reduced significantly ($p < 0.05$) in both normoglycemic and hyperglycemic SIN-1–treated animals in response to both low and high doses. The SIN-1 did not significantly affect the glucose response to insulin in the hypoglycemic groups (Table 1).

Infarction Volume

The decrease in serum glucose levels from 10 to 3.3 mmol/L resulted in a significant ($p < 0.001$) reduction in cortical infarction volume by 80%, from 95.8 ± 12 to 19.1 ± 11 mm^3, compared with the normoglycemic group (Fig. 2). Hyperglycemia (glucose level 22.4 mmol/L) resulted in exacerbation of cortical ischemic damage by 178%, to 170.3 ± 14 mm^3 ($p < 0.005$ compared with normoglycemic animals). When compared with the normoglycemic controls, SIN-1 at 0.1 mg/kg significantly decreased infarction volume

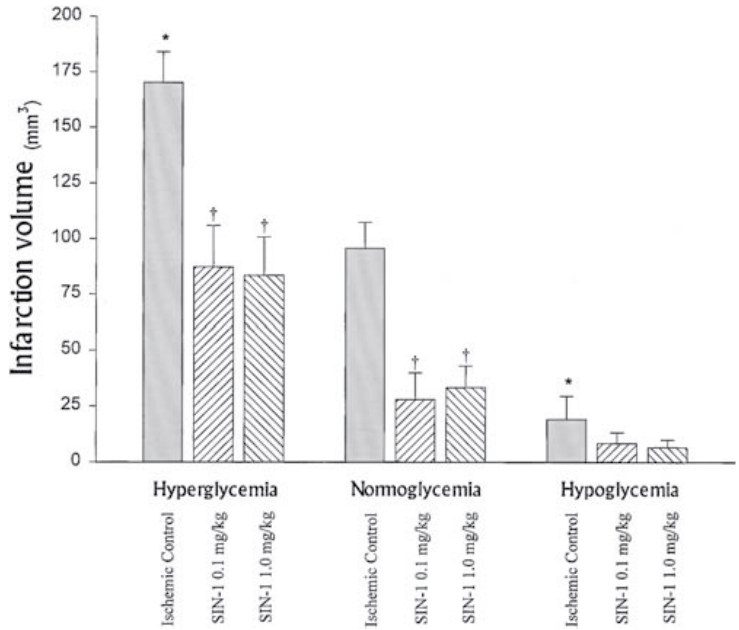

Figure. 2. Bar graph of infarction volumes shown in cubic millimeters comparing animals treated with SIN-1 at 0.1 and 1 mg/kg with ischemic controls during moderate hypoglycemia, normoglycemia, and hyperglycemia. The percentage differences in infarction volumes between treated and nontreated animals were 45 ± 11% and 51 ± 10% in the hyperglycemic group and 71 ± 13% and 69 ± 10% in the normoglycemic group for 0.1 mg/kg and 1 mg/kg SIN-1, respectively. Therefore, SIN-1 was less effective in reducing infarction volume as brain pHi became more acidic. During moderate hypoglycemia, differences between the treated and nontreated groups were 56 ± 27% and 66 ± 19% for 0.1 and 1 mg/kg SIN-1, respectively; this was not statistically significant. Data are expressed as the means ± standard errors of the means. *Statistically different from normoglycemic ischemic control values ($p < 0.005$). †Statistically different from respective ischemic control values ($p < 0.025$, analysis of variance).

by 71% ($p < 0.003$), from 95.8 ± 12 to 27.9 ± 12 mm^3. Increasing the dose of SIN-1 to 1 mg/kg also caused a significant reduction in ischemic damage by 69% ($p < 0.018$), to 33.4 ± 10 mm^3, compared with the normoglycemic control group. Infarction volumes between the two normoglycemic SIN-1–treated groups were not significantly different. In hyperglycemic animals SIN-1 treatment at 0.1 and 1 mg/kg significantly reduced cortical infarction volume by 45% ($p < 0.025$) and 51% ($p < 0.023$), from 170.3 ± 14 mm^3 to 87.3 ± 18.7 and 83.5 ± 17.3 mm^3, respectively. Increasing the SIN-1 treatment dose in hyperglycemic animals from 0.1 to 1 mg/kg did not significantly decrease cortical infarction volume. The differences in infarction volumes between the SIN-1- treated normoglycemic groups (69-71%) and SIN–treated hyperglycemic groups (45–51%) was statistically significant ($p < 0.05$). In the insulin-induced moderately hypoglycemic animals, SIN-1 tended to reduce infarction volume at both dosages of 0.1 mg/kg (8.4 ± 4.9 mm^3) and 1 mg/kg (6.6 ± 3.4 mm^3), but this reduction did not reach statistical significance ($p < 0.22$ and $p < 0.29$, respectively).

