Nitric Oxide–Related Brain Damage in Acute Ischemic Stroke

José Castillo, MD, PhD; Ramón Rama, PhD; Antoni Dávalos, MD, PhD

Background and Purpose—The neurotoxic and neuroprotective role of nitric oxide (NO) in experimental cerebral ischemia has generated considerable debate. The aim of this study was to analyze the relationship between NO metabolite (NO-m) concentrations in cerebrospinal fluid (CSF) and clinical and neuroimaging parameters of brain injury in patients with acute ischemic stroke.

Methods—We studied 102 patients and 24 control subjects who were included in a larger previous study conducted to analyze risk factors of progressing stroke. NO generation was calculated by quantifying nitrates and nitrites with a colorimetric assay in CSF samples obtained within the first 24 hours from symptoms onset. Early neurological deterioration was defined as a fall of 1 or more points in Canadian Stroke Scale score between admission and 48 hours after inclusion. Infarct volume was measured on days 4 to 7 by cranial CT.

Results—Median NO-m concentrations [quartiles] were 2.1 [1.0, 4.5] μmol/mL in patients and 1.0 [1.0, 1.0] μmol/mL in control subjects ($P<0.0001$). In 45 patients with subsequent early neurological deterioration, NO-m levels in CSF were significantly higher than in those with stable stroke (4.0 [1.7, 7.8] versus in 1.6 [1.0, 2.5] μmol/mL, $P<0.0001$). There was a moderate correlation between NO-m and infarct volume (coefficient 0.39, $P<0.001$). NO-m concentrations $>5.0$ μmol/mL were significantly associated with early neurological worsening (OR 5.7, 95% CI 1.2 to 27.4; $P=0.030$) independent of other important factors related to progressing stroke, such as CSF glutamate levels.

Conclusions—Our clinical findings suggest an important role of NO generation in acute ischemic stroke. Increased NO-m in CSF are associated with a greater brain injury and early neurological deterioration. (Stroke. 2000;31:852-857.)

Key Words: excitotoxicity ■ nitric oxide ■ stroke outcome ■ stroke, acute ■ tomography, x-ray computed

Nitric oxide (NO) is an inorganic gas that plays a part in the control of cerebral blood flow, thrombogenesis, and modulation of neuronal activity. NO is produced in the endothelial cells, neurons, glia, and macrophages by 3 different isoforms of the enzyme nitric oxide synthase (NOS). NOS mediates the conversion of L-arginine and oxygen to NO and citrulline. Under cerebral ischemia, high concentrations of NO generated by the calcium-dependent activation of the constitutive neuronal NOS (nNOS) and by the activation of the inducible form of NOS (iNOS) in macrophages and other cell types intervene in inflammatory and cytotoxic actions that lead to neuronal death. In contrast, NO generated by the activation of the constitutive endothelial NOS (eNOS) may have protective effects that decrease the ability of platelets to aggregate, prevent leukocyte endothelial adhesion, and increase vascular dilation and cerebral blood flow.

Because NO has an extremely short half-life, its detection in the brain is difficult. Current assays in vivo include fluorimetric measurements of its stable metabolite nitrite and indirect methods such as nitrate concentrations and citrulline concentrations as markers of NOS activity. NO generation is predominantly associated with increased or reduced brain injury in human stroke, and consequently to support the use, and to evaluate the effect, of future treatment with specific NOS antagonists in clinical trials.

In this study we analyzed the relationship between NO metabolite concentrations in the cerebrospinal fluid (CSF) of patients during the acute phase of cerebral ischemia and clinical and neuroimaging parameters of brain injury.

Subjects and Methods

For the purpose of this investigation, we studied 102 patients (63% males, mean age 67.3±10.9 years), from a larger series of 249 patients admitted consecutively with a first episode of hemispheric ischemic stroke within 24 hours after the onset of symptoms, and 24 individuals (46% males, mean age 54.3±15.9 years) taken from a group of 50 control subjects. The total number of patients and controls were included in a prospective clinical investigation conducted between October 1992 and December 1996 to analyze clinical, biochemical, and radiological factors related to progressing...
stroke. The protocol was approved by the Ethics Committee of Hospital Xeral de Galicia, and informed consent was given by the control subjects, the patients themselves, or by relatives in cases of patients’ inability to understand our information. Only patients and controls from whom we had obtained and stored (frozen) CSF samples for possible future laboratory determinations were evaluated in the present study. Availability of samples relied exclusively on the amount of CSF obtained at the time of inclusion and not on other clinical or biochemical factors. Therefore, this selection was performed without knowledge of stroke outcome and excitatory amino acid concentrations and before determination of NO metabolites.

