Is neuroprotective efficacy of nNOS inhibitor 7-NI dependent on ischemic intracellular pH?

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Coert, Bernard A., Robert E. Anderson, and Fredric B. Meyer. Is neuroprotective efficacy of nNOS inhibitor 7-NI dependent on ischemic intracellular pH? Am J Physiol Heart Circ Physiol 284: H151–H159, 2003. First published September 19, 2002; 10.1152/ajpheart.00580.2002.—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 h of focal cerebral ischemia under 1% halothane anesthesia. 7-NI, 10 and 100 mg/kg in 0.1 ml/kg DMSO, was administered 30 min 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 confirmed in a group of acute experiments utilizing umbelliferone fluorescence. The animals were euthanized at 72 h 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 hypoglycemia in itself dramatically reduced infarction volume. We hypothesize that a mechanism to explain the published discrepencies on the effects of neuronal NOS inhibitors in vivo may be due to the effects by differences in ischemic brain acidosis on the production of NO.

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 L-N-monomethyl-L-arginine (L-NMMA), Nω-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, 48, 49, 53, 59, 66, 67, 70) depending on the ischemia model, isozyme 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 and nNOS inhibitor AR-R17477. 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 pH on NOS 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 intracerebral 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 arteries for blood pressure monitoring and collecting samples for determination of serum glucose concentrations. The cerebral circulation was occluded by stereotaxic placement of a microinjection cannula (PE-10) into the MCA (bregma: 0.48 mm; AP: 5.0 mm; L: 1.5 mm) in the right hemisphere. The right external carotid artery was ligated and the right common carotid artery was cut. After the 2-h occlusion period, the MCA was infused with a mixture of heparinized saline, 4% glucose, and 4% bovine serum albumin (BSA) at a rate of 0.5 ml/min through the injection cannula. The BSA prevents coagulation in the cerebral microcirculation, which occurs during reperfusion. After reperfusion, the animals were allowed to recover for 3 h. The animals were euthanized at 72 h 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 hypoglycemia in itself dramatically reduced infarction volume. We hypothesize that a mechanism to explain the published discrepancies on the effects of neuronal NOS inhibitors in vivo may be due to the effects by differences in ischemic brain acidosis on the production of NO.

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into the femoral artery to monitor arterial blood pressure and for arterial blood sampling [arterial Pco2 (Paco2), arterial PO2 (PaO2), 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, and after 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 is returning to its normal size and bright red coloration. A nonpatent vessel would exhibit a dark blue colorization. In three animals used for fluorescent imaging, a 4 × 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 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-quality microclip of the technique (86%) was found in the moderate hypoglycemic 7-NI-treated group (100 mg/kg), which then promptly reduced the 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 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 those of the literature (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 pH 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), pH, 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 Humulin-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⁻¹ h⁻¹ over 20 min followed by a 1.2 g kg⁻¹ h⁻¹ infusion for maintenance. Serum glucose manipulations of pH. Confirmation of the effects of serum glucose manipulations on brain pH, was confirmed in a group of three acute experiments using fluorescent imaging before, during, and after focal cerebral ischemia (see DISCUSSION). As published previously, pH, 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 pH, 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 fluorophores, anionic and isoesthetic, 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 isoesthetic fluorescence varies with concentration. Brain pH 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 pH, 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 × 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 pre-
sented as means ± SE for all groups, with exception of region-of-interest data (brain pH<sub>i</sub>, 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, pH<sub>i</sub>, 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 pH<sub>i</sub>, rCBF, and NADH redox state. In three typical animals, brain pH<sub>i</sub>, rCBF, and NADH redox state were determined (Figs. 1 and 2). In normoglycemic animals [serum glucose, 10.7 mmol/l; PaCO<sub>2</sub>, 45 mmHg; arterial pH (pHa), 7.329; and mean arterial blood pressure (MABP) 86 mmHg] baseline brain pH<sub>i</sub> was 7.01 ± 0.03, NADH redox state measured 100%, and rCBF was 66.0 ± 15.5 ml·100 g<sup>-1</sup>·min<sup>-1</sup>. Brain pH<sub>i</sub> 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<sup>-1</sup>·min<sup>-1</sup>. Thirty minutes after restoration of flow, brain pH<sub>i</sub> was 6.69 ± 0.03, NADH redox state decreased to near baseline levels, and rCBF increased to 85.7 ± 13.8 ml·100 g<sup>-1</sup>·min<sup>-1</sup>.

In moderate hypoglycemic rats (serum glucose, 5.2 mmol/l; PaCO<sub>2</sub>, 43 mmHg; pHa, 7.397; and MABP, 86 mmHg) baseline brain pH<sub>i</sub> 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<sup>-1</sup>·min<sup>-1</sup>. Therefore, although CBF declined significantly from baseline, there was no significant brain acidosis during ischemia. Thirty minutes after restoration of flow, brain pH<sub>i</sub> 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<sup>-1</sup>·min<sup>-1</sup>

In hyperglycemic animals (serum glucose, 19 mmol/l; PaCO<sub>2</sub>, 48 mmHg; pHa,7.436; and MABP, 89 mmHg) after 2 h of ischemia, brain pH<sub>i</sub> 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 16.4 ± 10.7 ml·100 g<sup>-1</sup>·min<sup>-1</sup>. Thirty minutes after flow restoration, brain pH<sub>i</sub> was 6.45 ± 0.10, NADH redox state decreased by 60%, and rCBF increased to 46.4 ± 17.4 ml·100 g<sup>-1</sup>·min<sup>-1</sup>. The difference in brain pH<sub>i</sub> during ischemia between the normoglycemic and hyperglycemic groups was significant (P < 0.005).

