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L-Arginine Levels in Blood as a Marker of Nitric Oxide–Mediated Brain Damage in Acute Stroke: A Clinical and Experimental Study

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Summary: There are no useful markers in blood of nitric oxide (NO)-mediated brain damage. Because L-arginine (L-arg) is the only known substrate for NO generation, the authors investigated the plasma profile of L-arg after cerebral ischemia, and the relationship of L-arg concentrations in blood with stroke outcome and infarct volume in a clinical and experimental study. L-Arg levels were determined with high-performance liquid chromatography in blood and CSF samples obtained on admission, and in blood 48 hours after inclusion, in 268 patients admitted with a hemispheric ischemic stroke lasting 8.2 ± 5.9 hours. Infarct volume was measured by days 4 to 7 using computed tomography. Plasma L-arg profiles were analyzed in a separate group of 29 patients seen within 8 hours of onset (median, 4.5 hours) and in 24 male Fischer rats treated with subcutaneous vehicle or 20-mg/kg 1400W (a specific inducible NO synthase inhibitor) every 8 hours for 3 days after performing sham or permanent middle cerebral artery occlusion. Plasma L-arg concentrations decreased after the ischemic event, both in patients and rats, and peaked between 6 and 24 hours. In patients, there was a highly correlation between L-arg levels in CSF and plasma at 48 hours ($r = 0.85$, $P<0.001$). CSF and plasma L-arg concentrations negatively correlated with infarct volume ($r = -0.40$ and $r = -0.35$, respectively, $P<0.001$), and were significantly lower in patients with early neurologic deterioration and in those with poor outcome (Barthel index <85) at 90 days ($P<0.001$). In rats, the administration of 1400W resulted in a 55% significant reduction of infarct volume measured 72 hours after permanent middle cerebral artery occlusion, an effect that correlated with the inhibition caused by 1400W on the ischemia-induced decrease of plasma L-arg concentrations at 6 to 24 hours after the onset of the ischemia. Taken together, these data indicate that determination of L-arg levels in blood might be useful to evaluate the neurotoxic effects of NO generation. These findings might be helpful to guide future neuroprotective strategies in patients with ischemic stroke. **Key Words:** L-Arginine—Nitric oxide—Cerebral infarct—Rats—iNOS—1400W—Neuroprotection.

There is a growing body of evidence that nitric oxide (NO) is involved in the mechanisms of cerebral ischemia (Iadecola et al., 1997a). NO is synthesized from L-arginine (L-arg) by three different isoforms of the enzyme NO synthase. Overproduction of NO from either the neuronal (nNOS) or the inducible (iNOS) isoforms intervenes in the cytotoxic actions that lead to neuronal death, whereas NO generation from the endothelial (eNOS) isoform protects brain tissue by maintaining regional cerebral blood flow (Samdani et al., 1997).

Although experimental evidence indicates that NO has both a neurotoxic and neuroprotective role, NO generation has recently been related to brain damage in acute ischemic stroke. High NO metabolites (NOx) in CSF within the first 24 hours of the onset of symptoms were associated with higher stroke severity at admission, early neurologic deterioration, larger infarct volumes, and poor outcome at 3 months in a series of 102 patients with a hemispheric ischemic stroke (Castillo et al., 2000). These findings suggest predominant cytotoxic effects of NO in human stroke, and open new

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therapeutic avenues with specific nNOS and iNOS selective inhibitors.

Markers of NO-mediated brain injury could be useful to guide neuroprotective strategies. Because the taking of CSF samples is not feasible in clinical practice, molecular markers in blood that reflect the importance of NO generation are needed. NOx concentrations in plasma are subjected to high variability due to the influence of diet and renal function (Moshage, 1997). However, the plasma value of the NO precursor, L-arg, in predicting ischemic cerebral lesion has not been studied. We have found a highly significant negative correlation between NOx and L-arg concentrations in CSF, suggesting a consumption of extracellular L-arg for the synthesis of NO in patients with greater cerebral damage (Castillo et al., 2000). Therefore, plasma L-arg concentrations might decrease as a result of NO generation after acute ischemic stroke.

In this study, we investigated the relationship of concentrations of L-arg in CSF and plasma with stroke outcome and infarct volume in patients with an acute ischemic stroke. In addition, the blood profile of L-arg was analyzed in a group of patients admitted within the first 8 hours of the onset of symptoms, and in a rat stroke model of permanent middle cerebral artery occlusion (pMCAO) after the administration of a vehicle or 1400W, a specific iNOS inhibitor.