The characteristics of the control group and the inclusion criteria for the whole series of patients have already been published. In summary, all patients had a persistent focal neurological deficit and absence of mass effect or cerebral hemorrhage on the cranial CT criteria, early neurological deterioration was diagnosed when the functional capacity at 3 months. Following previously published controls from whom we had obtained and stored (frozen) CSF samples for possible future laboratory determinations were evaluated in the present study. Availability of samples relied exclusively on the amount of CSF obtained at the time of inclusion and not on other clinical or biochemical factors. Therefore, this selection was performed without knowledge of stroke outcome and excitatory amino acid concentrations and before determination of NO metabolites.

Laboratory Determinations
CSF samples were prepared by centrifugation (2000g for 10 minutes) and immediately stored at −80°C. Quantification of glutamate and l-arginine was performed by HPLC following the method described elsewhere. Amino acid determinations were performed with blinding to clinical and neuroimaging findings and to stroke outcome.

NO generation was calculated by quantifying the final products of its reactions, nitrates and nitrates, by a colorimetric assay (Cayman Chemical Co.). This procedure was carried out after the end of the inclusion period in a different laboratory, which had no knowledge of patient or control origin of the CSF sample, stroke characteristics, or amino acid concentrations. The nitrates in the CSF were enzymatically converted into nitrates by incubation with nitrate reductase and NADPH at room temperature for 2 hours. The levels of nitrates were then calculated. Nitrite concentration was measured with use of the Griess reaction by adding 100 μL of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine in 5% phosphoric acid) and mixed in 96-well plates and shaken gently for 20 minutes at room temperature. The addition of the Griess reagent results in a colorimetric product measured at 540 nm. The measurement was taken in a microplate reader at a standard curve generated by dilutions of nitrate. The assay is sensitive to nitrite concentrations of approximately 1 μmol/L. Interassay variability was 6%, and intra-assay variability was 2.9%. The final product of NO metabolites (NO-m) was used in this clinical investigation.

Statistical Analyses
NO-m concentrations are expressed as median [quartiles], because they were not normally distributed. Comparison of NO-m between 2 groups was performed with the Mann-Whitney test and between ≥2 groups with the Kruskal-Wallis test. Spearman analysis was used for bivariate correlations between NO-m and CSS score, inclusion delay, l-arginine, glutamate concentrations, and infarct volume.

The independent relationship between NO-m and infarct volume was evaluated by multiple linear regression analysis. A log transformation of infarct volume was performed to achieve a normal distribution of the dependent variable. The importance of NO-m concentrations for subsequent early neurological deterioration (0=no, 1=yes) and poor outcome (0=no, 1=yes) was assessed by logistic regression analysis based on the maximum likelihood ratio. Model 1 was fitted with a stepwise procedure, adjusting for age, gender, median (45) versus 1.0 [1.0, 1.0] μmol/mL, P<0.0001). Forty-three patients (42.1%) were diagnosed as having a large-artery atherosclerotic cerebral infarct, 33 (32.4%) a cardioembolic infarct, 13 (12.7%) a lacunar infarct, and 13 (12.7%) a cerebral infarct of unknown origin. No significant differences were observed in NO-m concentrations between stroke subtypes (P=0.71).

Results
NO-m concentrations in CSF were significantly higher in patients than in control subjects (2.1 [1.0, 4.5] versus 1.0 [1.0, 1.0] μmol/mL, P<0.0001). Forty-three patients (42.1%) were diagnosed as having a large-artery atherosclerotic cerebral infarct, 33 (32.4%) a cardioembolic infarct, 13 (12.7%) a lacunar infarct, and 13 (12.7%) a cerebral infarct of unknown origin. No significant differences were observed in NO-m concentrations between stroke subtypes (P=0.71).

Clinical Outcome
NO-m concentrations correlated to CSS score at admission (coefficient −0.54, P<0.001; Figure 1). A subsequent early neurological deterioration was observed in 45 patients (44%),
21 with cortical infarcts, 10 with deep infarcts, 12 with cortical and deep infarcts, and 2 with normal CT. Median NO-m concentration was 4.0 [1.7, 7.8] μmol/mL in the group with early neurological worsening and 1.6 [1.0, 2.5] μmol/mL in the group with nonprogressing cerebral infarct (P<0.0001). The significant difference between progressing and nonprogressing strokes was observed in patients with cortical infarcts (4.5 [1.8, 14.2] versus 1.6 [1.0, 3.0] μmol/mL, P<0.002), deep infarcts (4.5 [1.6, 12.1] versus 1.6 [1.0, 2.5] μmol/mL, P=0.019), and with infarcts involving cortical and subcortical regions (3.5 [1.6, 7.7] versus 1.0 [1.0, 2.1] μmol/mL, P=0.029).