Table 1 The mean systemic parameters in the study groups

Group	$PaCO_2$ (mm Hg)	PaO_2 (mm Hg)	pH_a	MABP (mm Hg)	Glucose (mmol/L)	Hematocrit (%)	Temperature (°C)	Weight Loss (%)
hypoglycemia								
ischemic control group								
before occlusion	36.7 ± 0.8	210.3 ± 7.6	7.437 ± 0.012	79.8 ± 2.7	4.0 ± 0.6†	39.5 ± 1.0	37.0 ± 0.1	
at 1 hr ischemia	40.7 ± 2.2	190.4 ± 7.3	7.408 ± 1.015	80.7 ± 1.0	3.0 ± 0.6†	38.9 ± 0.5	37.0 ± 0.1	
at 2 hrs ischemia	39.1 ± 1.9	180.7 ± 7.0	7.435 ± 0.017	83.2 ± 2.9	3.0 ± 0.5†	38.4 ± 0.6	36.9 ± 0.1	0.6 ± 1.9
SIN-1 0.1 mg/kg treatment group								
before occlusion	34.8 ± 1.7	194.7 ± 19.0	7.454 ± 0.020	84.0 ± 3.9	4.2 ± 0.4†	36.2 ± 1.1	38.3 ± 0.4	
at 1 hr ischemia	42.4 ± 1.5	193.3 ± 9.0	7.416 ± 0.016	89.1 ± 2.7	2.7 ± 0.6†	36.0 ± 1.5	38.2 ± 0.4	
at 2 hrs ischemia	41.0 ± 2.3	181.3 ± 7.0	7.412 ± 0.021	89.5 ± 3.0	2.4 ± 0.3†	36.2 ± 1.2	38.2 ± 0.4	5.6 ± 2.7
SIN-1 1 mg/kg treatment group								
before occlusion	39.7 ± 1.5	212.9 ± 3.7	7.442 ± 0.013	81.4 ± 2.2	3.5 ± 0.6†	39.2 ± 0.6	37.0 ± 0.0	
at 1 hr ischemia	36.4 ± 1.6	207.1 ± 5.9	7.450 ± 0.015	84.4 ± 2.9	2.3 ± 0.2†	38.1 ± 1.1	37.0 ± 0.1	
at 2 hrs ischemia	38.9 ± 1.5	195.1 ± 6.7	7.431 ± 0.016	84.9 ± 2.8	1.9 ± 0.3†	39.2 ± 0.5	37.0 ± 1.0	6.4 ± 2.3
normoglycemia								
ischemic control group								
before occlusion	37.8 ± 2.6	185.0 ± 10.2	7.442 ± 0.047	81.9 ± 1.9	9.8 ± 1.0	37.7 ± 1.0	36.9 ± 0.1	
at 1 hr ischemia	37.7 ± 4.1	163.4 ± 10.7	7.463 ± 0.041	80.8 ± 2.1	9.5 ± 1.1	36.9 ± 1.1	36.9 ± 0.1	
at 2 hrs ischemia	36.1 ± 2.5	158.1 ± 13.3	7.479 ± 0.040	87.4 ± 3.6	9.7 ± 0.8	35.1 ± 1.7	37.0 ± 0.2	15.2 ± 2.3
SIN-1 0.1 mg/kg treatment group								
