Nitric oxide in focal cerebral ischemia, an experimental study

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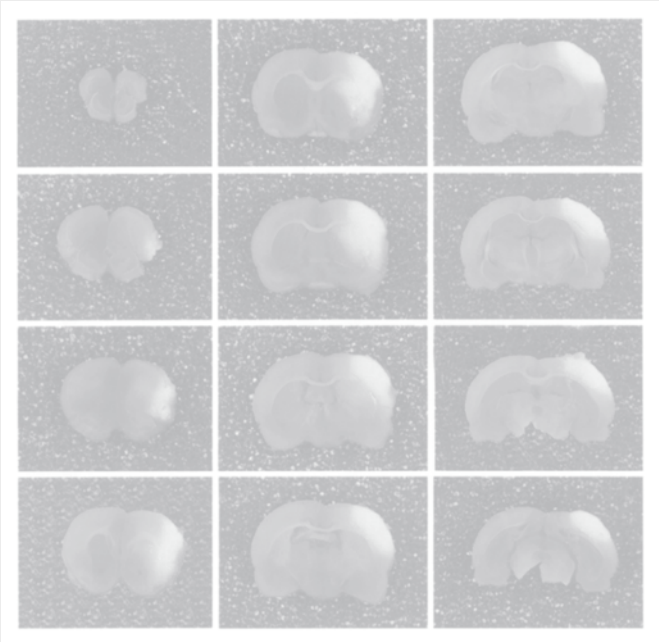
Chapter 5

Is the Neuroprotective Efficacy of nNOS Inhibitor 7-NI Dependent on Ischemic Intracellular pH?



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ABSTRACT

The purpose of this study was to test the hypothesis that the efficacy of 7-nitroindazole (7-NI), a selective neuronal nitric oxide (NO) synthase (NOS) inhibitor, is pH dependent in vivo during focal cerebral ischemia. Wistar rats underwent 2 hours of focal cerebral ischemia under 1% halothane anesthesia. 7-NI, 10 and 100 mg/kg in 0.1 ml/kg DMSO, was administered 30 minutes before occlusion. Ischemic brain acidosis was manipulated by altering serum glucose concentrations. Confirmation of the effects of these serum glucose manipulations on brain intracellular pH (pHi) was obtained in a group of acute experiments utilizing umbelliferone fluorescence. The animals were euthanized at 72 hours for histology. 7-NI significantly ($P < 0.05$) reduced infarction volume in both the normoglycemic by 93.3% and hyperglycemic animals by 27.5%. In the moderate hypoglycemic animals, the reduction in infarction volume did not reach significance because moderate hypoglycaemia in itself dramatically reduced infarction volume. We hypothesize that a mechanism to explain the published discrepancies of the results with neuronal NOS inhibitors in vivo may be effects of different levels of ischemic brain acidosis on the production of NO.

INTRODUCTION

Elaborate studies on the role of nitric oxide (NO) in the pathophysiology of stroke have revealed complex and contradictory results[34,55]. The effects of NO synthase (NOS) inhibitors such as N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine methyl ester (L-NAME), and 7-nitroindazole (7-NI) for treatment of cerebral ischemia vary from protective to cytotoxic[1,2,3,7,10,17,19,22,26,27,30,35,41,44,38,49,53,59,66,67,70] depending on the ischemia model, isoenzyme specificity, the timing, and dosage administered. In the early phase of cerebral ischemia, NO has been shown to be beneficial[69] by increasing collateral circulation and inhibiting platelet aggregation. Treatment with NO donors[69] and with L-arginine [47]during this phase has been shown to be effective in reducing ischemic brain damage. Neuronal NOS (nNOS) activation appears to increase ischemic damage, whereas selective inhibition reduces cerebral infarct volume[17,19,22,26,30,35,49,67]. It has been reported that nNOS-deficient mice develop smaller infarcts after middle cerebral artery (MCA) occlusion[33]. However, the severity of the ischemic insult may determine the neuroprotective efficacy of NOS inhibitors[3,30]. For example, severe ischemia resulted in loss of protective effects by the nonselective NOS inhibitor L-NAME[3] and nNOS inhibitor AR-R17477[30]. It has been hypothesized that increasing severity of ischemia results in more pronounced intracellular acidosis, which subsequently attenuates NOS enzyme activity[3]. Three in vitro studies[25,31,54] have demonstrated a biphasic pH sensitivity for NOS enzymes with a pH optimum for nNOS of 6.7-7.0[25,31,54]. Accordingly, the variable published effects of NOS inhibition might not only be related to the ischemia model, dosage, and timing but also to the effects of intra- and peri-ischemic intracellular pH (pHi) on NOS activity. To investigate the possible influence of brain pHi on nNOS inhibition in vivo, we utilized hyperglycemic and moderate hypoglycemic conditions during cerebral ischemia to exacerbate and attenuate intracellular acidosis and compared the protective effects of selective nNOS inhibition on ischemic brain damage. Given the current technology, it is not possible to simultaneously measure intracellular brain pH and NO in vivo. Therefore, a comparative analysis of the literature is made to elucidate the possible effects of pHi on NOS activity.

