ELSEVIER

European Journal of Pharmacology 354 (1998) 161–165

ejp

# Protective effect of *N*-(3-(aminomethyl)benzyl)acetamidine, an inducible nitric oxide synthase inhibitor, in brain slices exposed to oxygen–glucose deprivation

Antonio Cárdenas, Jorge De Alba, María A. Moro, Juan C. Leza, Pedro Lorenzo, Ignacio Lizasoain *

*Departamento de Farmacología, Facultad de Medicina, Universidad Complutense de Madrid, 28040 Madrid, Spain*

Received 1 April 1998; revised 10 June 1998; accepted 12 June 1998

**Abstract**

It has been suggested that large amounts of nitric oxide (NO) produced by inducible NO synthase are involved in the mechanisms of neurotoxicity after cerebral ischaemia. We have recently demonstrated that inducible NO synthase was expressed within hours after rat forebrain slices were exposed to oxygen–glucose deprivation. Therefore, we sought to determine whether NO produced by inducible NO synthase contributes to tissue damage in this model, by using a new, highly selective, inhibitor of inducible NO synthase, *N*-(3-(aminomethyl)benzyl)acetamidine (1400W). We found that incubation with 1400W from the start of the oxygen–glucose deprivation period until the end of the experiment decreases tissue damage determined as lactate dehydrogenase (LDH) efflux 4 h after the oxygen–glucose deprivation period, the time at which inducible NO synthase expression is maximal in this model. This effect may be a result of direct inhibition of inducible NO synthase activity, raising the possibility of a clinical use of selective inhibitors of this NO synthase isoform in the management of cerebral ischaemia. 

*Keywords:* Brain; Forebrain slice; Ischaemia; Nitric oxide (NO) synthase; (Rat); 1400W (*N*-(3-(aminomethyl)benzyl)acetamidine)

## 1. Introduction

There is increasing evidence that nitric oxide (NO) is involved in the mechanisms of neurotoxicity after cerebral ischaemia: NO production is enhanced at all stages of cerebral ischaemia and this increase in NO production is accompanied by up regulation of both NO synthase activity and gene expression (for review, see Iadecola, 1997).

It has been suggested that large amounts of NO produced by de novo expression of inducible NO synthase contribute to the progression of the damage and would thus be neurotoxic: for example, knockout mice lacking inducible NO synthase present smaller infarcts after an ischaemic injury than do wild-type mice (Iadecola, 1997). In addition, the administration of a relatively selective inducible NO synthase inhibitor (aminoguanidine) 24 h after occlusion of the rat middle cerebral artery has been shown to reduce the extent of the brain damage associated with focal ischaemia, suggesting that NO production by inducible NO synthase contributes to the tissue damage associated with late stages of focal cerebral ischaemia (Iadecola et al., 1995a).

We have recently demonstrated that inducible NO synthase is expressed in several cell types including neurones after oxygen–glucose deprivation in rat forebrain slices. In our model, maximal inducible NO synthase expression was found 180 min after the end of a 20-min oxygen–glucose deprivation period, suggesting that NO might play an important pathogenic role in the tissue damage that occurs in early stages of cerebral ischaemia (Moro et al., 1998).

It now remains to be established whether selective inhibition of inducible NO synthase with no effect on the constitutive NO synthase isoforms decreases tissue damage in this model. Aminoguanidine has been used, but it also inactivates the constitutive isoforms of NO synthase in the simultaneous presence of $Ca^{2+}$, calmodulin and other co-factors (Wolff and Lubeskie, 1995). In this context, *N*-(3-

* Corresponding author. Tel.: +34-1-394-1478; Fax: +34-1-394-1463; E-mail: nlfucm@eucmax.sim.ucm.es

0014-2999/98/$19.00 

(aminomethyl)benzyl)acetamidine (1400W) is the most selective inhibitor of inducible NO synthase isoform described to date. Furthermore 1400W is an irreversible inhibitor or an extremely slowly reversible inhibitor of inducible NO synthase; in addition, its inhibitory effect on constitutive NO synthase isoforms is relatively weaker, is rapidly reversible and competitive with that of L-arginine (Garvey et al., 1997).