before occlusion	36.3 ± 1.4	191.3 ± 8.8	7.429 ± 0.013	82.0 ± 2.9	8.2 ± 0.5	38.5 ± 0.9	37.0 ± 1.0	
at 1 hr ischemia	41.8 ± 0.6	190.5 ± 11.1	7.401 ± 0.013	86.3 ± 2.9	7.6 ± 0.4	38.4 ± 1.7	37.0 ± 1.0	
at 2 hrs ischemia	40.4 ± 0.7	178.3 ± 17.0	7.404 ± 0.008	77.7 ± 6.0	7.3 ± 0.5	37.2 ± 1.4	37.0 ± 1.0	2.5 ± 1.6‡
SIN-1 1 mg/kg treatment group								
before occlusion	34.0 ± 1.1	163.0 ± 12.6	7.424 ± 0.018	82.6 ± 2.2	8.4 ± 0.5	37.6 ± 0.4	37.0 ± 1.0	
at 1 hr ischemia	38.3 ± 1.6	161.9 ± 10.9	7.418 ± 0.016	84.3 ± 1.5	8.1 ± 0.7	37.6 ± 0.8	37.0 ± 1.0	
at 2 hrs ischemia	39.5 ± 1.9	162.4 ± 10.9	7.421 ± 0.012	90.8 ± 1.5	8.4 ± 0.9	37.4 ± 1.2	37.0 ± 1.0	6.4 ± 1.8‡
hyperglycemia								
ischemic control group								
before occlusion	42.5 ± 3.1	166.0 ± 21.0	7.372 ± 0.017	86.0 ± 1.0	15.9 ± 3.8†	37.5 ± 0.9	36.9 ± 0.1	
at 1 hr ischemia	41.1 ± 1.1	160.2 ± 20.2	7.362 ± 0.024	87.0 ± 1.0	26.5 ± 0.7†	37.1 ± 0.7	37.0 ± 0.1	
at 2 hrs ischemia	39.8 ± 1.1	155.5 ± 18.3	7.400 ± 0.018	86.0 ± 1.0	18.5 ± 2.2†	36.3 ± 0.5	37.0 ± 0.1	15.7 ± 2.2
SIN-1 0.1 mg/kg treatment group								
before occlusion	40.7 ± 2.2	168.3 ± 7.9	7.377 ± 0.020	86.8 ± 1.5	22.9 ± 2.2†	38.5 ± 1.0	37.0 ± 1.0	
at 1 hr ischemia	39.7 ± 1.7	154.7 ± 15.8	7.384 ± 0.019	84.6 ± 2.9	21.4 ± 1.7†	37.0 ± 1.6	37.0 ± 1.0	
at 2 hrs ischemia	43.2 ± 1.8	145.8 ± 11.4	7.372 ± 0.016	87.2 ± 1.7	18.3 ± 0.3†	38.8 ± 1.6	37.0 ± 1.0	6.4 ± 1.6‡
SIN-1 1 mg/kg treatment group								
before occlusion	35.0 ± 2.1	190.1 ± 4.0	7.403 ± 0.013	82.2 ± 1.4	22.4 ± 2.1†	35.1 ± 1.3	37.0 ± 1.0	
at 1 hr ischemia	41.0 ± 1.9	181.7 ± 9.8	7.390 ± 0.014	87.6 ± 2.1	22.7 ± 1.1†	35.4 ± 1.2	37.0 ± 1.0	
at 2 hrs ischemia	39.5 ± 3.0	188.7 ± 5.4	7.410 ± 0.022	87.0 ± 0.9	20.9 ± 1.2†	36.0 ± 1.1	37.0 ± 1.0	8.0 ± 1.9‡