MATERIALS AND METHODS

After we received approval by the Institutional Animal Care and Use Committee, 41 adult male Wistar rats weighing 300–450 g were fasted overnight and allowed free access to water. The animals were induced with halothane in a mixture of oxygen and air through a face mask at 2.0% during the surgical procedure and 1.0% during the occlusion period. Atropine was administered subcutaneously (0.08 mg/kg) preoperatively to reduce

respiratory secretions. Core body temperature was maintained at 36.5-37.5°C by using a heating pad and monitored continuously with a rectal temperature probe. Polyethylene catheters (PE-50) were inserted into the femoral artery to monitor arterial blood pressure and for arterial blood sampling [arterial PCO_2 ($PaCO_2$), arterial PO_2 (PaO_2), pH, hematocrit, and serum glucose] and inserted into a vein for administration of drugs. These physiological parameters were recorded 30 and 60 min before, during the 2-h ischemic period, and 30 min after flow restoration. Antibiotics (Durapen, 30,000 units) were administered intramuscularly before wound closure.

Model of focal cerebral ischemia

A modification by Coert et al. [16] of the technique described by Tamura et al. [60] was used. A 2-cm ventral coronal incision was made in the neck to expose and place snares (silk no. 2) around both common carotid arteries. A 2-cm skin incision was then made between the right outer canthus and the tragus. 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 to the foramen ovale. With the use of a high-speed drill (Hall Surgical), a small 3- to 4-mm craniectomy was made just anterior and superior to the foramen ovale. The dura was then opened with a sharp needle and the MCA freed of arachnoid. The MCA was temporarily occluded for 2 h at its crossing with the olfactory tract with the use of a no. 3 Sundt arteriovenous malformation microclip (Johnson and Johnson Professional). Simultaneously with occlusion of MCA, both common carotid arteries (CCAs) were temporarily occluded with the use of the snares. After restoration of flow, the MCA was observed for patency. Criteria for a patent vessel was the return to its normal size and bright red colorization. A nonpatent vessel would exhibit a dark blue colorization. In three animals used for fluorescent imaging, an extra 4 x 5-mm craniectomy was made over the frontal and parietal area of cortex. The dura was then removed, and edges were carefully cauterized at the margins of the craniectomy and then covered with Saran Wrap to prevent surface oxygenation and to keep the brain moist.

Experimental groups

Animals were divided into seven groups. Three control groups: 1) normoglycemic (n = 7), 2) moderate hypoglycemic (n = 7), and 3) hyperglycemic (n = 7), and three treatment groups that were given 7-NI intraperitoneally at 100 mg/kg in 0.1 ml/kg DMSO 1 h before ischemia for the normoglycemic (n = 6) and hyperglycemic (n = 7) groups and 10 mg/kg for the moderate hypoglycemic group (n = 7). A high mortality rate (86%) was found in the moderate hypoglycaemic 7-NI-treated group (100 mg/kg), which then prompted a reduction in the 7-NI dose to 10 mg/kg. Because of its lipid solubility, MacKenzie et al. [43] and Bush and Pollack [11] demonstrated that the preferred route of administration of 7-NI

is by intraperitoneal (i.p.) injection. DMSO was the preferred carrier as opposed to peanut oil or arachis oil because of its higher rate of absorption. The dosages used in this study were based on our previous study[17] and others[35,67]. Ishida et al. [35] demonstrated that 7-NI at 50 mg/kg transiently inhibited nNOS activity by 40% at 1 h, and 100 mg/kg inhibited nNOS activity by 56% at 1 h with significantly sustained reduction. Although 7-NI has been demonstrated to inhibit bovine endothelial NOS in vitro[8], 7-NI has not been shown to alter blood pressure at the dosages used in this study[1,45,67]. Therefore, 7-NI is a selective nNOS inhibitor at these dosages. In the seventh group, three animals were studied to confirm the relationship between pHi and moderate hypoglycemia, normoglycemia, and hyperglycemia using in vivo fluorescent imaging. This group was done to demonstrate the expected levels of regional cerebral blood flow (rCBF), pHi, and NADH redox state under moderate hypoglycemic, normoglycemic, and hyperglycemic conditions in the six chronic study groups as described above. Moderate hypoglycemia (3-5 mmol/l) was obtained by using a single dose of Humilin-N U-100 (Lilly) 1.0 U/kg administered subcutaneously 1 h before ischemia. Hyperglycemia (17- 27 mmol/l) was achieved by intravenous infusion of dextrose 0.5 g/ml (50%) at 6 g/kg/h over 20 min followed by a 1.2 g/kg/h infusion for maintenance.