Death and poor outcome at 3 months was observed in 9 (9%) and 44 (43%) patients, respectively. NO-m concentrations on admission were significantly higher in patients with poor outcome (4.2 [2.0, 9.8] μmol/mL) than in those who survived with good functional capacity at 3 months (1.6 [1.0, 2.8] μmol/mL) (P<0.0001).

**Neuroimaging Findings**

NO-m concentrations were significantly higher in the 65 patients with early signs of cerebral infarct on the initial CT scan than in the 37 patients without early CT signs (3.0 [1.6, 5.7] versus 1.6 [1.0, 2.4] μmol/mL) (P=0.001). There was a significant correlation between NO-m and ultimate infarct volume on days 4 to 7 (coefficient 0.39, P<0.001). No differences were observed on NO-m depending on infarct topography in the 97 patients who showed a corresponding lesion on the second CT; CSF NO-m concentrations were 2.2 [1.0, 4.6] μmol/mL in 46 cortical infarcts, 1.6 [1.4, 4.4] μmol/mL in 34 deep infarcts, and 2.2 [1.3, 5.2] μmol/mL in 17 infarcts that involved the cortical and subcortical regions (P=0.84).

**Multivariate Analyses for Brain Injury**

NO-m concentrations were not independently related to the log-transformed infarct volume in a multiple linear regression model, after adjustment for age, delay to inclusion, CSS score, body temperature, and serum glucose on admission (Table 1). Table 2 shows the result of logistic regression analyses for early neurological deterioration as a dependent variable. NO-m concentrations in CSF >5.0 μmol/mL were independently and significantly associated with early neurological worsening (OR 5.3, 95%CI 1.5 to 18.5; model 1). These findings did not change after adjustment for glutamate concentrations in CSF (model 2). NO-m over the 5.0 μmol/mL cut point did not predict death or dependency at 3 months. Age, stroke severity on admission, inclusion delay, and body temperature were the only factors selected by the logistic model.

**Biochemical Studies**

In the total series of patients, there was a positive correlation between NO-m concentrations in CSF and the inclusion delay (coefficient 0.41, P<0.001). In patients with stable stroke, NO-m values did not change significantly with time, although a slight increase was observed in those studied after the first 12 hours from stroke onset (P=0.063). In contrast, NO-m concentrations were significantly higher in CSF samples obtained after the first 6 hours from stroke onset in the group of patients who had a subsequent neurological deterioration (P=0.005). Furthermore, in patients who deteriorated, the median values determined at any time interval during the first 24 hours from onset were higher than those obtained in the stable stroke and control groups (Figure 2).

In patients, but not in the control group, NO-m concentrations correlated negatively to l-arginine concentrations in CSF (coefficient −0.85, P<0.0001). The levels of NO-m were particularly high when l-arginine concentrations were <6 μmol/L (Figure 3). As occurred with NO-m, l-arginine concentrations in CSF were significantly higher in patients with early neurological worsening on the initial CT scan (4.8 [1.8, 14.2] versus 1.6 [1.0, 2.8] μmol/mL, P<0.0001) and in those with nonprogressing cerebral infarct (4.8 [1.6, 12.1] versus 1.6 [1.0, 2.5] μmol/mL, P<0.02).

**Table 1. Multiple Linear Regression Analysis With Dependent Variable: Log-Transformed Infarct Volume**

<table>
<thead>
<tr>
<th>Variable</th>
<th>β</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time delay</td>
<td>−0.145878</td>
<td>−1.249</td>
<td>0.214</td>
</tr>
<tr>
<td>Age</td>
<td>−0.130289</td>
<td>−1.329</td>
<td>0.187</td>
</tr>
<tr>
<td>Temperature</td>
<td>0.217308</td>
<td>1.880</td>
<td>0.063</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.332674</td>
<td>3.211</td>
<td>0.001</td>
</tr>
<tr>
<td>CSS score</td>
<td>−0.251620</td>
<td>−2.494</td>
<td>0.014</td>
</tr>
<tr>
<td>NO-m, μmol/mL</td>
<td>0.094301</td>
<td>0.854</td>
<td>0.395</td>
</tr>
</tbody>
</table>
concentrations correlated with the inclusion delay. This fact was observed in patients with progressing stroke (coefficient $-0.53, P<0.001$), and in those with stable stroke (coefficient $-0.35, P=0.008$). A moderate positive correlation was found between NO-m and glutamate concentrations in CSF (coefficient 0.24, $P=0.01$).