* Values are expressed as the means ± standard errors of the means.

† Statistically different from normoglycemic control values ($p < 0.05$).

‡ Statistically different from respective ischemic control values ($p < 0.05$).

DISCUSSION

Three hypotheses can be supported by this study. First, SIN-1, an NO donor that has been used in the treatment of myocardial infarction may also be useful as an intraoperative neuroprotectant and as a treatment for stroke. Second, the efficacy of SIN-1 is dependent on brain pHi, and may be more effective in cases of moderate ischemia than in those of severe ischemia. Third, when evaluating the potential effects of pharmaceutical agents on the treatment of central nervous system disorders, such as cerebrovascular disease and brain tumors, brain pHi must be considered to be a possible influencing factor.

The NO Donor SIN-1

The NO donor SIN-1 is currently used in interventional cardiology because it produces antispastic and vasodilatory effects without inducing tolerance[17]. In human acute coronary syndromes, titration of SIN-1 to the desired antiischemic effect was suggested to lie within the flow rate of 0.2 and 1.6 mg/hour, and titration to the desired vasodilatory

effect, without untoward action on filling pressures, cardiac index, or heart rate was said to lie with doses of approximately 1 mg/hour. Comparing these suggested dosages with the dosages used in the present study and in other animal studies[39,50,51,52] we have to conclude that the amounts used in this study, 0.1 and 1 mg/kg in rats weighing between 300 and 450 g, are relatively high. In this analysis of SIN-1, Feelisch, et al. [16], reported that simultaneous with NO release, SIN-1 generates superoxide. Bohn and Schönafinger[8] identified two factors that influence NO release, PO_2 and pH, and found that the oxidative capacity in vivo was high enough to guarantee NO release under ischemic conditions. Singh, et al. [41], reported that the pure NO donor behaviour exhibited by SIN-1 in vivo, without superoxide production, occurred because of the presence of biological electron acceptors that outcompete O_2. Combining the data from Noack and Feelisch[32] on time dependent formation of various metabolites and their velocity of initial NO liberation, the overall half-life for the NO donation of SIN-1 was estimated to be 230 minutes. As we reported earlier[13] and in the present study, we observed a temporary reduction in MABP after intravenous administration of SIN-1, but MABP returned to baseline within 30 minutes between injection of the drug and onset of ischemia. Although reports of the protective effect of NO donors such as SIN-1 have been limited primarily to permanent ischemia models, NO donors have been demonstrated to be efficacious in the present study and in the work of Salom, et al. [39], in which a model of temporary focal cerebral ischemia was used. Proposed mechanisms of protection include the following: the reduction of activation and recruitment of neutrophils that produce cytokines and proteases, amplifying endothelial dysfunction and promoting tissue damage, and the loss of vasomotor control contributing to the "no reflow" phenomenon[24]. The agent SIN-1 also has a negative insulinotropic action[5,42], whereas L-arginine was found to induce insulin secretion from pancreatic ß cells[40]. In their study, Zhang, et al. [52], reported an approximately 20% increase in glucose levels in SIN-1- and phenylephrine-treated groups, but found in a separate group that SIN-1 (3 mg/kg) without phenylephrine did not significantly alter serum glucose levels, thus attributing that effect to phenylephrine. In our experiment in normoglycemic, moderately hypoglycemic, and hyperglycemic rats, SIN-1 treatment did not result in glucose levels that were different from those measured in the moderately hypo-, normo-, and hyperglycemic control groups. In this study, the efficacy of SIN-1, expressed as a percentage of reduction in cortical infarction volume, was approximately 70% in rats with normoglycemia, approximately 61% in rats with hypoglycemia, and 48% in rats with hyperglycemia. Therefore, SIN-1 was less effective under more acidic brain conditions. These data closely mirror the endothelial cNOS response curve that was demonstrated in vitro by Hecker, et al.[22]. In their study, the concentration of hydrogen ions was found to be an important determinant of endothelial cNOS activity, whereas pH sensitivity was lost one unit above or below the optimum pH 7.6). Similar studies on pH sensitivity of the neuronal isoform of cNOS revealed an optimal pH of 6.7 within

a bell-shaped curve similar to endothelial cNOS[23]. Combining their data on both cNOS enzymes, we can conclude that, within the pH range encountered (6.12- 6.82), enzyme activity would vary considerably. The following supports this hypothesis: 1) increased intracellular acidosis achieved by augmenting the severity of ischemia caused a loss of the neuroprotective effect by the nonselective NOS inhibitor, L-NAME, suggesting that NOS inhibition was less effective because of inhibition of NOS activity in acidosis[1]; 2) in a separate study (Chapter 5), administration of 7-NI, a selective nNOS inhibitor, was far less effective during periods of hyperglycemia (27.5% reduction compared with approximately 48% in this study) and more effective during periods of both normoglycemia (93.5% reduction compared with approximately 70% in this study) and hypoglycemia (72.6% reduction compared with approximately 61% in this study). Based on the data the following hypothesis can be supported. In the healthy brain, NO is produced to maintain basal tone. In the brain with cerebral ischemia, NO production increases as the brain becomes ischemic, to a pH optimum of NO (approximately 6.7- 6.8). Thereafter, production decreases as the brain becomes more acidotic. Therefore, it follows that, with worsening acidosis, a pH below approximately 6.7, NO donors become more effective compared with NOS inhibitors, and with improving acidosis, a pH above approximately 6.7, inhibition of nNOS becomes more effective.