Serum glucose manipulations of pHi

Confirmation of the effects of serum glucose manipulations on brain pHi was confirmed in a group of three acute experiments using fluorescent imaging before, during, and after focal cerebral ischemia (see discussion). As published previously, pHi as well as rCBF and NADH redox state can be measured in vivo by using umbelliferone, a fluorescent indicator [5]. A PE-10 catheter was placed in the right external carotid artery with the tip at the carotid bifurcation for retrograde injection of umbelliferone. A video-fluorescent microscope was focused on the parietal cortex for brain pHi, rCBF, and NADH redox state measurements. Umbelliferone solution (0.2 g in 200 ml of 5% glucose) was injected into the external carotid line at 30- to 60-min 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 pH, whereas the isobestic fluorescence varies with concentration. Brain pHi can be then calculated from the 340-to-370 nm ratio. NADH fluorescent images excited at 370 nm are acquired before umbelliferone injection for correction of background fluorescence and for analysis of mitochondrial function[61]. The scale factor for the percent change in NADH fluorescence from baseline is set so that 100% represents the level of NADH fluorescence in the normal brain, whereas an increase to 300% represents brain death[61]. The images from the 340-nm excitation were processed to compute rCBF by using the 1-min initial slope index using a partition coefficient of unity for umbelliferone[5]. All images of pHi,

rCBF, and NADH redox states are stored on tape for analysis. rCBF as measured by umbelliferone is defined as those areas that are relatively avascular and contain primarily arterioles and capillary beds[5]. The imaging system allows the measurement of rCBF by allowing the investigator to outline cortical areas of interest, which are devoid of major surface conducting vessels.

Histopathology

Seventy-two hours after flow restoration, the animals were reanesthetized with pentobarbital and then intracardially perfused with warm (37°C) 2% 2,3,5,-triphenyltetrazolium chloride (TTC) solution. The brains were quickly removed and immersed in the TTC solution for 15 min to enhance staining after which they were then placed in a 10% buffered formaldehyde solution for 5 days. During this time period there was no diminution of TTC staining. Eleven serial coronal sections were cut from each brain at 1-mm intervals and photographed. Total cortical infarction volume was measured and calculated in a blinded fashion by integrating the infarct areas of all 11 sections (area of infarction in square millimeter x thickness of section). The total infarction volume was multiplied by the ratio of the total left hemisphere volume to that of the total right hemisphere volume to correct for cerebral edema[24].

Statistical analysis

ANOVA followed by Fisher PLSD post hoc test was used to test the statistical significance of differences between groups. A P value of < 0.05 was considered significant. Polynomial regression was performed by using the individual data points from each group. Data are presented as means ± SE for all groups, with exception of region-of-interest data (brain pHi, rCBF, and NADH redox state), which from the video images are presented as means ± SD. The region-of-interest data are intended to show the expected alterations in rCBF, pHi, and NADH state under different glycemic states used in the six chronic groups in this study. All analysis was conducted using SATVIEW statistical software.

RESULTS

In vivo fluorescence imaging of brain pHi, rCBF, and NADH redox state

In three typical animals, brain pHi, rCBF, and NADH redox state were determined (Figs. 1 (for colour version see back cover) and 2). In normoglycemic animals [serum glucose, 10.7 mmol/l; $PaCO_2$, 45 mmHg; arterial pH (pHa), 7.329; and mean arterial blood pressure (MABP) 86 mmHg] baseline brain pHi was 7.01 ± 0.03, NADH redox state measured 100%, and rCBF was 66.0 ± 15.5 ml /100 g / min. Brain pHi declined significantly in the 2 h of ischemia to 6.58 ± 0.07, whereas NADH redox state markedly increased by 44%. rCBF

significantly declined to 23.7 ± 13.4 ml / 100 g / min. Thirty minutes after restoration of flow, brain pHi was 6.69 ± 0.03, NADH redox state decreased to near baseline levels, and rCBF increased to 85.7 ± 13.8 ml /100 g / min. In moderate hypoglycemic rats (serum glucose, 5.2 mmol; $PaCO_2$,43 mmHg; pHa, 7.397; and MABP, 86 mmHg) baseline brain pHi was 7.01 ± 0.08 and then decreased to 6.79 ± 0.06 after 2 h of ischemia, whereas NADH redox state increased by 14% and rCBF declined from 79.8 ± 14.4 to 32.8 ± 10.3 ml / 100 g / min. Therefore, although CBF declined significantly from baseline, there was no significant brain acidosis during ischemia. Thirty minutes after restoration of flow, brain pHi was 6.89 ± 0.05, NADH redox state decreased by 17% but still remained elevated, and rCBF increased to 89.1 ± 20.1 ml / 100 g/ min. In hyperglycemic animals (serum glucose, 19 mmol/l; $PaCO_2$, 48 mmHg; pHa, 7.436; and MABP, 89 mmHg) after 2 h of ischemia, brain pHi decreased from 7.01 ± 0.07 to 6.12 ± 0.05, NADH redox state increased by 75%, and rCBF declined from 75.3 ± 24.4 to 16.44 ± 10.7 ml / 100 g / min. Thirty minutes after flow restoration, brain pHi was 6.45 ± 0.10, NADH redox state decreased by 60%, and rCBF increased to 46.4 ± 17.4 ml / 100 g / min. The difference in brain pHi during ischemia between the normoglycemic and hyperglycaemic groups was significant ($P < 0.005$).