**Discussion**

To our knowledge, this is the first study supporting at the clinical setting an important role of NO generation in acute ischemic stroke. We have found a significant increase of NO-m in the CSF of patients studied within the first 24 hours from stroke onset in comparison to controls. Furthermore, we have demonstrated a significant relationship between NO-m concentrations and clinical and neuroimaging indicators of brain injury. In fact, high NO-m concentrations were associated with deleterious effects in cerebral ischemia, that is, higher stroke severity at admission, early neurological deterioration, larger infarct volumes, and poor outcome at 3 months. Although these findings suggest predominant cytotoxic effects as a result of NO generation, we cannot rule out the possibility that the high levels of NO-m obtained in patients with greater brain injury result in part from an increased endothelial NO generation with neuroprotective, but failed, purposes.

NOS activity and NO release are greatly increased in the acutely human ischemic brain, but opposite effects, neurotoxic and neuroprotective, have been demonstrated in experimental stroke models. The effect of selective antagonists of NOS isoforms on acute cerebral ischemia and the effect of cerebral ischemia on mouse models in which a single NOS isoform gene is not expressed (knockout mice) have clarified the protective role of eNOS activity and the neurotoxic role of nNOS and iNOS activity. The former is associated with smaller cerebral infarcts and the latter with increased infarct volume. A further example of this dual action is the time-dependent opposite effects of L-arginine, a precursor amino acid of NO that is the principal natural substrate for each of the 3 NOS isoforms. In animal models, L-arginine has neuroprotective effects when it is administered up to 2 hours after onset of cerebral ischemia, but it is associated with increased infarct volume when its administration is delayed by 24 hours. The deleterious consequence of the late administration is thought to result from delayed neuronal injury due to the appearance of inducible NOS in the ischemic penumbra, after a time lag of 6 to 12 hours.

**Figure 2.** Median values and quartiles (25% and 75%) of CSF NO-m in patients with stable (○) and progressing (●) ischemic stroke. Numbers indicate the number of patients studied. Dotted line indicates the median values and quartiles of NO-m in control subjects. Note that patients with early neurological deterioration had higher levels at all time intervals during the first 24 hours from stroke onset, which were particularly increased after the first 6 hours from stroke onset.

**TABLE 2.** Logistic Regression With Dependent Variable: Early Neurological Deterioration*

<table>
<thead>
<tr>
<th>Variable</th>
<th>b</th>
<th>SE</th>
<th>OR</th>
<th>CI 95%</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1 (Stepwise)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Temperature</td>
<td>3.0787</td>
<td>0.7830</td>
<td>21.73</td>
<td>4.68–100.82</td>
<td>0.0001</td>
</tr>
<tr>
<td>Serum glucose</td>
<td>0.0311</td>
<td>0.0092</td>
<td>1.03</td>
<td>1.01–1.05</td>
<td>0.0007</td>
</tr>
<tr>
<td>CSS on admission</td>
<td>0.4773</td>
<td>0.2209</td>
<td>1.61</td>
<td>1.04–2.48</td>
<td>0.0307</td>
</tr>
<tr>
<td>CSF NO-m$&gt;5.0 \mu$mol/mL</td>
<td>1.6688</td>
<td>0.6382</td>
<td>5.30</td>
<td>1.51–18.53</td>
<td>0.0089</td>
</tr>
<tr>
<td>Model 2 (Enter)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature</td>
<td>1.7842</td>
<td>0.9567</td>
<td>5.95</td>
<td>0.91–38.83</td>
<td>0.0622</td>
</tr>
<tr>
<td>Serum glucose</td>
<td>0.0411</td>
<td>0.0153</td>
<td>1.04</td>
<td>1.01–1.07</td>
<td>0.0074</td>
</tr>
<tr>
<td>CSS on admission</td>
<td>0.1988</td>
<td>0.2836</td>
<td>1.22</td>
<td>0.69–2.52</td>
<td>0.4833</td>
</tr>
<tr>
<td>CSF NO-m$&gt;5.0 \mu$mol/mL</td>
<td>1.7371</td>
<td>0.8032</td>
<td>5.68</td>
<td>1.17–27.42</td>
<td>0.0306</td>
</tr>
<tr>
<td>CSF glutamate$&gt;8.3 \mu$mol/mL</td>
<td>2.4372</td>
<td>0.8365</td>
<td>11.44</td>
<td>2.22–58.95</td>
<td>0.0036</td>
</tr>
</tbody>
</table>