In the present study we therefore used 1400W to determine whether NO produced by inducible NO synthase contributes to tissue damage in rat forebrain slices exposed to oxygen–glucose deprivation.

## 2. Materials and methods

### 2.1. Preparation and incubation of slices

Male Sprague–Dawley rats (200–250 g) were killed by decapitation (according to procedures approved by Committee of Animal Care at the Universidad Complutense of Madrid), and the slices were prepared and preincubated as described (Moro et al., 1998). Slices were then incubated in a modified Krebs–Henseleit solution (incubation solution) containing (mM): NaCl (120), KCl (2), $CaCl_2$ (2), $NaHCO_3$ (26), $MgSO_4$ (1.19), $KH_2PO_4$ (1.18), glucose (11) and 5,6,7,8-tetrahydrobiopterin ($BH_4$, 10 μM) bubbled with 95% $O_2$/5% $CO_2$. The slices corresponding to the control group were then incubated 10 min further under the same conditions. Slices corresponding to the 'ischaemic' (oxygen–glucose-deprived) group were incubated 10 min in the solution without glucose and equilibrated with 95% $N_2$/5% $CO_2$ to mimic an ischaemic condition. After these periods of 10 min, the medium from both groups was replaced every 30 min with fresh incubation solution containing glucose and equilibrated with 95% $O_2$/5% $CO_2$ to simulate a 'reperfusion' period. In some experiments, 1400W (20 μM) was included in the incubation solution during both 'ischaemic' and 'reperfusion' periods. Slices were taken out at different times: 120, 180, 210 and 240 min after the oxygen–glucose deprivation period and frozen immediately with liquid nitrogen.

### 2.2. NO synthase activity

Two different measurements of NO synthase were performed.

(1) NO synthase activity was determined after sonication of both control and oxygen–glucose-deprived forebrain slices (Labsonic 2000) at 4°C in 5 volumes of buffer containing 320 mM sucrose, 1 mM EDTA, 1 mM DL-dithiothreitol, 10 μg/ml leupeptin, 100 μg/ml phenylmethylsulphonyl fluoride, 10 μg/ml soybean trypsin inhibitor, 2 μg/ml aprotinin and 50 mM Tris brought to pH 7.0 at 20°C with HCl. The homogenate was centrifuged at 4°C at 12 000 × $g$ for 20 min and the pellet was discarded. NO synthase activity was then determined in the post mitochondrial supernatant by monitoring the conversion of L-[U-$^{14}$C]arginine into [U-$^{14}$C]citrulline as described by Salter et al. (1991) with modifications by Rees et al. (1995), according to which the co-factors NADPH (100 μM), $BH_4$ (3 μM), FAD (3 μM) and FMN (3 μM) are included in the enzyme assay. Using this modification, we obtained a significant improvement (10–20-fold) in the values for constitutive NO synthase, but not those for inducible NO synthase. The activity of the $Ca^{2+}$-dependent isoforms (constitutive NO synthases) was determined from the difference between the [$^{14}$C]citrulline produced from control samples and from samples containing 1 mM EGTA; the activity of the $Ca^{2+}$-independent isoform (inducible NO synthase) was determined from the difference between samples containing 1 mM EGTA and samples containing 1 mM EGTA and 1 mM $N^G$-monomethyl-L-arginine (L-NMMA).

(2) To test the effect of 1400W on inducible NO synthase activity, we used slices exposed to oxygen–glucose deprivation to obtain the enzyme from the homogenate. To examine the effects of 1400W on constitutive NO synthase activity, freshly cut forebrain slices were used to obtain the enzyme from the homogenate. The activity was determined as described before, where the concentration of L-arginine in the assay buffer (Rees et al., 1995) was 20 μM. In addition, to study the L-arginine-induced reversal of 1400W-induced inhibition of NO synthase isoforms, 200 μM L-arginine was used. The effect of 1400W (2–200 μM) on inducible NO synthase activity was determined from the difference between samples containing 1 mM EGTA and samples containing 1 mM EGTA and 1400W (2–200 μM). The effect of 1400W on constitutive NO synthase activity was determined by comparing [$^{14}$C]citrulline produced from control samples and from samples containing 1400W (20 μM).

