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ORIGINAL ARTICLE

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Fructose-1,6-bisphosphate inhibits the expression of inducible nitric oxide synthase caused by oxygen-glucose deprivation through the inhibition of glutamate release in rat forebrain slices

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Abstract Fructose-1,6-bisphosphate (FBP) is a glycolytic pathway intermediate with a neuroprotective action in animal models of brain ischaemia. We addressed the question of whether FBP acts through inhibiting inducible nitric oxide synthase (iNOS) expression via reduction of glutamate release, since we have recently demonstrated that glutamate is involved in the expression of iNOS. FBP (5 mM) added to the incubation solution of rat forebrain slices subjected to oxygen-glucose deprivation (OGD) inhibited glutamate release significantly (around 40%). FBP also inhibited the induction of the calcium-independent NOS activity and reduced the levels of iNOS protein in rat forebrain slices subjected to OGD. We conclude that the action of FBP by reducing glutamate release and iNOS expression, both of which have been implicated in cell damage, is a reason for further evaluation of FBP as a neuroprotectant.

Key words ATP levels · Glutamate · Neuroprotective · Nitric oxide · Oxygen-glucose deprivation

Introduction

There is increasing evidence that nitric oxide (NO) may play complex roles in the pathophysiology of cerebral ischaemia. However, the precise role that NO plays in the mechanisms of ischaemic brain damage has been a source of controversy, as this agent may be either beneficial or detrimental to the ischaemic brain (for review, see Dirnagl et al. 1999). Inducible NO synthase (iNOS), a high-output isoform of NOS, might contribute to the tissue damage that occurs after cerebral ischaemia, since its expression has been demonstrated in blood and glial cells present in the post-ischaemic brain of in vivo models of cerebral ischaemia-reperfusion (Endoh et al. 1994; Wallace and Bisland 1994; Iadecola et al. 1995a), and also in neurones in rat forebrain slices exposed to oxygen and glucose deprivation (OGD) (Moro et al. 1998). In support of this idea, aminoguanidine, a selective inhibitor of iNOS, decreases the damage caused by focal cerebral ischaemia (Iadecola et al. 1995b) and we have found recently that 1400 W, a more selective inhibitor of iNOS (Garvey et al. 1997) ameliorates the necrotic tissue damage produced by OGD in rat forebrain slices (Cárdenas et al. 1998). Indeed, mice lacking the iNOS gene are less susceptible to ischaemic damage (for a review, see Dirnagl et al. 1999).

The mechanisms of iNOS expression after cerebral ischaemia are a subject of current research. Cytokines expressed after ischaemia activate the expression of iNOS in central nervous system (CNS) cells (Galea et al. 1992; Simmons and Murphy 1992, 1993; Minc-Golomb et al. 1994, 1996). In addition, oxidative stress produced in this situation might itself trigger the expression of iNOS (Melillo et al. 1995). On the other hand, activation of glutamate receptors has also been linked to a diversity of long-term changes in the CNS, including the rapid induction of a number of immediate-early genes (Ghosh et al. 1994; Lerea 1997). Moreover, stimulation of glutamate receptors can activate the transcription factor NF-κB in neurones (Guerrini et al. 1995; Kaltschmidt et al. 1995) and, interestingly, NF-κB has been recognised as an essential requirement for the expression of iNOS gene (Xie et al. 1994). Indeed, we have found recently that glutamate is involved in the expression of iNOS in our model (Cárdenas et al. 2000).

Glutamate can be released after ischaemia by exocytosis and by the reversal of glutamate reuptake pumps when the intracellular ATP level falls (Szatkowski and Attwell 1994). Fructose-1,6-bisphosphate (FBP) is a gly-

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colytic pathway intermediate that is neuroprotective in animal models of brain ischaemia (Farias et al. 1986). The main hypothesis for this effect is that FBP alters metabolic production of ATP, probably increasing it, and serves as a glycolytic intermediate (Bickler and Buck 1996). We therefore decided to study whether FBP, by reducing glutamate release, might inhibit iNOS expression in our model. We also discuss the beneficial effects of FBP possibly associated with decreased production of NO.