Physiological parameters. There were no significant differences in PaCO<sub>2</sub>, pHa, body temperature, hematocrit, and MABP (Table 1). Glucose levels in moderate hypoglycemic and hyperglycemic treatment and control groups were significantly different (P < 0.05) from the normoglycemic control and treatment groups. Weight loss was significantly increased only in the moderate hypoglycemic 7-NI-treated group compared with the control group.

Infarction volume. A significant (P = 0.0009) reduction in cortical infarction volume (95.8 ± 11.7% to 19.1 ± 10.4 mm<sup>3</sup>) 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 by 177.8% to 170.3 ± 13.8 mm<sup>3</sup> (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 mm³). 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 mm³). Under moderate hypoglycemic conditions treatment with 10 mg/kg ip 7-NI resulted in a 72.6% reduction in cortical ischemic damage compared with their moderate hypoglycemic controls (5.3 ± 4.1 vs. 19.1 ± 10.4 mm³). 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 pH 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...
Table 1. Physiological parameters

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<th>P_{\text{aCO}}_2, mmHg</th>
<th>P_{\text{aO}}_2, mmHg</th>
<th>pH</th>
<th>MABP, mmHg</th>
<th>Glucose, mmol/l</th>
<th>Hct, %</th>
<th>Temperature, °C</th>
<th>Weight Loss, %</th>
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<td>Before occlusion</td>
<td>37 ± 1</td>
<td>210 ± 8</td>
<td>7.437 ± 0.012</td>
<td>80 ± 3</td>
<td>4.0 ± 0.6*</td>
<td>39.5 ± 1.0</td>
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<td>1 h ischemia</td>
<td>41 ± 2</td>
<td>190 ± 7</td>
<td>7.408 ± 0.015</td>
<td>81 ± 1</td>
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<td>38.9 ± 0.5</td>
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<td>2 h ischemia</td>
<td>39 ± 2</td>
<td>181 ± 7</td>
<td>7.435 ± 0.017</td>
<td>83 ± 3</td>
<td>3.0 ± 0.5*</td>
<td>38.4 ± 0.6</td>
<td>36.9 ± 0.1</td>
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<td>234 ± 11</td>
<td>7.448 ± 0.010</td>
<td>87 ± 5</td>
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<td>208 ± 9</td>
<td>7.364 ± 0.010</td>
<td>92 ± 4</td>
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<td>43.0 ± 1.2</td>
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<td>198 ± 9</td>
<td>7.404 ± 0.015</td>
<td>95 ± 6</td>
<td>3.1 ± 0.0*</td>
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<td>37.0 ± 0.1</td>
<td>7.3 ± 1.3†</td>
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<td>1 h ischemia</td>
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<td>86 ± 1</td>
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<td>184 ± 6</td>
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<td>89 ± 2</td>
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<td>1 h ischemia</td>
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<td>162 ± 11</td>
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<td>89 ± 2</td>
<td>25.5 ± 5.1*</td>
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<td>22.9 ± 4.4*</td>
<td>38.4 ± 1.0</td>
<td>37.0 ± 1.0</td>
<td>11.7 ± 1.6</td>
</tr>
</tbody>
</table>

Values are means ± SE. P_{\text{aCO}}_2, arterial PCO; P_{\text{aO}}_2, arterial PO; pH, 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 mm³) 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.

less effective at lower brain pH_i conditions. 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 the data with those of Hecker et al. (31), Riveros-Moreno et al. (54), Gorren et al. (25), and ours, 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 nonselective NOS inhibitor, N-Nitro-L-NAME (3); and 2) in a separate study (18) administration of 3-morpholinosyndinmine 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 hypoglycemia (~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, then 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 pH 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 pH 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 pH becomes increasingly acidic during ischemia declining from ~6.7 to <6.0 concomitant with serum glucose levels (~6.5 to >28 mmol/l). Conversely, brain pH becomes less acidic (6.7 to 7.0) as serum glucose levels decrease 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 mm³ from 95.8 ± 11.7 mm³ in the normoglycemic group, whereas the hyperglycemic animals resulted in a 178% increase in infarction volume to 170 ± 14 mm³. To confirm the effects of serum glucose concentrations on brain pH 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 hypoglycemia 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 citrulline 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 pH becomes less acidic, 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 expression 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 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 pH.

NOS inhibition in cerebral ischemia. 7-NI is found to be a potent inhibitor of the neuronal constitutive isoform 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 ip, 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 ip in young male Wistar rats, NOS activity was reduced maximally (85%) at 1 h, whereas after 4 h was at 60%, and at 24 h was 20% (59). Studying the pharmacokinetics of 7-NI, Bush and Pollack (11) discovered a marked nonlinearity consistent with saturable elimination after intra-peritoneal administration of 7-NI dissolved in peanut.
oil. At a higher dose this resulted in a disproportionately 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). Margail 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.

In a model of neonatal hypoxia, Muramatsu et al. (48) showed a trend toward neuroprotection using 7-NI. In contrast, using 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). Margail et al. (44) reported protective effects of treatment with L-NAME (1 mg/kg) up to 8 h after transient focal ischemia.

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 pH. This apparent effect of pH on NO is consistent with in vitro data (25, 31, 54). We propose that brain pH 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 pH 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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IS nNOS Activity Dependent on Ischemic Brain pH?