The protein content of the homogenate from each slice was determined using bicinchoninic acid (Hill and Straka, 1988).

### 2.3. Lactate dehydrogenase (LDH) assay

Tissue damage was measured by examining the LDH efflux to the incubation solution. Samples of this solution were taken every 30 min during 240 min of reperfusion and the enzyme activity was measured spectrophotometrically at 340 nm by following the oxidation of NADH (decrease in absorbance) in the presence of pyruvate (Koh and Choi, 1987), using a Spectronic 601 spectrophotometer or a Molecular Devices microplate reader.

### 2.4. Chemicals and statistical analyses

L-[U-$^{14}$C]arginine was obtained from Amersham, $BH_4$ [(6$R$)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride] was obtained from RBI (Research Biochemicals International),

*N*-(3-(aminomethyl)benzyl) acetamidine (1400W) was obtained from Glaxo-Wellcome (UK) and other chemicals were from Sigma or as indicated in previous sections. Results are expressed as means ± S.E.M. of the indicated number of experiments, and statistical comparisons were made using a Newman–Keuls test. $P < 0.05$ was considered as statistically significant.

## 3. Results

### *3.1. Inducible and constitutive NO synthase activity in rat forebrain slices exposed to oxygen–glucose deprivation*

Oxygen–glucose deprivation for 10 min caused the appearance of an inducible NO synthase activity in the rat forebrain slices, which increased time dependently during the period studied. As shown in Fig. 1, inducible NO synthase activity appeared approximately 180 min after the oxygen–glucose deprivation period, reaching its maximal activity 210 min after the end of this period. During the same period, inducible NO synthase activity was not detected in control slices (data not shown). On the other hand, constitutive NO synthase activity in oxygen–glucose deprived slices was not significantly modified at the times studied (Fig. 1, $P > 0.05$).

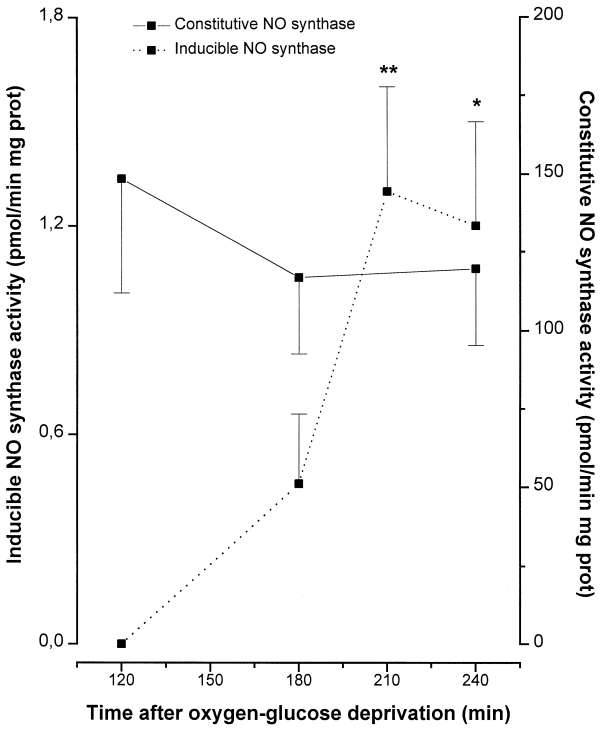


Fig. 1. Time-course of inducible and constitutive NO synthase activity from rat forebrain slices exposed to oxygen–glucose deprivation. NO synthase activity was measured by monitoring the conversion of L-[U-$^{14}$C]arginine into [U-$^{14}$C]citrulline (see Section 2). The data represent the means ± S.E.M. of 16 independent experiments. * $P < 0.05$; ** $P < 0.01$ (Newman–Keuls test).