Methods

Preparation and incubation of slices. Male Sprague-Dawley rats (200–250 g) were killed by decapitation (according to procedures approved by The Committee of Animal Care at the Universidad Complutense of Madrid) and forebrain slices prepared as described earlier (Moro et al. 1998). Briefly, slices were preincubated in a sucrose-free preincubation solution equilibrated with 95% O_2/5% CO_2 in a shaking water-bath at 37 °C for 45 min. After this preincubation, slices were then incubated in a modified Krebs-Henseleit 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 (6*R*)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride (BH_4, 10 μM) and bubbled with 95% O_2/5% CO_2. The slices corresponding to the control group were incubated for a further 20 min under the same conditions. Another set of slices was incubated in the same conditions but with 100 μM glutamate added. Slices corresponding to the "ischaemic" group were incubated 20 min in incubation solution without glucose and equilibrated with 95% N_2/5% CO_2 to mimic ischaemia (OGD). After the "ischaemic" period, the medium was replaced with fresh incubation solution equilibrated with 95% O_2/5% CO_2 to simulate reperfusion. The slices were taken out 180 min after the OGD period and frozen immediately in liquid N_2. The incubation solution was sampled at 0, 30, 60 and 120 min after the OGD period for determination of excitatory amino acids (EAA). FBP (5 mM) was added during both preincubation and ischaemic periods.

NOS activity. NO synthase activity was determined after sonication of the forebrain slice (Labsonic 2000, Barcelona, Spain) at 4 °C in 5 vol buffer containing 320 mM sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM D,L-dithiothreitol, 10 μg/ml leupeptin, 100 μg/ml phenylmethylsulphonyl fluoride, 10 μg/ml soybean trypsin inhibitor, 2 μg/ml aprotinin and 50 mM tris(hydroxymethyl)aminomethane (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 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 cofactors NADPH (100 μM), BH_4 (3 μM), flavin adenine dinucleotide (FAD, 3 μM) and flavin mononucleotide (FMN, 3 μM) are included in the enzyme assay. The activity of the calcium-dependent NOS was calculated from the difference between the [^{14}C]citrulline produced from control samples and samples containing 1 mM ethyleneglycol-bis(β-aminoethyl-ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA); the activity of the calcium-independent isoform 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).

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

Determination of iNOS protein by Western blot. Slices were homogenised in lysis buffer (10 mM TRIS pH 8.0, 0.2% Nonidet P-40, 1 mM dithioerythritol) and, after centrifugation in a microcentrifuge for 15 min, the proteins present in the supernatant were loaded (10 μg) and size-separated in 10% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE, 50 mA). The gels were blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore) and incubated with a specific polyclonal anti-iNOS antibody (Transduction Laboratories; 1:1000 dilution). The iNOS isoform was visualized using a commercially available kit (ECL, Amersham Iberica) following the manufacturer's instructions.

HPLC determination of EAA concentration. The EAAs aspartate and glutamate in each sample were analysed by HPLC with fluorimetric detection (Perkin Elmer Binary LC Pump 250 and fluorescence detector LC 240) following pre-column derivatisation with the *o*-phthaldialdehyde procedure (Lindroth and Mopper 1979). EAA derivatives were separated isocratically on a reverse-phase column (4.6×150 mm, 5 μm particle diameter, Nucleosil 100-C18) using a mobile phase consisting of sodium acetate buffer (0.05 M pH6.5), 20% methanol and 2% tetrahydrofuran. The area of each peak was determined with a Perkin Elmer Nelson Model 1020 integrator (Phoenix 8088 ROM BIOS Version 2.52 software) and compared with the peak area of the corresponding external standard to determine the EAA concentration.

ATP concentrations. ATP concentrations in individual slices were measured with a firefly luciferin-luciferase assay with a luminometer (Fluoroskan Ascent FL, BioAnalysis Labsystems). Calibration of the luminometer with FBP present in the standards was checked to ensure that FBP did not interfere with the assay. Data are expressed as the percentage of control levels in picomole/milligram protein.

Chemicals and statistical analyses. L-[U-^{14}C]Arginine was obtained from Amersham, BH_4 was obtained from RBI (Natick, Mass., USA) and other chemicals were from Sigma or as indicated in the previous sections. Results are expressed as mean±SEM of the indicated number of experiments and the significance of differences was evaluated using a Newman-Keuls test.

Results

EAA efflux from rat forebrain slices exposed to OGD or glutamate. Effect of FBP

OGD increased the concentration of glutamate and aspartate in the incubation solution of rat forebrain slices 0 and 30 min after the "ischaemic" period (P<0.05, n=4; Table 1). The addition of FBP (5 mM) to the incubation solution of slices exposed to OGD inhibited glutamate release significantly at both times (43% inhibition at 0 min and 39% inhibition at 30 min, P<0.05, n=4; Table 1).