MATERIALS AND METHODS

Clinical study

We studied 268 patients (61% men; mean age, 68 ± 10 years) admitted consecutively with an acute hemispheric ischemic stroke within 24 hours of the onset of symptoms, and 50 control subjects. The purpose of this investigation was to analyze clinical, biochemical, and radiologic factors related to early neurologic deterioration. This series has been the core of recent investigations (Dávalos et al., 2000; Vila et al., 2000) and the characteristics of the control group and the inclusion criteria for patients have already been described (Castillo et al., 1996, 1997). In summary, controls were subjects without neurologic disorders subjected to epidural anesthesia (60% men; mean age, 56 ± 17.5 years), and patients had a persistent focal neurologic deficit and absence of mass effect or cerebral hemorrhage on the cranial computed tomography (CT) performed before inclusion. The mean time from the onset of symptoms to the arrival at the hospital was 8.2 ± 5.9 hours (range, 1.5–23 hours). Blood samples were taken, and stroke severity was quantified using the Canadian Stroke Scale (CSS) on admission and 48 hours after hospitalization. As soon as informed consent was obtained and the cerebral CT was completed, a spinal tap was performed in 242 patients. The average time between taking the first blood sample and the CSF sample was 1.2 ± 0.8 hours. A second brain CT scan was performed between days 4 and 7 after clinical onset. Early signs of infarction were carefully evaluated in the first examination. In the control CT, infarct volume ($0.5 \times a \times b \times c$, where a and b are largest perpendicular diameters measured on the computed tomography, and c is the slice thickness) was calculated. The same radiologist who was blind to clinical and biochemical results performed all CT evaluations. Patients received standard treatment according to published guidelines (Adams et al., 1994). Two clinical outcome measures were evaluated: (1) early neurologic deterioration (END), as a potential sign of enlarging brain injury, and (2) functional capacity at 3 months. Following already published criteria (Dávalos et al., 1990), END was diagnosed when the CSS score dropped 1 or more points within the first 48 hours of hospitalization. We used the Barthel index to assess patients' functional capacity at 3 months. Poor outcome was defined as death or Barthel index lower than 85 (Sluter et al., 1999).

To analyze the plasma L-arg profile, we studied a separate group of 29 patients (mean age, 69 ± 8 years) with an acute ischemic stroke of less than 8 hours duration. Blood samples were collected at admission (mean time from stroke onset, 4.9 ± 1.4 hours) and at 12, 24, 36, 48, and 120 hours from the onset of symptoms. Cerebrovascular risk factors and stroke subtypes were similar to those of the total series. The median [quartile] CSS score at admission was 5.5 [3.0,8.0]. One patient died on the second day of hospitalization, so the complete profile was available in 28 patients.

Experimental study

Experiments were performed on male Fischer rats weighing 225 to 275 g. Rats were anesthetized with 2.5% halothane in a mixture of 70% nitrogen/30% oxygen, and permanent focal cerebral ischemia was induced by ligature of the left common carotid artery and occlusion of the ipsilateral distal middle cerebral artery as described previously (Puig et al., 2000). During the process, body temperature was maintained at 37.5°C ± 0.5°C. All procedures conformed to the Committee of Animal Care at the Universidad Complutense of Madrid according with European Union rules (DC86/609/CEE).

Three groups were used for determinations of L-arg levels and infarct volume. Rats in which the middle cerebral artery was exposed but not occluded (sham-operated controls, n = 8), rats with pMCAO (n = 8), and rats which had received 20-mg/kg N-(3-(aminomethyl)benzyl) acetamidine (1400W; GlaxoSmithKline, UK) at onset of ischemia and at 8-hour intervals for 3 days after pMCAO by a subcutaneous injection volume of 1 mL/100 g body weight (MCAO + 1400W; n = 8). The dose and time of administration of 1400W was chosen according to our previous data (Cárdenas et al., 1998; Menchén et al., 2001). Blood samples were obtained from the tail before pMCAO (time 0) and 1, 2, 4, 6, 24, 48, and 72 hours after pMCAO in the presence or absence of 1400W.

The brains were removed 72 hours after pMCAO, and series of 2-mm coronal brain slices were obtained (Brain Matrix, WPI, UK) and stained in 1% TTC (2,3,5-triphenyl-tetrazolium chloride, Merck) in 0.1-mol/L phosphate buffer. The infarcted area, which is not stained, was quantified by image analysis (Scion Image for Windows 2000, Scion Corporation, Frederick, MD, U.S.A.).