Role of NO in Cerebral Ischemia

Direct measurements of NO production in vivo have revealed both increased[28] and decreased NO concentration during cerebral ischemia[29]. Using a porphyrinic microsensor, Malinski, et al. [28], reported an increase from a baseline level of lower than 10^{-8} M to an approximate level of 10^{-6} M in cases of focal ischemia. Using Nafion and porphyrine-coated carbon fiber electrodes, Lin, et al. [26], recorded NO production in rats during 40 minutes of combined MCA and bilateral CCA occlusion. Basal extracellular NO concentration increased to a mean of 18.8 ± 3.4 nmol/L. The differences between these values and those reported by Malinski, et al.[28], were explained by the smaller tip of the probe, a different occlusion technique, more superficial cortical measurements, and the higher selectivity of the probe[26]. In a cat model of focal cerebral ischemia, NO concentrations were shown to increase during the first 10 minutes in regions exhibiting depolarization. The course of NO production after this was found to be variable[34] and heterogeneous, ranging from a continuous reduction to a sustained overproduction[35]. It was suggested that NO production could be pH dependent[35]. Altogether, these data suggest that outcome is determined by the individual contributions of eNOS and nNOS through their specific and local effects rather than by the absolute concentration of NO during ischemia. A limited number of in vivo studies have been performed in which the effect of NO donors in focal cerebral ischemia was shown to be primarily protective [30,39,50-52]. Differences in methodology, including animal models, anesthetic agents, occlusion techniques, duration of ischemia, and

drug delivery and dosing complicate a comparison of effects in these different studies. In the study by Morikawa, et al. [30], in which 300 mg/kg L-arginine was used as a treatment dose, a 28 to 35% reduction in ischemic damage was reported. Sodium nitroprusside was used in three studies[39,50,52] in dosages ranging from 0.11 mg/kg/hr (total dose 0.22 mg/kg/hr) to 3 mg/kg/hr (total dose 3 mg/kg/hr), resulting in reductions in infarction volume of 67 and 27%, respectively. In a study by Salom, et al. [39], a high dose of SNP (1.1 mg/kg/hr administered intravenously for 2 hours), which did not significantly reduce regional cortical blood flow but significantly reduced MABP, did not attenuate ischemic damage compared with a lower dose (0.11 mg/kg/hr), which improved treatment outcome. In contrast, a high dose of SNP with addition of phenylephrine (10–100 µg/hour intracarotid infusion) to prevent hypotension was protective in the study by Zhang, et al. [52] At 3 mg/kg/hr SIN-1 was shown to attenuate ischemic damage in a permanent model of focal ischemia when administered up to 60 minutes after onset of ischemia[51]. In a previous study by Coert, et al. [13], intravenous administration of SIN-1 at 1 mg/kg reduced the mean cortical infarction volume, but this reduction was not statistically significant. Reducing occlusion times from 3 to 2 hours in the present study did not significantly change cortical infarction volume or variability, although a reduction in the occlusion time from 3 hours to 1 hour of ischemia in this same model did just that[14]. Initiating intravenous SNP (0.19 µg/kg/min) in patients with white-matter lacunar (four patients) or cortical infarcts (18 patients) at a mean of 21.3 hours (range 9.3–27 hours) after onset of stroke, Butterworth, et al. [11], were able to improve cerebral blood flow, although MABP was reduced. A reduction in platelet function was also found in patients who were not on a regimen of aspirin prior to their ischemic strokes. Overall outcome in the SNP-treated group, however, was not different from that of the control group[11].The neuroprotectiveness of NO donors can be attributed to either a parenchymal or vascular effect. It has been demonstrated that NO donors enhance regional cortical blood flow by vasodilation, which reduces neuronal damage in the area surrounding the ischemic core[50-52]. These studies used either SNP or SIN-1, which was given by intracarotid infusion, whereas in three other studies[12,30,39] these donors were given intravenously and demonstrated no significant changes in regional cortical blood flow. This may suggest that intracarotid infusion may result in a higher concentration of NO donor in the brain, thereby exerting a profound dilatory effect, or there could be a loss of vascular reactivity because of cerebral ischemia. In this study we chose the intravenous route for administration of SIN-1 because clinically it is routinely given intravenously. On the other hand, a parenchymal effect of NO could, for example, decrease neuronal death by attenuating the rise in intracellular calcium[27] or by reducing free radical formation[47]. Inhibition of NOS also has been demonstrated in a number of published reports to be either neuroprotective[2,6,9,10,13,31,38] or neurotoxic[20,25,33,49]. This suggests in part that NO can be neuroprotective or neurotoxic, depending on the ischemic environment, which in part may be pH dependent.