Table 1 Effect of fructose-1,6-bisphosphate (*FBP*; 5 mM) on excitatory amino acid efflux from rat forebrain slices exposed to oxygen-glucose deprivation (*OGD*). Slices were examined 0 and 30 min after the end of the ischaemic period. Means±SEM, n=4

Time (min)	Control	OGD	OGD+FBP
	Glutamate (ng/ml)		
0	72± 8	997±43*	573±96$^{\#}$
30	67±10	116± 9*	71± 6$^{\#}$
	Aspartate (ng/ml)		
0	44±1	340±34*	143±25$^{\#}$
30	53±7	78±13*	31± 5$^{\#}$

*P<0.05 vs. control, $^{\#}P$<0.05 vs. OGD

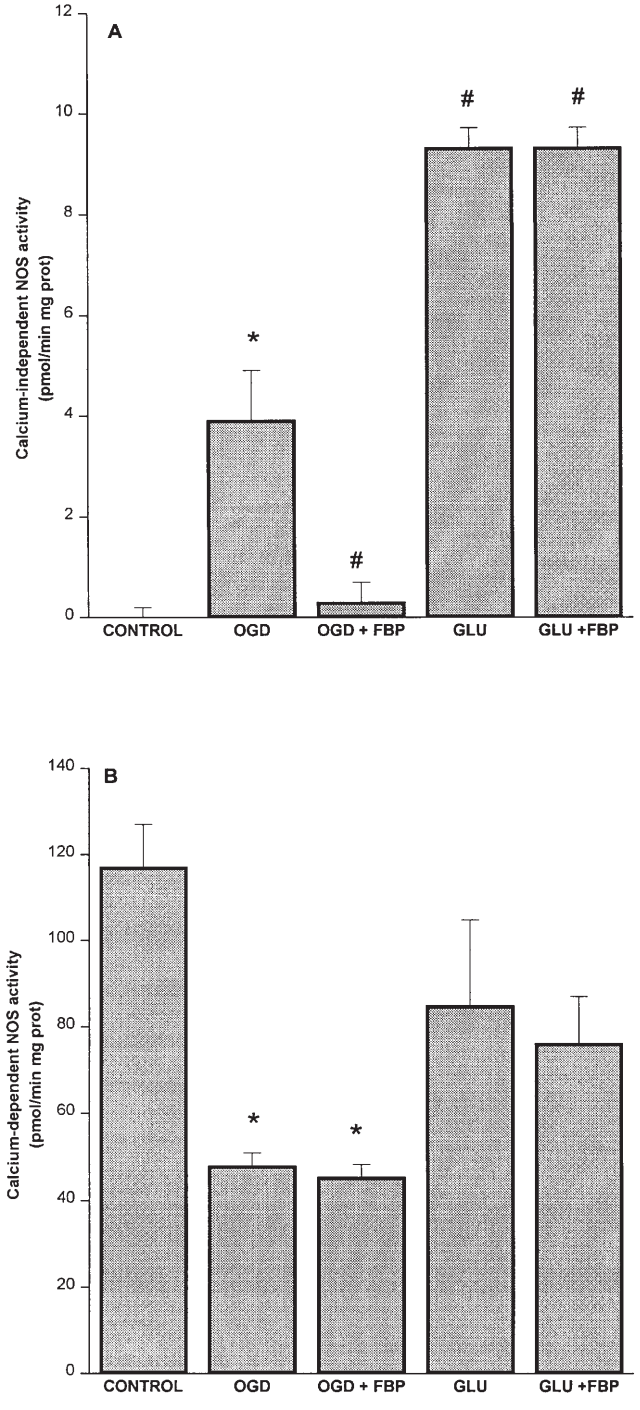

Fig. 1A, B Effect of fructose-1,6-bisphosphate (*FBP*) on nitric oxide synthase (NOS) activities in rat forebrain slices exposed to oxygen-glucose deprivation (*OGD*) or glutamate (*GLU*, 100 μM). **A** Calcium-independent NOS activity. **B** Calcium-dependent NOS activity. Means±SEM, n=16; *P<0.05 vs. control, $^{\#}P$<0.05 vs. OGD

NOS activity in rat forebrain slices exposed to OGD or glutamate. Effect of FBP

As we have shown previously (Moro et al. 1998; Cárdenas et al. 2000), OGD for 20 min caused the appearance of a calcium-independent NOS activity 180 min after the onset of the "reperfusion" period (Fig. 1). Calcium-independent NOS activity was not detectable in control forebrain slices (Fig. 1A). The addition of FBP (5 mM) inhibited the induction of the calcium-independent NOS activity in the rat forebrain slices exposed to OGD (P<0.05, n=16, Fig. 1A). On the other hand, the incubation of rat forebrain slices with glutamate (100 μM) for 20 min induced a calcium-independent NOS activity 180 min after the onset of the "reperfusion" period (P<0.05, n=16, Fig. 1A) and the addition of FBP (5 mM) failed to inhibit this response (n=16, Fig. 1A).