Laboratory determinations

Blood and CSF samples were centrifuged and immediately stored at −80°C until L-arg determination. Quantification of L-arg was performed by high-performance liquid chromatography following the method described elsewhere (Castillo et al., 1996). Amino acid determinations from patients and animals were done in the same laboratory and were blinded to the experimental group, clinical and neuroimaging findings, and to stroke outcome.

Statistical analyses

L-Arg concentrations are expressed as median [quartiles], because they were not normally distributed. The comparison of

L-arg concentrations between two groups was performed with the Mann-Whitney test, and comparisons were made among more than two groups with the Kruskal-Wallis test. Comparisons of repeated measures were done with the Friedman test. Spearman analysis was used for bivariate correlations between L-arg and CSS score, time from stroke onset to inclusion, and infarct volume.

We used logistic regression analysis to study the importance of L-arg concentration on END and stroke outcome. The effect on infarct volume was analyzed by multiple linear regression analysis, after a log-transformation of infarct volume to achieve a normal distribution. The models were adjusted for the time from onset of symptoms to hospitalization, and for the variables associated with END and poor outcome in our previous investigations conducted in the same series of patients: age, body temperature, serum glucose, CSS score on admission, and early CT signs of cerebral infarct (Castillo et al., 1996, 1997; Dávalos et al., 2000; Vila et al., 2000). One logistic model was built for each L-arg parameter (CSF levels on admission and plasma concentrations at 48 hours), so the odds ratios are given after adjusting for the same six covariates. In the same way, the effect of each L-arg parameter on infarct volume was studied in a separate linear regression model.

RESULTS

Clinical study

Plasma L-arg concentrations on admission were lower in the 268 patients than in the 50 control subjects, although the difference was not statistically significant ($P = 0.068$). L-Arg concentrations determined 48 hours after admission in 254 patients were significantly lower in comparison with the admission levels ($P<0.001$), and then the difference with the values of the control group was significant ($P<0.001$). Median values of L-arg in CSF on admission were also lower in patients than in control subjects ($P<0.001$) (Table 1). We found that the longer the interval from onset of the symptoms to the time CSF and blood samples were taken, the lower the CSF ($r = 0.54$, $P<0.001$) and plasma ($r = 0.15$, $P = 0.015$) L-arg levels.

There was a highly significant correlation between L-arg concentrations in CSF and plasma in the control group ($r = 0.90$, $P<0.001$), and a moderate correlation in the patient group on admission ($r = 0.44$, $P<0.001$). In contrast, there was a stronger correlation between CSF levels on admission and plasma concentrations at 48 hours in the patient group ($r = 0.85$, $P<0.001$).

TABLE 1. *L-Arginine concentrations in plasma and cerebrospinal fluid (CSF) in patients and controls*

	Patients on admission (n = 268)	Patients at 48 h (n = 254)	Control group (n = 50)
L-Arginine in plasma	67 [60,76]	48 [35,67]	74 [63,88]
L-Arginine in CSF*	13.6 [6,16.2]	—	17.6 [16,20.6]

Values are median [quartiles] in μmol/L.
* CSF was obtained in 242 patients.