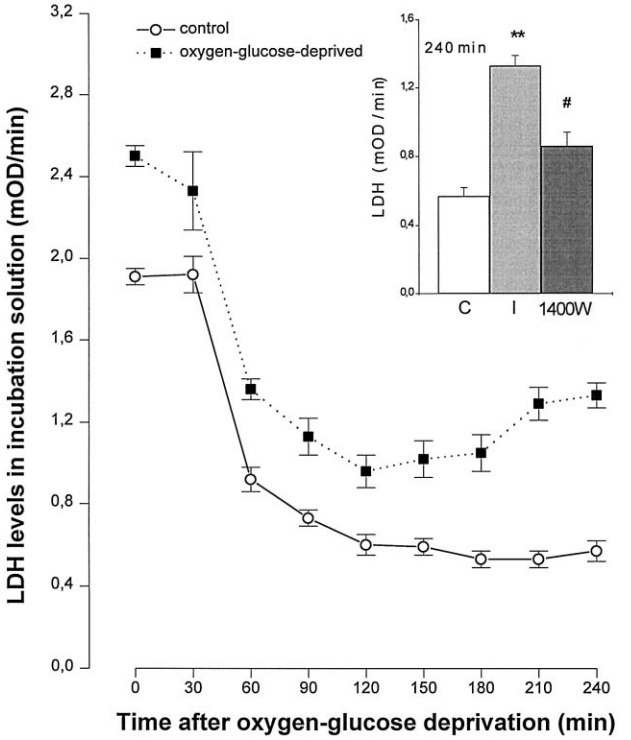


Fig. 2. LDH levels in incubation solution of rat forebrain slices exposed to oxygen–glucose deprivation vs. control tissue ($P < 0.05$). LDH levels were measured by monitoring the oxidation of NADH in the presence of pyruvate (see Section 2). Inset: Effect of the addition of 1400 W (20 μM) to the slices exposed to oxygen–glucose deprivation on LDH release at 240 min. ** $P < 0.01$ control (C) vs. oxygen–glucose-deprived (I); # $P < 0.05$ oxygen–glucose-deprived vs. oxygen–glucose-deprived + 1400W (1400W) (Newman–Keuls test).

### *3.2. LDH levels in incubation solution from control and oxygen–glucose-deprived rat forebrain slices*

Since LDH levels remaining in control and oxygen–glucose-deprived slices at 240 min of reperfusion were very high and not significantly different from each other (8375 ± 69 vs. 8512 ± 293 mOD/min in control and oxygen–glucose-deprived slices, respectively, $n = 4$, $P > 0.05$), LDH efflux was merely expressed as the LDH activity (mOD/min) present in the incubation solution. After 10 min of oxygen–glucose deprivation, LDH levels in the incubation solution were significantly higher ($P < 0.05$) than those found in control slices during the whole period of reperfusion. The difference in LDH levels was stable (ΔmOD/min: 0.4 ± 0.03) from 30 to 150 min of reperfusion. However, this difference then increased (ΔmOD/min: 0.76 ± 0.01), to reach a maximum level at the latest times of reperfusion (Fig. 2).

### *3.3. Effect of 1400W on LDH efflux*

The addition of 1400W (20 μM) to the incubation solution from slices exposed to oxygen–glucose deprivation caused a significant inhibition of LDH release at 240

Table 1
Effect of 1400W (2–200 μM) on inducible NO synthase activity found in brain slices exposed to oxygen–glucose deprivation and on constitutive NO synthase activity from freshly cut slices

| | Inducible NO synthase activity (%) | Constitutive NO synthase activity (%) |
|---|---|---|
| Control | 100 | 100 |
| 1400W 2 μM | 49 ± 9[a] | – |
| 1400W 20 μM | 11 ± 4[b] | 71 ± 4[a] |
| 1400W 200 μM | 11 ± 4[b] | – |
| 1400W 20 μM + L-Arg 200 μM | 11 ± 4[b] | 93 ± 8 |

[a] $P < 0.05$.
[b] $P < 0.01$ vs. control.
$n = 4–14$ (Newman–Keuls test).
NO synthase activity was measured by monitoring the conversion of L-[U-$^{14}$C]arginine into [U-$^{14}$C]citrulline (see Section 2).
Data are expressed as % of NO synthase activity in the absence of 1400W and are means ± S.E.M.

min of reperfusion (35% inhibition, $P < 0.05$) (Fig. 2, inset), but not at earlier times (data not shown).