Physiological parameters

There were no significant differences in $PaCO_2$, PaO_2, pHa, body temperature, hematocrit, and MABP (Table 1). Glucose levels in moderate hypoglycemic and hyperglycemic treatment and control groups were significantly ($P < 0.05$) different from the normoglycemic control and treatment groups. Weight loss was significantly ($P < 0.05$) increased only in the moderate hypoglycemic 7-NI-treated group compared with the control group.

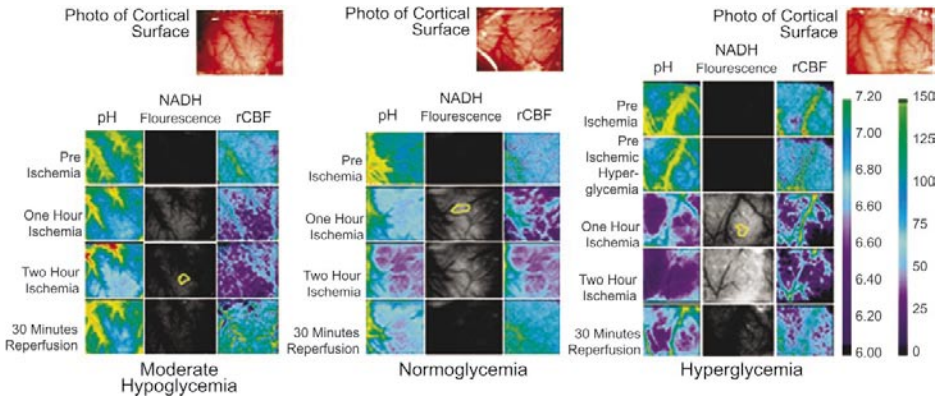

Fig. 1. Video pictures of brain intracellular pH (pHi), NADH redox state, and regional cerebral blood flow (rCBF) in three typical animals during moderate hypoglycemia (serum glucose 5.2 mmol/l) (A), normoglycemia (serum glucose 10.7 mmol/l) (B), and hyperglycemia (serum glucose 19 mmol/l) (C). (For colour version see back cover) Calibration bars for pHi and rCBF are to the right of the three sets of images. Regions of interest (≈13,600 μm^2) for comparison of brain pHi, NADH redox state, and rCBF in these experiments are outlined in yellow. Each video frame is ≈ 0.5 x 0.5 cm.

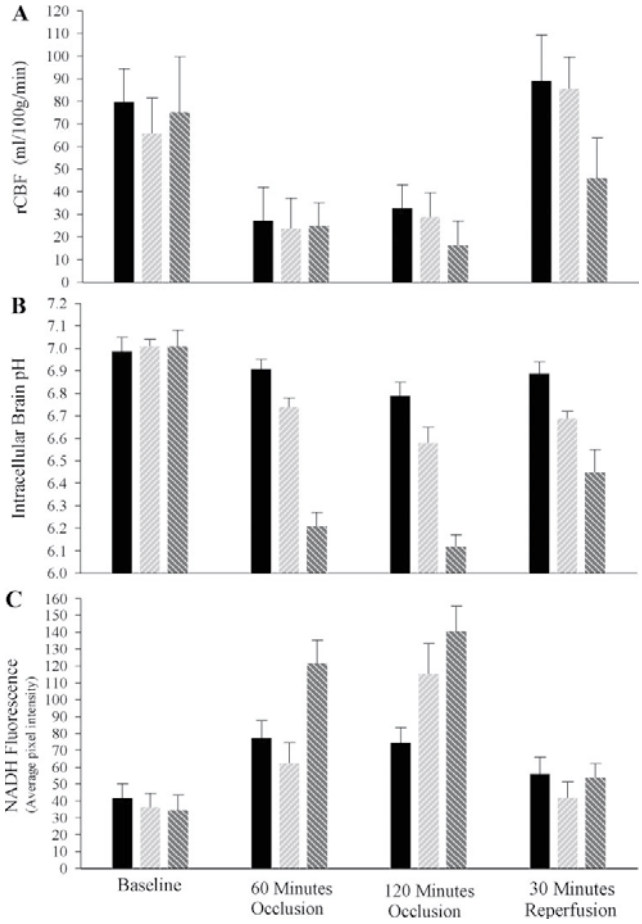

Fig. 2. Bar graph of rCBF, pHi, and NADH redox state from a moderate hypoglycemic (solid bar), normoglycemic (front slashed bar), and hyperglycaemic (back slashed bar) animal. Data depicted are from the region of interest from each video image at their respective time points from each animal. Graph demonstrates the findings of rCBF, pHi, and NADH redox state under different glycemic conditions. Note that before ischemia, the values of rCBF, pHi, and NADH redox state are quite similar among the different glycemic states. During the ischemic period there are distinct gradated levels of pHi and NADH redox state according to the level of glycemia. The worsening of rCBF at 2 h of ischemia and its slow recovery after restoration of flow are due mainly to edema. Data are expressed as means ± SD.