*Yes=1, no=0.
The method we used in this clinical study did not allow determination of which portion of the NO-m concentration derived from endothelial NO, from neuronal NO, or from NO generated in other cells by the action of iNOS. However, we believe that the nitrites and nitrates measured in our patients were metabolites of the NO, because they showed an extremely high negative correlation to \( L\)-arginine concentration in CSF. This finding is in agreement with the higher consumption of \( L\)-arginine reported in patients with greater cerebral damage by our group.\(^{38}\) We hypothesize that the exponential negative relationship between NO-m and \( L\)-arginine values found in our study (Figure 3) may be explained by the fact that the synthesis of small quantities of NO may be supplied by the consumption of intracellular \( L\)-arginine, which would not affect levels in the CSF. However, the activation of NOS isoforms during cerebral ischemia would increase the demand for \( L\)-arginine and would rapidly reduce extracellular levels.

In our opinion, some findings in this study support the hypothesis that high levels of NO-m derived mainly from a delayed inducible NOS activation, a process that is not mediated by glutamate release and calcium influx.\(^{35}\) First, we found only a moderate correlation between glutamate and NO-m concentrations in CSF, and the effect of NO-m on stroke worsening was independent of the glutamate effect. Second, in patients with neurological deterioration, NO-m levels were higher in CSF samples obtained after 12 hours from symptom onset than in those obtained earlier, which suggests a delayed generation of NO by the iNOS. Although the time course of iNOS expression in the human brain has not yet been defined, iNOS synthesis in rodents begins at 12 hours after permanent middle cerebral artery occlusion.\(^{36}\) Furthermore, although the cerebral dynamics of NO-m are unknown, the delayed appearance of NO-m in CSF differs from the early increase of CSF glutamate reported by our group in patients with progressing stroke.\(^{39}\) Third, the late increase of NO-m was consistent with a delayed consumption of \( L\)-arginine, which suggests the activation of iNOS. Finally, NO-m concentrations were equally high in patients with cortical and subcortical cerebral infarcts in the whole group and in patients with early neurological deterioration. This argues against a glutamate-calcium–dependent generation of NO, because glutamate release is significantly higher in cortical than in subcortical infarcts, probably owing to a higher number of glutamatergic neurons in the gray matter.\(^{40}\)

The involvement of NO in the progression of cerebral infarction is an appealing hypothesis. The present study shows that NO plays a part in early neurological deterioration, a fact that has been attributed to the expansion of the ischemic area.\(^{41-43}\) As occurred with glutamate,\(^{17}\) this relationship was independent to other important predictors and associated factors with progressing stroke, such as hyperthermia, high serum glucose concentrations, early infarct signs on CT, infections detected within the first week of stroke, and ultimate infarct volume. These findings suggest a direct neurotoxic effect of NO on the propagation of ischemic penumbra and neuronal death, a process that is supported by some experimental findings. It has been shown that immediately after ischemia, NOS is much more active in the core than in the surrounding area, which gives rise to an increasing concentration of NO that gradually extends from the core to the vulnerable neighboring neurons in the penumbra.\(^{44}\) This phenomenon may explain in part the progressive expansion of the brain injury in the absence of relevant changes in cerebral blood flow. Although the neurotoxic mechanisms mediated by NO have been only partially elucidated, free radical damage by formation of peroxynitrite may have an important role.\(^{45}\) Peroxynitrite decomposes to other reactive oxygen species, such as radical hydroxyl and nitrogen dioxide, which cause lipid peroxidation and thus destroy cell membranes. This mechanism may be particularly important in the peri-infarct region, because oxygen delivery during reperfusion facilitates a delayed generation of NO and a higher production of peroxynitrite due to the reduction of NO by the superoxide anion.\(^{46,47}\)

In conclusion, although current experimental studies show that NO can play a dual role in cerebral ischemia, our clinical findings suggest that increased NO generation is predominantly associated with cytotoxic effects. High NO metabolites in CSF within the first hours of acute stroke predict subsequent early neurological deterioration regardless other biochemical mechanisms mediated by glutamate. The possibility of establishing markers for NOS isoforms\(^{48}\) would provide

![Figure 3. Scatterplot shows NO-m and L-arginine concentrations within the first 24 hours from stroke onset. Note that NO-m concentrations were particularly high when L-arginine levels were <6 \( \mu\)mol/L.](http://stroke.ahajournals.org/)

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the opportunity to determine precisely the mechanism for neuronal lesion and how it can be treated.

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References


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