Model of Graded Focal Cerebral Ischemia

To ascertain the relationship between intracellular brain acidosis and NO, animals were subjected to moderate hypo-, normo-, or hyperglycemia to provide models of three graded levels of ischemia. There have been several studies[4,43] in which brain pHi has been measured before, during, and after global and focal cerebral ischemia. Brain pHi becomes more acidotic during ongoing ischemia, declining from approximately 6.7 to less than 6 in response to increasing serum glucose levels (approximately 6.5 mmol/L to > 28 mmol/L). Conversely, as serum glucose levels become more hypoglycemic (approximately 6.7- 7 mmol/L to approximately 7 mmol/L), brain pHi becomes less acidotic (6.7–7.0) [4,43]. Cerebral infarction is reduced under moderate hypoglycemic conditions, whereas it becomes exacerbated under hyperglycemic conditions[4,21]. In our present study, hypoglycemia (serum glucose level of approximately 3 mmol/L) reduced the cortical infarction volume by approximately 80%, from 95.8 ± 12 to 19.1 ± 10 mm^3 in the normoglycemic control group, whereas in the hyperglycemic animals there was a 178% increase in infarction volume (to 170.3 ± 14 mm^3).

CONCLUSIONS

In this experiment the protective effect of NO enhancement by SIN-1 during focal cerebral ischemia was altered by serum glucose concentrations, which in effect reflected a manipulation of brain pHi. As a neuroprotectant in this study SIN-1 was significantly effective in reducing infarction volume in animals with hyperglycemia and, to a greater extent, in animals with normoglycemia. The successful use of NO donors in the treatment of myocardial ischemia makes them attractive candidates for use as neuroprotective agents. The apparent effect of pHi on NO is consistent with in vitro data[22,23]. We propose that brain pHi is an important factor for determining NO activity and that the observed variability in effects of NO enhancement indifferent models of cerebral ischemia is partly due to differences in brain pHi during ischemia. The effect of pH on NO enhancement provides an additional mechanism by which acidosis contributes to ischemic brain damage. We also propose that, depending on the environment, brain pHi might influence how pharmaceutical agents perform as a treatment modality. For example, brain tumors have been shown to alter pHi[18,28]. Further investigations will need to be undertaken to elucidate the mechanism by which the concentration of H^+ affects NO activity.

REFERENCES

1. Anderson RE, Meyer FB: Is intracellular brain pH a dependent factor in NOS inhibition during focal cerebral ischemia? Brain Res 856:220–226, 2000
2. Anderson RE, Meyer FB: Nitric oxide synthase inhibition by LNAMEduring repetitive focal cerebral ischemia in rabbits. Am J Physiol 271:H588–H594, 1996
3. Anderson RE, Meyer FB, Tomlinson FH: Focal cortical distribution of blood flow and brain pHi determined by in vivo fluorescent imaging. Am J Physiol 263:H565–H575, 1992
4. Anderson RE, Tan WK, Martin HS, et al: Effects of glucose and PaO2 modulation on cortical intracellular acidosis, NADH redox state, and infarction in the ischemic penumbra. Stroke 30: 160–170, 1999
5. Antoine MH, Ouedraogo R, Hermann M, et al: 3-Morpholinosydnonimine as instigator of a glibenclamide-sensitive reduction in the insulin secretory rate. Biochem Pharmacol 53: 1211–1213, 1997
6. Ashwal S, Cole DJ, Osborne TN, et al: Dual effects of LNAME during transient focal cerebral ischemia in spontaneously hypertensive rats. Am J Physiol 267:H276–H284, 1994
7. Batteur-Parmentier S, Margaill I, Plotkine M: Modulation by nitric oxide of cerebral neutrophil accumulation after transient focal ischemia in rats. J Cereb Blood Flow Metab 20: 812–819, 2000
8. Bohn H, Schönafinger K: Oxygen and oxidation promote the release of nitric oxide from sydnonimines. J Cardiovasc Pharmacol 14 (Suppl 11):S6–S12, 1989
9. Buisson A, Margaill I, Callebert J, et al: Mechanisms involved in the neuroprotective activity of a nitric oxide synthase inhibitor during focal cerebral ischemia. J Neurochem 61:690–696, 1993
10. Buisson A, Plotkine M, Boulu RG: The neuroprotective effect of a nitric oxide inhibitor in a rat model of focal cerebral ischemia. Br J Pharmacol 106:766–767, 1992
11. Butterworth RJ, Cluckie A, Jackson SHD, et al: Pathophysiological assessment of nitric oxide (given as sodium nitroprusside) in acute ischaemic stroke. Cerebrovasc Dis 8:158–165, 1998
12. Chi OZ, Wei HM, Weiss HR: Effects of CAS 754, new nitric oxide donor, on regional cerebral blood flow in focal cerebral ischemia. Anesth Analg 80:703–708, 1995
13. Coert BA, Anderson RE, Meyer FB: A comparative study of the effect of two nitric oxide synthase inhibitors and two nitric oxide donors on temporary focal ischemia in the Wistar rat. J Neurosurg 90:332–338, 1999
14. Coert BA, Anderson RE, Meyer FB: Reproducibility of cerebral cortical infarction in the Wistar rat after middle cerebral artery occlusion. J Stroke Cerebrovasc Dis 8:380–387, 1999
15. Dalkara T, Yoshida K, Irikura K, et al: Dual role of nitric oxide in focal cerebral ischemia. Neuropharmacology 33: 1447–1452, 1994
16. Feelisch M, Ostrowski J, Noack E: On the mechanism of NO release from sydnonimines. J Cardiovasc Pharmacol 14 (Suppl 11):S13–S22, 1989
17. Foucher-Lavergne A, Kolsky H, Spreux-Varoquaux O, et al: Hemodynamics, tolerability, and pharmacokinetics of linsidomine (SIN-1) infusion during the acute phase of uncomplicated myocardial infarction. J Cardiovasc Pharmacol 22: 779–784, 1993
18. Gerweck LE, Seetharaman K: Cellular pH gradient in tumor versus normal tissue: potential exploitation for the treatment of cancer. Cancer Res 56:1194–1198, 1996