### 3.4. Effect of 1400W on NO synthase activity

1400W (2–200 μM) caused a concentration-dependent inhibition of the inducible NO synthase activity of the solution from oxygen–glucose-deprived slices at 20 μM L-arginine (Table 1, $n = 14$, $P < 0.05$). The effect of 1400W (20 μM) on inducible NO synthase activity was not reversed in the presence of 200 μM L-arginine (Table 1, $n = 4–14$, $P > 0.05$ vs. 20 μM 1400W alone). On the other hand, 20 μM 1400W partially inhibited constitutive NO synthase activity in the presence of 20 μM L-arginine (Table 1, $n = 4$, $P < 0.05$) but this effect was reversed by 200 μM L-arginine (Table 1, $n = 4$, $P > 0.05$ vs. control).

## 4. Discussion

We have demonstrated that the selective inhibitor of inducible NO synthase, 1400W, attenuates LDH efflux into the incubation solution of rat forebrain slices exposed to oxygen–glucose deprivation as compared the efflux from control tissue. These findings suggest that pharmacological inhibition of inducible NO synthase activity after ischaemia reduces tissue damage, indicating that NO production by inducible NO synthase participates in cerebral ischaemia.

We have previously shown that 20 min of oxygen–glucose deprivation leads to the appearance of $Ca^{2+}$-independent NO synthase activity in rat forebrain slices as early as 120 min after the hypoxic insult, which corresponded to the expression of the inducible isoform of NO synthase, as shown by detection of both inducible NO synthase message and protein in these tissues (Moro et al., 1998). Different authors have found inducible NO synthase expression in in vivo models of ischaemia-reperfusion but this expression did not take place before 1–3 days (Endoh et al., 1994; Wallace and Bisland, 1994; Iadecola et al., 1995b). Since it is known that much of the tissue damage following cerebral ischaemia occurs within the first few hours (Dereski et al., 1993; Garcia et al., 1993), our data suggest that NO could play an important pathogenic role not only in the late stages of ischaemia but also in the initiation of this process.

We have now shown that the time of appearance of inducible NO synthase activity is dependent on the duration of the oxygen–glucose deprivation period since, when this period lasts 10 min, inducible NO synthase activity appears 180 min after the 'ischaemic' injury, reaching a maximum at 210 min in our model.

Oxygen–glucose deprivation causes cytotoxicity in our model, as revealed by the increase during the reperfusion period of LDH release to the incubation solution of oxygen–glucose-deprived slices as compared with control tissue. However, the participation of inducible NO synthase in tissue damage does not seem to occur before 180 min. The addition of 1400W causes a significant inhibition of LDH release from slices exposed to oxygen–glucose deprivation at the latest time studied. The effect of 1400W on LDH levels may be a result of direct inhibition of the inducible isoform of NO synthase and not of the constitutive isoforms, since 1400W caused significant inhibition of the inducible NO synthase activity in slices exposed to oxygen–glucose deprivation, and this inhibition was not reversed in the presence of L-arginine concentrations (200 μM) in the physiological range (Barbul, 1986). Although 1400W partially inhibited constitutive NO synthase activity, the effect was reversed in the presence of physiological concentrations of L-arginine. This might have been expected since the inhibition of $Ca^{2+}$-dependent NO synthase isoforms by 1400W is relatively weak and rapidly reversible with L-arginine (Garvey et al., 1997).

On the other hand, 1400W had significant effect on LDH release later after the oxygen–glucose-deprivation period. This is consistent with our data on the time profile of $Ca^{2+}$-independent NO synthase activity, suggesting both that inducible NO synthase could account for cytotoxicity at those stages in which its activity is maximal, and that other phenomena such as excitotoxicity were responsible for the damage at earlier times.

## 5. Conclusion

All these data suggest that our model may be considered a useful in vitro method since it is easily accessible and is suitable for pharmacological studies trying to determine the therapeutic role of different tools such as NO synthase inhibitors in the management of cerebral ischaemia.

Although 1400W is closely related to bisisothioureas, compounds which are acutely toxic, it has been reported that toxicity is only observed at doses higher than the therapeutic ones (reviewed in Garvey et al., 1997).