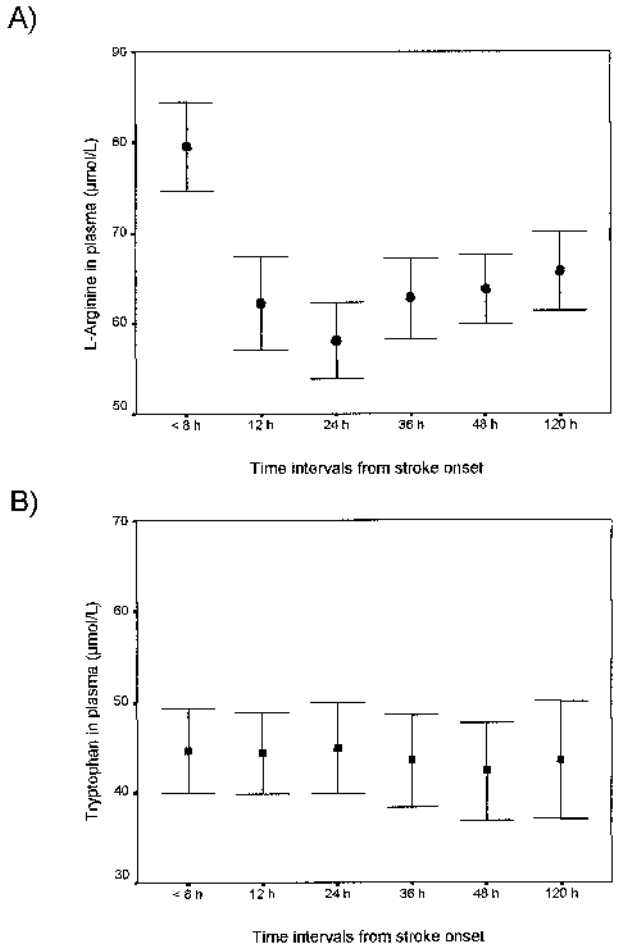

FIG. 1. Error bars showing mean values (dots) and 2 × SEM (vertical lines). **(A)** L-Arginine concentrations in plasma at fixed intervals from stroke onset. Note that L-arginine concentrations decreased within the fist 12 hours and slowly increase after 24 hours (Friedman text, $P<0.001$). **(B)** Tryptophan concentrations in plasma did not show significant changes after stroke onset ($P = 0.67$).

Plasma L-arg profile after stroke

In the series of patients in whom repeated blood samples were taken, plasma L-arg concentrations showed a fall within the first 12 hours of stroke onset, the lowest concentration being at 24 hours. Subsequently, a smooth increase was observed, but L-arg levels still remained lower at day 5 in comparison with the admission levels ($P<0.001$). Plasma concentrations of tryptophan remained stable during the acute phase of stroke (Fig. 1).

L-Arg and clinical outcome

The median [quartiles] CSS score was 6.0 [3.5,8.0] on admission and 5.0 [3.0,8.0] at 48 hours. Eighty-nine (33.2%) patients showed END, and poor outcome was recorded in 126 patients (47.0%) at 3 months (27 patients died and 99 had a Barthel index lower than 85). The median [quartiles] ultimate infarct volume on the second

TABLE 2. *L-Arginine concentrations in plasma and cerebrospinal fluid (CSF) by early clinical course and stroke outcome*

	Early neurologic deterioration			Stroke outcome		
	Yes (n = 89)	No (n = 179)	*P* value	Poor (n = 126)	Good (n = 142)	*P* value
Plasma L-arginine						
On admission	65 [59,69]	68 [61,82]	0.002	67 [60,74]	67 [60,79]	0.950
At 48 h	38 [27,48]	54 [43,71]	<0.001	38 [25,51]	55 [44,69]	<0.001
L-Arginine in CSF*	6.4 [4.5,11.4]	15 [9.2,17.6]	<0.001	6 [4,11.3]	15.5 [14,17]	<0.001

Values are median [quartiles] in μmol/L.
* CSF was obtained in 242 patients on admission.

CT scan was 16.5 [4.5, 36] cc in the 263 patients who survived the first 4 days.

Plasma L-arg concentrations at inclusion had a marginal correlation with CSS score at admission ($r = 0.129$, $P = 0.03$), but not with the ultimate infarct volume. Lower median levels were found in patients with END than in those without END, but no significant differences were found between the poor-outcome and good-outcome groups (Table 2). In contrast, plasma L-arg levels at 48 hours were strongly correlated with CSS score at admission ($r = 0.60$, $P<0.001$) and at 48 hours ($r = 0.72$, $P<0.001$) (Fig. 2B), and moderately correlated with the ultimate infarct volume ($r = -0.35$, $P<0.001$). Patients with END and those with poor outcome had lower median values of L-arg at 48 hours than the groups without END and with good outcome at 3 months (Table 2).

In CSF, L-arg concentrations were strongly correlated with stroke severity on admission ($r = 0.72$, $P<0.001$) and at 48 hours ($r = 0.83$, $P<0.001$) (Fig. 2A), and with the ultimate infarct volume ($r = -0.40$, $P<0.001$). A significant association of low L-arg values with END and poor stroke outcome was found (Table 2). In the logistic regression analyses, low CSF L-arg levels on admission (odds ratio, 0.67; 95% CI, 0.56–0.80; $P<0.0001$) and low plasma L-arg concentrations at 48 hours (odds ratio, 0.91; 95% CI, 0.87–0.95; $P<0.0001$) were independently associated with END, but not with poor outcome at 3 months. In the multiple linear regression analysis, L-arg levels in CSF and plasma were not significantly correlated with the log-transformed infarct volume.

Experimental study

Basal plasma levels of L-arg were stable during the preischemic period. pMCAO produced a significant decrease in plasma concentrations of L-arg (Friedman test, $P<0.001$) (Fig. 3). This decrease was observed 4 hours after MCAO and reached its maximal value 6 hours after pMCAO. L-Arg concentration returned to basal levels 48 to 72 hours after the occlusion. Treatment with 20-mg/kg 1400W for 3 days caused a significant inhibition of the decrease in L-arg concentration at 6 and 24 hours after MCAO. Infarct volume measured at 72 hours after pMCAO was reduced by 55% in the group treated with 20-mg/kg of 1400W in comparison with the nontreated group (Fig. 4).