19. Golanov EV, Reis DJ: Contribution of cerebral edema to the neuronal salvage elicited by stimulation of cerebellar fastigial nucleus after occlusion of the middle cerebral artery in rat. J Cereb Blood Flow Metab 15:172–174, 1995

20. Hamada J, Greenberg JH, Croul S, et al: Effects of central inhibition of nitric oxide synthase on focal cerebral ischemia in rats. J Cereb Blood Flow Metab 15:779–786, 1995

21. Hamilton HG, Tranmer BI, Auer RN: Insulin reduction of cerebral infarction due to transient focal ischemia. J Neurosurg 82:262–268, 1995

22. Hecker M, Mülsch A, Bassenge E, et al: Subcellular localization and characterization of nitric oxide synthase(s) in endothelial cells: physiologic implications. Biochem J 299:247–252, 1994

23. Hecker M, Mülsch A, Busse R: Subcellular localization and characterization of neuronal nitric oxide synthase. J Neurochem 62:1524–1529, 1994

24. Johnson D, Freischlag JA, Lesniak R, et al: Endothelial damage due to ischemia and reperfusion is prevented with SIN-1. Cardiovasc Surg 6:367–372, 1998

25. Kuluz JW, Prado RJ, Dietrich WD, et al: The effect of nitric oxide synthase inhibition on infarct volume after reversible focal cerebral ischemia in conscious rats. Stroke 24: 2023–2029, 1993

26. Lin SZ, Chiou AL, Wang Y: Ketamine antagonizes nitric oxide release from cerebral cortex after middle cerebral artery ligation in rats. Stroke 27:747–752, 1996

27. Lipton SA, Choi YB, Pan ZH, et al: A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. Nature 364:626–632, 1993

28. Malinski T, Bailey F, Zhang ZG, et al: Nitric oxide measured by a porphyrinic microsensor in rat brain after transient middle cerebral artery occlusion. J Cereb Blood Flow Metab 13: 355–358, 1993

29. Mason RB, Pluta RM, Walbridge S, et al: Production of reactive oxygen species after reperfusion in vitro and in vivo: protective effect of nitric oxide. J Neurosurg 93:99–107, 2000

30. Morikawa E, Moskowitz MA, Huang Z, et al: L-arginine infusion promotes nitric oxide-dependent vasodilation, increases regional blood flow, and reduces infarct volume in the rat. Stroke 25:429–435, 1994

31. Nishikawa T, Kirsch JR, Koehler RC, et al: Nitric oxide synthase inhibition reduces caudate injury following transient focal ischemia in cats. Stroke 25:877–885, 1994