OGD for 20 min caused a decrease in calcium-dependent NOS activity compared with control slices (Fig. 1B). The addition of FBP (5 mM) did not modify the levels of calcium-dependent NOS activity found in the rat forebrain slices exposed to OGD (Fig. 1B). Incubation of rat forebrain slices with glutamate (100 μM) with or without FBP (5 mM) did not affect calcium-dependent NOS activity in control rat forebrain slices significantly (Fig. 1B).

Quantification of iNOS protein in rat forebrain slices exposed to OGD or glutamate. Effect of FBP

Western blot analysis using a specific anti-iNOS antibody showed that OGD induced the expression of iNOS protein in rat forebrain slices (Fig. 2), as described previously (Moro et al. 1998; Cárdenas et al. 2000). The addition of FBP (5 mM) attenuated this response (P<0.05, Fig. 2). Incubation of rat forebrain slices with glutamate (100 μM) for 20 min also induced expression of iNOS protein (Fig. 2), a response not inhibited by the addition of 5 mM FBP (Fig. 2).

ATP levels from rat forebrain slices exposed to OGD. Effect of FBP

OGD decreased the levels of ATP in rat forebrain slices (control 49 pmol/mg protein; P<0.05, n=8, Fig. 3). The addition of FBP (5 mM) to the incubation solution of slices exposed to OGD produced a significant recovery of ATP levels (P<0.05, n=8, Fig. 3).

Discussion

We have shown previously that OGD increases calcium-independent NOS activity, and that this response is inhibited by dexamethasone (Moro et al. 1998). This enzymatic activity is due to the expression of the inducible isoform, as demonstrated by immunohistochemistry and by quantification of both iNOS message and protein by Northern and Western blot analyses, respectively (Moro et al. 1998). Dexamethasone also decreases the levels of iNOS protein (De Alba et al. 1999). The present study showed that FBP abolished OGD-induced iNOS expression, as shown by the disappearance of a calcium-independent NOS activity and the absence of iNOS protein in brain

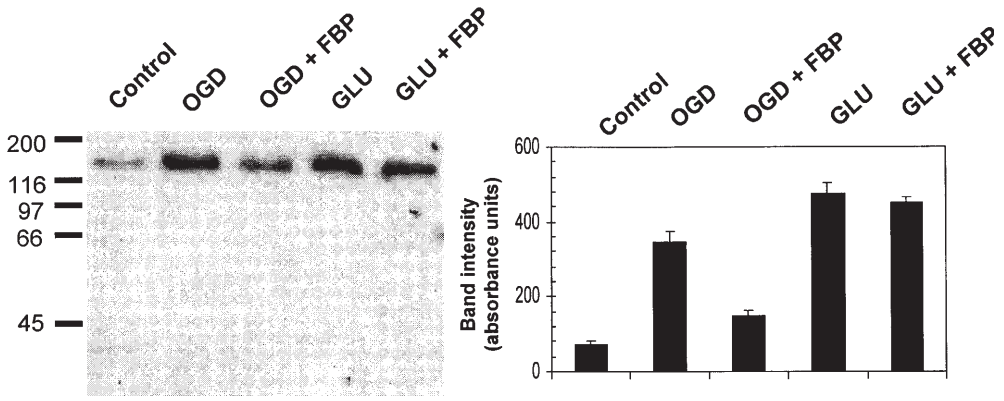

Fig. 2 Effect of FBP on expression of the inducible NOS isoform (iNOS) protein in rat forebrain slices exposed to OGD or GLU (100 μM). *Left* Western blot analysis of iNOS in soluble extracts. Commercial markers (Bio-Rad prestained) were used as molecular mass standards. *Right* Laser densitometric analysis of iNOS expression