Infarction volume

A significant (P = 0.0009) reduction in cortical infarction volume (95.8 ± 11.7 to 19.1± 10.4 mm^3) was achieved by lowering serum glucose levels from normoglycemia (≈10 mmol/l) to moderate hypoglycemia (≈3.3 mmol/l) (Fig. 3). Raising serum glucose levels to 22.4 ± 32 mmol/l resulted in an exacerbation of cortical ischemic damage to 177.8% to 170.3 ± 13.8 mm^3 (P = 0.0014 compared with normoglycemia animals). Treatment with 7-NI in the normoglycemic group resulted in a significant (P = 0.0001) 93.5% reduction in a cortical infarct volume compared with normoglycemic controls (6.4 ± 4.4 vs. 95.8 ± 11.7 mm^3).

Table 1. Physiological parameters

	Pa_{CO_2}, mmHg	Pa_{O_2}, mmHg	pH_a	MABP, mmHg	Glucose, mmol/l	Hct, %	Temperature, °C	Weight Loss, %
				Hypoglycemia				
Ischemic control group								
Before occlusion	37 ± 1	210 ± 8	7.437 ± 0.012	80 ± 3	4.0 ± 0.6*	39.5 ± 1.0	37.0 ± 0.1	
1 h ischemia	41 ± 2	190 ± 7	7.408 ± 0.015	81 ± 1	3.0 ± 0.6*	38.9 ± 0.5	37.0 ± 0.1	
2 h ischemia	39 ± 2	181 ± 7	7.435 ± 0.017	83 ± 3	3.0 ± 0.5*	38.4 ± 0.6	36.9 ± 0.1	0.6 ± 1.9
Treatment group (7-NI 10.0 mg/kg)								
Before occlusion	38 ± 2	234 ± 11	7.448 ± 0.010	87 ± 5	4.0 ± 0.2*	42.7 ± 0.9	36.9 ± 0.1	
1 h ischemia	42 ± 1	208 ± 9	7.364 ± 0.010	92 ± 4	3.4 ± 0.3*	43.0 ± 1.2	37.0 ± 0.0	
2 h ischemia	40 ± 1	198 ± 9	7.404 ± 0.015	95 ± 6	3.1 ± 0.0*	42.4 ± 1.6	37.0 ± 0.1	7.3 ± 1.3†
				Normoglycemia				
Ischemic control group								
Before occlusion	38 ± 3	185 ± 10	7.442 ± 0.047	82 ± 2	9.8 ± 1.0	37.7 ± 1.0	36.9 ± 0.1	
1 h ischemia	38 ± 4	163 ± 11	7.463 ± 0.041	81 ± 2	9.5 ± 1.1	36.9 ± 1.1	36.9 ± 0.1	
2 h ischemia	36 ± 3	158 ± 13	7.479 ± 0.040	87 ± 4	9.7 ± 0.8	35.1 ± 1.7	37.0 ± 0.2	15.2 ± 2.3
Treatment group (7-NI 100.0 mg/kg)								
Before occlusion	36 ± 2	191 ± 13	7.433 ± 0.013	83 ± 3	8.7 ± 0.6	41.8 ± 1.0	37.0 ± 0.0	
1 h ischemia	39 ± 2	198 ± 8	7.403 ± 0.017	86 ± 1	9.5 ± 0.7	41.2 ± 1.3	37.0 ± 0.0	
2 h ischemia	41 ± 2	197 ± 9	7.387 ± 0.017	89 ± 2	9.1 ± 0.9	40.3 ± 0.7	37.0 ± 0.0	8.1 ± 3.0
				Hyperglycemia				
Ischemic control group								
Before occlusion	43 ± 3	166 ± 21	7.372 ± 0.017	86 ± 1	15.9 ± 3.8*	37.5 ± 0.9	36.9 ± 0.1	
1 h ischemia	41 ± 1	160 ± 20	7.362 ± 0.024	87 ± 1	26.5 ± 0.7*	37.1 ± 0.7	37.0 ± 0.1	
2 h ischemia	40 ± 1	156 ± 18	7.400 ± 0.018	86 ± 1	18.5 ± 2.2*	36.3 ± 0.5	37.0 ± 0.1	15.7 ± 2.2
Treatment group (7-NI 100.0 mg/kg)								
Before occlusion	36 ± 1	184 ± 6	7.427 ± 0.011	89 ± 2	20.3 ± 5.3*	39.3 ± 1.5	37.1 ± 0.1	
1 h ischemia	42 ± 2	162 ± 11	7.373 ± 0.026	89 ± 2	25.5 ± 5.1*	40.3 ± 2.0	37.0 ± 0.1	
2 h ischemia	39 ± 2	163 ± 10	7.379 ± 0.016	89 ± 3	22.9 ± 4.4*	38.4 ± 1.0	37.0 ± 1.0	11.7 ± 1.6