32. Noack E, Feelisch M: Molecular aspects underlying the vasodilator action of molsidomine. J Cardiovasc Pharmacol 14 (Suppl 11):S1–S5, 1989

33. Nowicki JP, Duval D, Poignet H, et al: Nitric oxide mediates neuronal death after focal cerebral ischemia in the mouse. Eur J Pharmacol 204:339–340, 1991

34. Ohta K, Graf R, Rosner G, et al: Early nitric oxide increase in depolarized tissue of cat focal cerebral ischemia. Neuroreport 8:143–148, 1996

35. Ohta K, Graf R, Rosner G, et al: Profiles of cortical tissue depolarization in cat focal cerebral ischemia in relation to calcium ion homeostasis and nitric oxide production. J Cereb Blood Flow Metab 17:1170–1181, 1997

36. Radomski MW, Palmer RMJ, Moncada S: An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. Proc Natl Acad Sci USA 87:5193–5197, 1990

37. Rees DD, Palmer RMJ, Moncada S: Role of the endotheliumderived nitric oxide in the regulation of blood pressure. Proc Natl Acad Sci USA 86:3375–3378, 1989

38. Regli L, Held MC, Anderson RE, et al: Nitric oxide synthase inhibition by L-NAME prevents brain acidosis during focal cerebral ischemia in rabbits. J Cereb Blood Flow Metab 16: 988–995, 1996

39. Salom JB, Orti JM, Centeno JM, et al: Reduction of infarct size by the NO donors sodium nitroprusside and spermine/NO after transient focal cerebral ischemia in rats. Brain Res 865: 149–156, 2000

40. Schmidt HH, Warner TD, Ishii K, et al: Insulin secretion from pancreatic B cells caused by L-arginine-derived nitrogen oxides. Science 255:721–723, 1992

41. Singh RJ, Hogg H, Joseph J, et al: The peroxynitrite generator, SIN-1, becomes a nitric oxide donor in the presence of electron acceptors. Arch Biochem Biophys 361:331–339, 1999

42. Sjöholm Å: Nitric oxide donor SIN-1 inhibits insulin release. Am J Physiol 271:C1098–C1102, 1996

43. Smith ML, von Hanwehr R, Siesjö BK: Changes in extra- and intracellular pH in the brain during and following ischemia in hyperglycemic and in moderately hypoglycemic rats. J Cereb Blood Flow Metab 6:574–583, 1986

44. Snyder SH: Nitric oxide: first in a new class of neurotransmitters. Science 257:494–496, 1992

45. Tamura A, Graham DI, McCulloch J, et al: Focal cerebral ischaemia in the rat: 1. Description of technique and early neuropathological consequences following middle cerebral artery occlusion. J Cereb Blood Flow Metab 1:53–60, 1981

46. Tomlinson FH, Anderson RE, Meyer FB: Acidic foci within the ischemic penumbra of the New Zealand white rabbit. Stroke 24:2030–2040, 1993

47. Wink DA, Hanbauer I, Krishna MC, et al: Nitric oxide protects against cellular damage and cytotoxicity from reactive oxygen species. Proc Natl Acad Sci USA 90:9813–9817, 1993

48. Winter PM, Poptani H, Bansal N: Effects of chemotherapy by 1,3-bis(2-chloroethyl)-1-nitrosourea on single-quantum- and triple-quantum-filtered 23Na and 31P nuclear magnetic resonance of the subcutaneously implanted 9L glioma. Cancer Res 61:2002–2007, 2001

49. Yamamoto S, Golanov EV, Berger SB, et al: Inhibition of nitric oxide synthesis increases focal ischemic infarction in rat. J Cereb Blood Flow Metab 12:717–726, 1992

50. Zhang F, Iadecola C: Nitroprusside improves blood flow and reduces brain damage after focal ischemia. Neuroreport 4: 559–562, 1993

51. Zhang F, Iadecola C: Reduction of focal cerebral ischemia by delayed treatment with nitric oxide donors. J Cereb Blood Flow Metab 14:574–580, 1994

52. Zhang F, White JG, Iadecola C: Nitric oxide donors increase blood flow and reduce brain damage in focal ischemia: evidence that nitric oxide is beneficial in the early stages of cerebral ischemia. J Cereb Blood Flow Metab 14:217–226, 1994