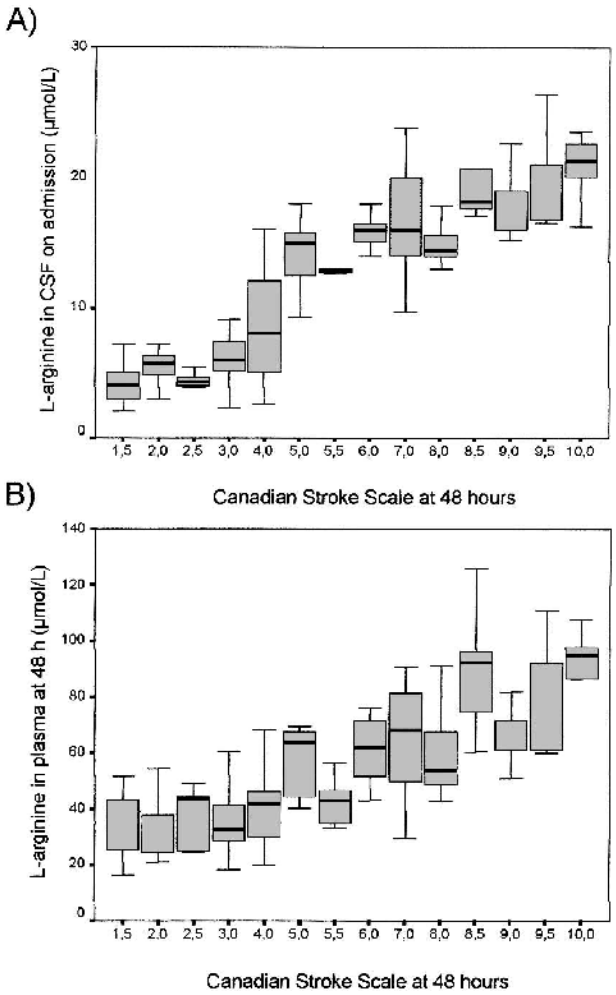

FIG. 2. Boxplots showing median values (horizontal line inside the box), quartiles (box boundaries), and the largest and smallest observed values (lines drawn from the end of the box. **(A)** L-Arginine levels in CSF on admission grouped by Canadian Stroke Scale scores at 48 hours. **(B)** L-Arginine concentrations in plasma grouped by Canadian Stroke Scale scores at 48 hours.

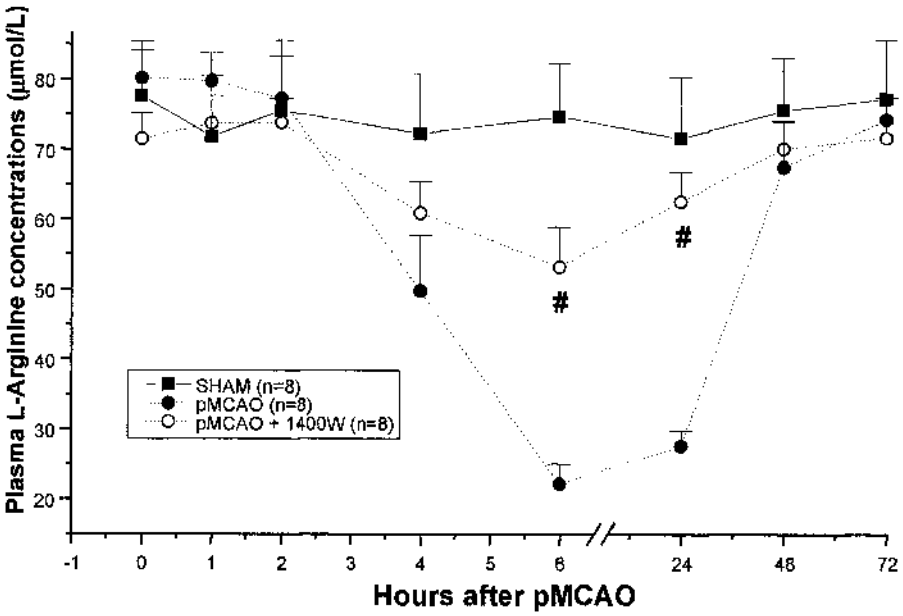

FIG. 3. Effect of 1400W (20 mg/kg at 8-hour intervals) on plasma L-arginine concentrations in rats. Data are mean values (dots) and SD (vertical lines) at fixed intervals after permanent middle cerebral artery occlusion (pMCAO) and in sham-operated animals. #P<0.05 pMCAO + 1400W versus pMCAO.