Values are means ± SE. ($PaCO_2$, arterial PCO_2; PaO_2, arterial PO_2; pHa, arterial pH; MABP, mean arterial blood pressure; Hct, hematocrit; 7-NI, 7-nitroindazole.) *Statistically different from normoglycemic control values ($P < 0.05$); † Statistically different from respective ischemic control groups ($P < 0.05$).

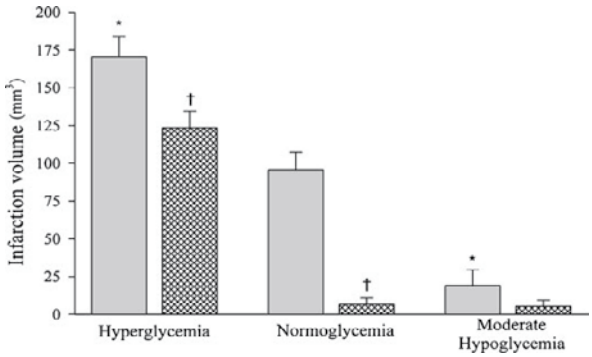

Fig. 3. Bar graph of infarction volume (in mm^3) comparing 7-nitroindazole (7-NI)-treated animals (crosshatched bars) with ischemic controls (solid bars) at moderate hypoglycemia, normoglycemia, and hyperglycemia. Percent difference in infarction volumes between treated and nontreated animals in the hyperglycemic group was 27.5% ($P = 0.0216$) and in the normoglycemic group it was 93.3% ($P = 0.0001$). Therefore, 7-NI was less effective in reducing infarction volume in the hyperglycemic group. Although there was a difference of 72.6% during moderate hypoglycemia between the treated and nontreated groups, it was not statistically significant ($P = 0.277$). Data are expressed as means ± SE. †Statistically different from respective ischemic control values, $P < 0.05$. *Statistically different from normoglycemic ischemic control values, $P < 0.005$.

Hyperglycemic 7-NI-treated animals also demonstrated a significant ($P = 0.0216$) 27.5% reduction in cortical infarct volume compared with their hyperglycemic controls (123.6 ± 11.1 vs. 170.3 ± 13.8 mm^3). Under moderate hypoglycemic conditions treatment with 10 mg/kg i.p. 7-NI resulted in a 72.6% reduction in cortical ischemic damage compared

with their moderate hypoglycaemic controls (5.3 ± 4.1 vs. 19.1 ± 10.4 mm^3). This was not statistically significant (P = 0.277).

DISCUSSION

This study provides evidence that one of the effects of nNOS may be dependent on brain pHi in a model of focal cerebral ischemia. The protective effect of 7-NI expressed as percent reduction of cortical infarct volume was 93.3% in normoglycemia and 27.5% in hyperglycemia. In the moderate hypoglycemic animals, the reduction in infarction volume did not reach significance because moderate hypoglycemia in itself dramatically reduced infarction volume. Therefore, 7-NI was less effective at lower brain pHi. The results of this study mirror that of the in vitro study by Hecker et al.[31] in which they demonstrated that the activity of microsomal constitutive NOS obtained from rat cerebellum was pH dependent in a biphasic fashion, where pH sensitivity was lost 1 unit above or below the optimum pH of 6.7. Two other in vitro studies, Riveros-Moreno et al. [54] and Gorren et al. [25], also demonstrated a biphasic response between nNOS activity and pH. Combining our data with those of Hecker et al.[31], Riveros-Moreno et al.[54], and Gorren et al.[25], we can conclude that within the encountered pH range (6.12- 6.82), enzyme activity would vary considerably. Support for this includes the following: 1) increased intracellular acidosis achieved by augmenting the severity of ischemia resulted in loss of the neuroprotective effect by the non-selective NOS inhibitor, L-NAME[3]; and 2) in a separate study[18] administration of 3-morpholinosydnonimine hydrochloride (SIN-1), a NO donor, was far more effective during hyperglycemia (≈ 48% reduction vs. 27.5% in this study), and less effective during normoglycemia (≈ 71% reduction vs. 93.5% in this study) and moderate hypoglycaemia (≈ 61% reduction vs. 72.6% in this study). Consideration of the binding properties of 7-NI to nNOS under different pH conditions must be taken into account. To our knowledge, the pKa of 7-NI and its sensitivity to pH in neurons are unknown. However, because SIN-1, an NO donor, was far more effective than 7-NI during hyperglycemia[18], it is more than likely that if 7-NI had greater binding properties at more acidotic levels, it would have been less effective in reducing infarction volume. On the basis of this study, the following suggestion can be made. In the normal brain, NO is produced to maintain basal tone. In cerebral ischemia, NO production increases as the brain becomes ischemic to a pH optimum (≈ 6.7- 6.8). As the brain becomes more acidotic, NO production decreases. Therefore, it follows that with worsening acidosis below pH ≈ 6.7- 6.8, NO donors become more effective compared with NOS inhibitors and that decreasing acidosis above pH ≈ 6.7- 6.8, inhibition of nNOS becomes more effective.