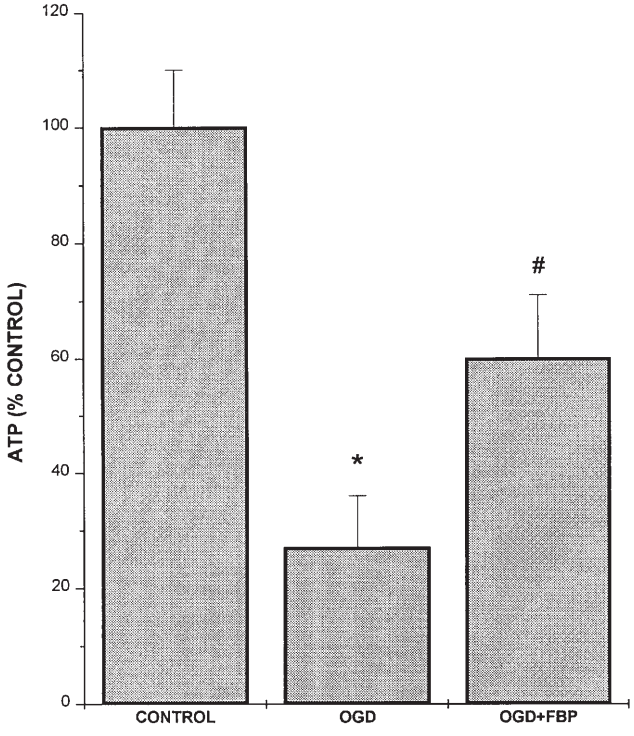

Fig. 3 Effect of FBP on ATP levels in rat forebrain slices exposed to OGD. Values are expressed as a percentage of control levels. Means±SEM, n=8; *P<0.05 vs. control, $^{\#}P$<0.05 vs. OGD

slices exposed to OGD. Finally, our results show that FBP effects were due, at least in part, to inhibition of glutamate release from brain slices exposed to OGD.

Regardless of whether FBP has an extra- or intracellular site of action, currently a matter of controversy (Edde et al. 1998), the administration of FBP has been effective in preventing ischaemic injury in brain (Bickler and Buck 1996; Sola et al. 1996; Espanol et al. 1998). The mechanism by which FBP reduces glutamate release is not clear. Glutamate accumulation after ischaemia results from an initial exocytotic release and, mainly, from the reversal of glutamate reuptake pumps as ATP levels fall (Szatkowski and Attwell 1994). FBP does not affect exocytotic release since FBP fails to block glutamate release after depolarisation with KCl (50 mM) for 2 min (data not shown). FBP might thus reduce glutamate release by blocking non-exocytotic release via maintenance of ATP levels, as has been reported previously (Bickler and Buck 1996; Espanol et al. 1998). The reduction in glutamate release by FBP occurs even when no glucose and oxygen are available (time 0 post-reperfusion), suggesting that this effect may be due not only to aerobic metabolism of FBP but also to other oxygen-independent mechanisms, such as stimulation of the pentose phosphate pathway (Bickler and Buck 1996). The implication of reduced glutamate release with FBP on cell damage is discussed below.

FBP also inhibits the induction of the calcium-independent NOS activity and abolishes iNOS expression in brain slices exposed to OGD. The mechanisms of iNOS expression in the post-ischaemic brain are also unknown. In this context, pro-inflammatory cytokines expressed after ischaemia cause the induction of iNOS in CNS cells (Galea et al. 1992; Simmons and Murphy 1992, 1993; Minc-Golomb et al. 1994, 1996). It is thus plausible that cytotoxic concentrations of glutamate might express or release cytokines (De Bock et al. 1996). Decreasing glutamate release by FBP would therefore inhibit iNOS expression. In addition, the oxidative stress produced in this situation might itself trigger the induction of iNOS (Melillo et al. 1995) and it has been shown that FBP inhibits oxidant production (Sun et al. 1990). We have recently demonstrated that activation of *N*-methyl-D-aspartate (NMDA) receptors by glutamate released after an ischaemic insult is involved in the expression of iNOS in rat forebrain slices via Ca^{2+}-dependent activation of the transcription factor NF-kB (Cárdenas et al. 2000). Therefore, FBP might abolish iNOS expression by reducing glutamate release and thus attenuating the activation of NF-kB, as might be suggested by the absence of iNOS protein. Indeed, FBP failed to block iNOS expression when glutamate was added directly to the solution. Other mechanisms such as extracellular chelation of calcium (Galzinga et al. 1989) could also explain this effect. These data are in agreement in part with those previously reported in macrophages (Edde et al. 1998).

FBP did not modify the levels of calcium-dependent NOS activity found after OGD or glutamate. Measurements of calcium-dependent NOS activities in these samples show that calcium-independent NOS activity is 5–10% of total NOS activity 180 min after the end of the challenging period (OGD or glutamate). However, these

values reflect the activity under optimal conditions in the tissue homogenate, which are well above the basal intracellular calcium levels, at which calcium-dependent isoforms are inactive.

In summary, the mechanism by which FBP might be neuroprotective is not only the decrease of glutamate release, which is well known to play a central role in ischaemic brain injury (Choi 1988), but also inhibition of iNOS expression. Indeed, we have demonstrated that a selective inhibitor of iNOS such as 1400 W is protective in brain slices exposed to OGD (Cárdenas et al. 1998). We can conclude that the action of FBP on reducing glutamate release and iNOS expression is a reason for further evaluation of FBP as a neuroprotectant.

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