DISCUSSION

Our findings show that patients with acute ischemic stroke have lower levels of L-arg in plasma and CSF than control subjects, and that low concentrations of L-arg are associated with greater cerebral damage. L-Arg concentrations in CSF within 24 hours of the onset of symptoms, and in plasma 48 hours after acute stroke, had the highest correlation to the clinical signs of brain injury. In this study, two facts suggest that low L-arg levels resulted from the consumption of this amino acid in the molecular events triggered by cerebral ischemia. Firstly, we have observed that the longer the time from symptoms onset, the lower the CSF and plasma values of L-arg. Secondly, serial determinations in blood in a group of patients with acute stroke and in rats after pMCAO, showed a decrease in L-arg concentrations, with a peak value between 6 and 24 hours after the onset of ischemia.

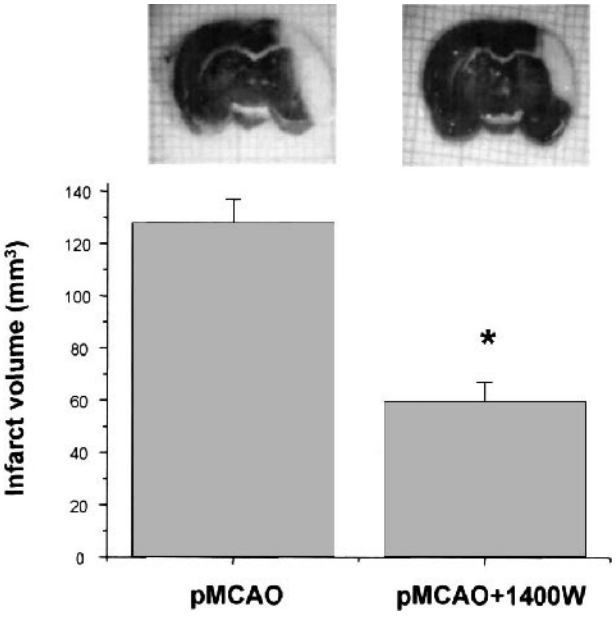

FIG. 4. Effect of 1400W (20 mg/kg at 8-hour intervals) on infarct volume after permanent middle cerebral artery occlusion (pMCAO) in rats. Data are mean values (columns) and SD (vertical lines); *P<0.05. Photomicrographs of representative animals are shown. A smaller cortical infarct is seen after 1400W administration.

The association between L-arg consumption and poor stroke prognosis may be explained by the neurotoxic effects of NO generation, because L-arg is the only known precursor in the synthesis of NO (Moncada and Higgs, 1993; Sessa, 1994). NO is an endogenous free radical, which has a double role in cerebral ischemia (Iadecola et al., 1994). In early stages, small quantities of NO generated by eNOS cause vasodilation and hence an increase in the collateral blood flow limiting the extent of brain injury, whereas in a second phase, a greater production of NO by nNOS and iNOS isoforms worsens cerebral damage (Huang et al., 1994; Iadecola et al., 1995b, 1997b). This explanation fits in with the time-dependent opposite effects of L-arg. In animal models, L-arg has a neuroprotective role when it is administered up to 2 hours after onset of cerebral ischemia, but increases infarct volume when its administration is delayed by 24 hours. The deleterious consequence of a delayed L-arg administration is thought to result from NO generation by iNOS expression after a time lag of 6 to 12 hours (Iadecola et al., 1995a; Zhang et al., 1995).

Some clinical findings support the idea that low L-arg levels in acute stroke are due to NO generation. In a previous study, we found a negative correlation between NO metabolites (NOx) and L-arg concentrations in CSF

within 24 hours of stroke onset (Castillo et al., 2000). CSF NOx were particularly high in patients with END and in those with L-arg levels below 6.0 μmol/L. The present findings replicate, in a larger series of patients, the results of our earlier investigation, because the median value of L-arg in CSF of patients with END was 6.4 μmol/L in contrast with 15 μmol/L in patients with good early and late outcome.