Relationship among systemic glucose and pHi, rCBF, and NO

To determine the relationship between intracellular brain pH and the efficacy of nNOS inhibition by 7-NI, animals were made moderately hypoglycemic, normoglycemic, and hyperglycemic to provide three graded levels of brain acidosis. In the last several decades numerous investigations have been performed to study the effects of intracellular acidosis on cerebral ischemia by modification of serum glucose levels via addition of glucose and/or insulin. Manipulation of serum glucose is a well-documented method to alter brain pHi during cerebral ischemia. For example, there have been several published studies[4,6,13,14,21,29,32,39,40,57,58,62,64] in which brain pHi has been altered in both models of global and focal cerebral ischemia under hypoglycemic, normoglycemic, and hyperglycemic conditions. Studies[37,39] have demonstrated that acidosis is one of the primary contributing factors of ischemic damage. Intracellular brain pHi becomes increasingly acidotic during ischemia declining from ≈ 6.7 to < 6.0 concomitant with serum glucose levels (≈ 6.5 to > 28 mmol/l). Conversely, brain pHi becomes less acidotic (6.7 to 7.0) as serum glucose levels decline toward moderately hypoglycemic levels (≈6.7 to ≈7.0 mmol/l) [4,58]. Cerebral infarction is reduced under moderate hypoglycemic conditions, whereas it becomes exacerbated under hyperglycemic conditions[4,28]. In our present study, moderate hypoglycemia (serum glucose ≈ 3 - 4 mmol/l) reduced cortical infarct volume by ≈ 80% to 19.1 ± 10.4 mm^3 from 95.8 ± 11.7 mm^3 in the normoglycemic group, whereas in the hyperglycemic animals this resulted in a 178% increase in infarction volume to 170 ± 14 mm^3. To confirm the effects of serum glucose concentrations on brain pHi in our rat model of focal cerebral ischemia used in this study, animals in the acute setting were studied and found to produce acidosis comparable to the different glycemic levels as described above. rCBF is not altered during moderate hypoglycaemia in serum glucose concentrations of ≈ 3.0 mmol/l and at lower concentrations, rCBF increases significantly[9,15]. Under hyperglycemic steady-state conditions, if the serum glucose concentration is ≈ 25 mmol/l or less, there is no significant change in rCBF[50,51]. In concentrations greater than ≈ 25 mmol/l, rCBF is significantly decreased. However, during ischemia, rCBF has been shown to be significantly lower compared with normoglycemic conditions mainly due to edema formation[38]. These findings support the expected rCBF results in the animals studied in the acute setting used in this study. Wei and Quast[63], using microdialysis in rats, showed that hyperglycemia did not alter citruline levels compared with normoglycemic rats before focal cerebral ischemia. Citruline is a by-product in the transformation of arginine to NO. Citruline levels increased during focal cerebral ischemia, however, not significantly at the majority of time points compared with normoglycemic rats. After restoration of flow, citruline increased significantly compared with normoglycemic rats. This follows that after restoration of flow, brain (pHi) becomes less acidotic, and then, according to the in vitro NOS data[25,31,54], NO production would increase. In nonischemic animals, Yu et al. [68] found that there were no differences in the activity and gene expres-

sion of nNOS between hyperglycemic and normoglycemic nonischemic rats. It should be noted that hyperglycemia may have additional effects on extracellular excitatory amino acids[23,42] and free fatty acids during ischemia[52]. With the use of in vivo microdialysis in rabbits, the effect of hyperglycemia on peri-ischemic extracellular glutamate concentration in global ischemia revealed decreased glutamate concentrations compared with normoglycemic ischemic controls[12]. A reduction in glutamate efflux under hyperglycemic conditions was also reported by Phyllis et al. [52]. Raising blood glucose levels from 90 to 373 mg/dl (= 5 to 21 mmol/l) during severe focal ischemia in the rat increased glutamate levels in the neocortex with twofold higher peak values[42]. Differences between studies were explained with the observation that glutamate release during ischemia varies with the experimental conditions and areas chosen for sampling[42]. However, in contrast, Yamamoto et al. [65] demonstrated in gerbils during hypothermia that ischemic damage was still reduced when given an intracerebral CA1 injection of glutamate. Nonetheless, it is conceivable that the measured effects of variable serum glucose on NO production in this study were multifactorial in addition to the alterations in brain pHi.