Together, these observations provide evidence allowing one to conclude that the administration of the highly selective inducible NO synthase inhibitor, 1400W, decreases the release of LDH in slices exposed to oxygen–glucose deprivation by direct inhibition of inducible NO synthase activity. The data indicate that NO production by inducible NO synthase contributes to the ischaemic damage from early to late stages. This raises the possibility of clinical use of inducible NO synthase inhibitors in the management of cerebral ischaemia to diminish neural tissue damage during the reperfusion period.

## Acknowledgements

We thank Dr. R.G. Knowles (Glaxo-Wellcome, U.K.) for helpful discussions and for supplying 1400W. This work was supported by grants from DGICYT PM95-0070 and Fundación Central Hispano. J.D.A. and A.C. are recipients of fellowships of the Ministry of Education and Science and Universidad Complutense of Madrid, Spain.

## References

Barbul, A., 1986. Arginine: biochemistry, physiology, and therapeutic implications. J. Parenter. Enter. Nutr. 10, 227–238.

Dereski, M.O., Chopp, M., Knight, R.A., Rodolosi, L.C., Garcia, J.H., 1993. The heterogeneous temporal evolution of focal ischemic neuronal damage in the rat. Acta Neuropathol. 85, 327–333.

Endoh, M., Maiese, K., Wagner, J., 1994. Expression of the inducible form of nitric oxide synthase by reactive astrocytes after transient global oxygen and glucose deprivation. Brain Res. 651, 92–100.

Garcia, J.H., Yoshida, Y., Chen, H., Li, Y., Zhang, Z.G., Lian, J., Chen, S., Chopp, M., 1993. Progression from ischemic injury to infarct following middle cerebral artery occlusion in the rat. Am. J. Pathol. 142, 623–635.

Garvey, E.P., Oplinger, J.A., Furfine, E.S., Kiff, R.J., Laszlo, F., Whittle, B.J.R., Knowles, R.G., 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.

Hill, H.D., Straka, J.G., 1988. Protein determination using bicinchoninic acid in the presence of sulfhydryl reagents. Anal. Biochem. 170, 203–208.

Iadecola, C., 1997. Bright and dark sides of nitric oxide in ischemic brain injury. Trends Neurosci. 20, 132–139.

Iadecola, C., Zhang, F.Y., Xu, X., 1995a. Inhibition of inducible nitric oxide synthase ameliorates cerebral ischemic damage. Am. J. Physiol. 268, R286–R292.

Iadecola, C., Zhang, F., Xu, S., Casey, R., Ross, M.E., 1995b. Inducible nitric oxide synthase gene expression in brain following cerebral ischemia. J. Cereb. Blood Flow Metab. 15, 378–384.

Koh, J.Y., Choi, D.W., 1987. Quantitative determinations of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. J. Neurosci. Methods 20, 83–90.

Moro, M.A., De Alba, J., Leza, J.C., Lorenzo, P., Fernández, A.P., Bentura, M.L., Boscá, L., Rodrigo, J., Lizasoain, I., 1998. Neuronal expression of inducible nitric oxide synthase after oxygen and glucose deprivation in rat forebrain slices. Eur. J. Neurosci. 10, 445–456.

Rees, D.D., Cunha, F.Q., Assreuy, J., Herman, A.G., Moncada, S., 1995. Sequential induction of nitric oxide synthase by *Corynebacterium parvum* in different organs of the mouse. Br. J. Pharmacol. 114, 689–693.

Salter, M., Knowles, R.G., Moncada, S., 1991. Widespread tissue distribution, species distribution and changes in activity of $Ca^{2+}$-dependent and $Ca^{2+}$-independent nitric oxide synthases. FEBS Lett. 291, 145–149.

Wallace, M.N., Bisland, S.K., 1994. NADPH-diaphorase activity in activated astrocytes represents inducible nitric oxide synthase. Neuroscience 59, 905–919.

Wolff, D.J., Lubeskie, A., 1995. Aminoguanidine is an isoform-selective, mechanism-based inactivator of nitric oxide synthase. Arch. Biochem. Biophys. 316, 290–301.