Interestingly, what we have observed in plasma may be a reflex of what happened to the CNS within the first 24 hours of stroke, given that there was a lineal correlation between levels of L-arg in the CSF at admission and the levels of L-arg at 48 hours in plasma. These findings, together with the high correlation between blood and CSF values in control subjects, suggest that NO generation after cerebral infarction by *de novo* expression of the inducible NOS isoform might increase the demand of L-arg, and presumably reduce the extracellular concentrations, firstly in CSF and subsequently in plasma, due to good diffusion of this amino acid through the blood–brain barrier. L-Arg has been found to cross blood–brain barrier through a transporter with specificity for amino acid analogues possessing cationic terminal guanidine groups, such as those contained in L-arg (Mahar et al., 2000), but its diffusion may be even easier as a result of stroke (del Zoppo, 1994; Sage et al., 1984). We can reasonably rule out a plasma L-arg decline due to a low L-arg intake in the acute phase of stroke because the concentrations of tryptophan, an amino acid not involved in the pathophysiology of cerebral ischemia, were stable during the same period. One of the major questions raised by our study is whether low L-arg levels in blood are the expression of brain ischemia or originate as a result of the acute-phase reaction or systemic causes like concomitant infections, these factors being responsible for the neurological worsening. Because recent infections have been associated with an increased risk of impending stroke and the release of proinflammatory molecules, NO generation and L-arg consumption could have been influenced by a recent infection (Grau et al., 1995). To partially control for such effects, we included in the analysis indirect markers of infections, such as body temperature, and other prognostic factors of brain damage.

Although this clinical study does not allow us to rule out the possibility of a reduction of plasma L-arg after stroke onset due to metabolic reactions other than the L-arg–NO pathway (Moncada et al., 1991; Moncada and Higgs, 1993; Guayao and Morris, 1998), the experimental study confirms our clinical hypothesis. The decrease in plasma L-arg concentrations after pMCAO was inhibited by the administration of one of the most selective inhibitors of iNOS isoform described to date, an effect that correlated with a significant reduction in infarct volume. In contrast with aminoguanidine (an iNOS inhibitor used to produce neuroprotection after focal ischemia in rats that also inactivates the constitutive isoforms of NO synthase in the simultaneous presence of Ca^{2+}, calmodulin, and other cofactors; Iadecola et al., 1995b; Wolff and Lubeskie, 1995), 1400W is an irreversible inhibitor or an extremely slowly reversible inhibitor of iNOS. Furthermore, physiologic concentrations of the substrate, L-arg, reverse the weak inhibition that 1400W exerts on the constitutive isoforms (nNOS and eNOS), while not affecting inhibition on iNOS activity. Although 1400W is closely related to bisisothioureas, compounds that are acutely toxic, it has been reported that toxicity is only observed at doses higher than the therapeutic ones (Garvey et al., 1997). Therefore, we can attribute the reduction of plasma L-arg concentration after stroke onset to the NO generation by the expression of the iNOS isoform.

In conclusion, this study has demonstrated L-arg consumption after acute ischemic stroke, particularly in patients with greater ischemic brain damage and worse stroke outcome. Taken together with the experimental results, our data indicate that determination of L-arg levels in blood might be useful to evaluate the neurotoxic effects of NO generation. These findings might be helpful to guide future neuroprotective strategies in patients with ischemic stroke.

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REFERENCES

Adams HP Jr, Brott TG, Crowell RM, Furlan AJ, Gomez CR, Grotta J, Helgason CM, Marler JR, Woolson RF, Zivin JA, et al (1994) Guidelines for the management of patients with acute ischemic stroke. A Statement for Healthcare Professionals from a Special Writing Group of the Stroke Council, American Heart Association. *Stroke* 25:1901–1914

Cárdenas A, De Alba J, Moro MA, Leza JC, Lorenzo P, Lizasoain I (1998) Neuroprotective effect of N-(3-(aminomethyl benzyl) acetamidine, an inducible nitric oxide synthase inhibitor, in brain slices exposed to oxygen-glucose deprivation. *Eur J Pharmacol* 354:161–165

Castillo J, Dávalos A, Naviero J, Noya M (1996) Neuroexcitatory amino acids and their relation to infarct size and neurological deficit in ischemic stroke. *Stroke* 27:1060–1065

Castillo J, Dávalos A, Noya M (1997) Progression of ischemic stroke and excitotoxic amino acids. *Lancet* 349:79–83

Castillo J, Rama R, Dávalos A (2000) Nitric oxide–related brain damage in acute ischemic stroke. *Stroke* 31:852–857

Dávalos A, Castillo J, Marrugat J, Fernandez-Real JM, Armengou A, Cacabelos P, Rama R (2000) Body iron stores and early neurologic deterioration in acute cerebral infarction. *Neurology* 54:1568–1574