NOS inhibition in cerebral ischemia

7-NI is found to be a potent inhibitor of the neuronal constitutive isoforms of NOS and causes a dose-related antinociception[45,46]. MacKenzie et al. [43] studied the time course of brain NOS inhibition after administration of 7-NI intraperitoneally. At 30 mg/kg i.p., maximal inhibition (85%) was reached 30 min after 7-NI administration. After 3 h, NOS activity was reduced to 29% of baseline and returned to baseline level at 24 h[43]. When 7-NI in arachis oil was administered at 10 mg/kg i.p. in young male Wistar rats, NOS activity was reduced maximally (85%) at 1 hour, whereas after 4 hours it was 60%, and at 24 hours it was 20%[59]. Studying the pharmacokinetics of 7-NI, Bush and Pollack[11] discovered a marked nonlinearity consistent with saturable elimination after intraperitoneal administration of 7-NI dissolved in peanut oil. At a higher dose this resulted in a disproportionally increased exposure to 7-NI. Clearly, a critical review of the literature demonstrates contradictory results regarding the protective effects of NOS inhibitors. A study on time-dependent effects of NOS inhibition on ischemic cerebral damage using the nonselective NOS inhibitor L-NAME at 3 mg/kg iv revealed that in a permanent focal ischemia model in the rat, early treatment (< 5 min of MCA occlusion) increased infarct volume, whereas given 3 h later, it slightly reduced infarct volume[70]. In contrast early treatment with L-NAME at 3 mg/kg iv repeated at 3, 6, 24, and 36 h in a similar MCA occlusion model reduced cortical infarct volume by 43%[10]. Margaill et al. [44] reported protective effects of treatment with L-NAME (1 mg/kg) up to 8 h after transient focal ischemia. Adachi et al. [1], using a gerbil model of global ischemia, demonstrated that both L-NAME and 7-NI exacerbated ischemic damage in the striatum after 5 min of occlusion compared with that of the saline control group. At 10 min of occlusion, L-NAME and 7-NI had no effect

on ischemic damage. This suggests that, at least in part, as ischemia worsens, these NOS inhibitors have reduced efficacy in reducing infarction. In a model of neonatal hypoxia, Muramatsu et al. [48] showed a trend toward neuroprotection using 7-NI only at high dosages of 50 mg/kg i.p. In contrast, using 7-NI, Yoshida et al. [67] revealed a reduction of cortical damage of 24% and 25% for doses of 25 and 50 mg/kg i.p. in peanut oil, respectively. Significant reductions in cortical infarct volume were previously reported by our group for doses of 10 and 100 mg/kg i.p. in 0.1 ml/kg DMSO[17]. The protective effects have been described for DMSO[20,56]; these studies used dosages (> 0.1 ml) much greater than the dosage used in this present study. DMSO at 0.1 ml/kg did not alter cortical infarct volume in the present study as well as in our previous study[17] and in another study by Jiang et al. [36]. At a dose of 100 mg/kg 7-NI, 60 min before occlusion, cortical infarct volume was reduced by 92% (from 92.5 to 9.0 mm^3) [17]. In the present study in which the occlusion time was reduced to 2 h, mean cortical infarct was reduced by 90% in the normoglycemic 7-NI-treated group when compared with the normoglycemic controls (from 102.3 to 9.9 mm^3). Comparison of the effects of 7-NI (50 mg/kg i.p. in peanut oil) and NG-nitro-L-arginine (L-NNA, 1 mg/kg i.p.) on focal ischemic damage in the mouse revealed that L-NNA still reduced ischemic damage when given just before reperfusion, whereas 7-NI did not provide protection at this point[26]. Ninety minutes into ischemia, 7-NI (60 mg/kg i.p.) was also shown not to be neuroprotective, whereas the same dose was neuroprotective when given 5 min after MCA occlusion[22]. Although 7-NI did not reduce ischemic damage, it did reduce nitrotyrosine formation (a measure of peroxynitrite formation) to an extent comparable to L-NNA[26]. It was proposed that the partial inhibition of endothelial NOS provided the optimum benefit by inhibiting peroxynitrite formation without significantly increasing intravascular clogging[26].

In conclusion, in this experiment the protective effect of selective nNOS inhibition by 7-NI during focal cerebral ischemia was altered by serum glucose concentrations, which in effect was manipulation of brain pHi. This apparent effect of pHi on NO is consistent with in vitro data[25,31,54]. We propose that brain pHi may be an important factor for determining NOS activity and that the observed variability in effects of NOS inhibition in different models of cerebral ischemia is partly due to differences in brain pHi during ischemia. The effect of pH on nNOS activity provides an additional mechanism by which acidosis contributes to ischemic brain damage. Further investigations will be needed to elucidate the mechanism by which hydrogen ion concentration affects nNOS enzyme activity.

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