Dávalos A, Cendra E, Teruel J, Martinez M, Genís D (1990) Deteriorating ischemic stroke: risk factors and prognosis. *Neurology* 140:1865–1869

del Zoppo GJ (1994) Microvascular changes during cerebral ischemia and reperfusion. *Cerebrovasc Brain Metab Rev* 6:47–96

Garvey EP, Oplinger JA, Furfine ES, Kiff RJ, Laszlo F, Whittle BJR, Knowles RG (1997) 1400W is a slow, tight binding, and highly

selective inhibitor of inducible nitric-oxide synthase *in vitro* and *in vivo*. *J Biol Chem* 272:4959–4963

Grau AJ, Buggle F, Heindl S, Steichen-Wienh C, Banerjee T, Maiwald M, Rohlfs M, Suhr H, Fiehn W, Becher H, Hacke W (1995) Recent infection as a risk factor for cerebrovascular ischemia. *Stroke* 26:373–379

Guayao WU, Morris MM (1998) Arginine metabolism: nitric oxide and beyond. *Biochem J* 336:1–17

Huang Z, Huang PL, Panahian N, Dalkara T, Fishman MC, Moskowitz MA (1994) Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. *Science* 265:1883–1885

Iadecola C, Pellegrino DS, Moskowitz MA, Lassen NA (1994) Nitric oxide synthase inhibition and cerebrovascular regulation. State of the art review. *J Cereb Blood Flow Metab* 14:175–192

Iadecola C, Zhang F, Xu S, Casey R, Ross ME (1995a) Inducible nitric oxide synthase gene expression in brain following cerebral ischemia. *J Cereb Blood Flow Metab* 15:378–384

Iadecola C, Zhang F, Xu X (1995b) Inhibition of inducible nitric oxide synthase ameliorates cerebral ischemic damage. *Am J Physiol* 268:R286-R292

Iadecola C (1997a) Bright and dark sides of nitric oxide in ischemic brain injury. *Trend Neurosci* 20:132–139.

Iadecola C, Zhang F, Casey R, Nagayama N, Ross ME (1997b) Delayed reduction of ischemic brain injury and neurological deficits in mice lacking the inducible nitric oxide synthase gene. *J Neurosci* 17:9157–9164

Mahar DKM, Lakhman SS, Boje KM (2000) Blood-brain barrier transport studies of organic guanidino cations using an *in situ* brain perfusion technique. *Brain Res* 876:141–147

Menchén L, Colón A, Moro MA, Leza JC, Lizasoain I, Menchén P, Alvarez E, Lorenzo P (2001) N-(3-(aminomethyl benzyl) acetamidine, an inducible nitric oxide synthase inhibitor, decreases colonic inflammation induced by trinitrobenzene sulphonic acid in rats. *Life Sci* 69:479–491

Moncada S, Higgs A (1993) The L-arginine-Nitric oxide pathway. *N Engl J Med* 329:2002–2012

Moncada S; Palmer RMJ, Higgs EA (1991) Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol Rev* 43:109–1042

Moshage H (1997) Nitric oxide determinations: much a do about NO°-Thing?. *Clin Chem* 43:553–556

Puig N, Dávalos A, Adan J, Piulats J, Martinez JM, Castillo J (2000) Serum amino acid levels after permanent middle cerebral artery occlusion in the rat. *Cerebrovasc Dis* 10:449–454.

Sage JI, van Uitert RL, Duffy TE (1984) Early changes in blood brain barrier permeability to small molecules after transient cerebral ischemia. *Stroke* 15:46–50

Samdani AF, Dawson TM, Dawson VL (1997) Nitric oxide synthase in models of focal ischemia. *Stroke* 28:1283–1288

Sessa WC (1994) The nitric oxide synthase family of proteins. *J Vas Res* 31:131–142

Sluter G, Steen C, De Keyser J (1999) Use of Barthel index and modified Rankin scale in acute stroke trials. *Stroke* 30:1538–1541

Vila N, Castillo J, Dávalos A, Chamorro A (2000) Proinflammatory cytokines and early neurological worsening in ischemic stroke. *Stroke* 31:2325–2329

Wolff DJ, Lubeskie A (1995) Aminoguanidine is an isoform-selective, mechanism-based inactivator of nitric oxide synthase. *Arch Biochem Biophys* 316:290–301

Zhang F, Xu S, Iadecola C (1995) Time dependence effect of nitric oxide synthase inhibition on cerebral ischemic damage. *J Cereb Blood Flow Metab* 15:595–601