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Mechanisms of the neuroprotective effect of aspirin after oxygen and glucose deprivation in rat forebrain slices

M.A. Moro [a,*], J. De Alba [a], A. Cárdenas [a], J. De Cristóbal [a], J.C. Leza [a], I. Lizasoain [a], M.J.M. Díaz-Guerra [b], L. Boscá [b], P. Lorenzo [a]

[a] *Departamento de Farmacología, Facultad de Medicina, Universidad Complutense de Madrid (UCM), 28040 Madrid, Spain*
[b] *Instituto de Bioquímica, Consejo Superior de Investigaciones Científicas, Facultad de Farmacia, UCM Madrid, Spain*

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Abstract

Acetylsalicylic acid (ASA, Aspirin) is an anti-inflammatory drug with a wide spectrum of pharmacological activities and multiple sites of action. Apart from its preventive actions against stroke due to its antithrombotic properties, recent data in the literature suggest that high concentrations of ASA also exert direct neuroprotective effects. We have used an in vitro model of brain ischaemia using rat forebrain slices deprived of oxygen and glucose to test ASA neuroprotective properties. We have found that ASA inhibits neuronal damage at concentrations lower than those previously reported (0.1–0.5 mM), and that these effects correlate with the inhibition of excitatory amino acid release, of NF-κB translocation to the nucleus and iNOS expression caused by ASA. All of these three mechanisms may mediate the neuroprotective effects of this drug. Our results also show that the effects of ASA are independent of COX inhibition. Taken together, our present findings show that ASA is neuroprotective in an in vitro model of brain ischaemia at doses close to those recommended for its antithrombotic effects.

Keywords: Aspirin; Brain ischaemia; Excitatory amino acids; Glutamate; iNOS; Neuroprotection; Nitric oxide; Nuclear factor-κB

1. Introduction

Acetylsalicylic acid (ASA, Aspirin) is a non-steroidal anti-inflammatory drug (NSAID) with a wide spectrum of pharmacological activities and multiple sites of action. It has been reported that ASA reduces the size of infarcts after ischaemic stroke (Grotta et al., 1985; Carolei et al., 1986; Joseph et al., 1992) and this was generally attributed to its antiplatelet actions through inhibition of the cyclooxygenase-dependent pathway; in this context, the maximal effectiveness appears to depend upon selective blockade of thromboxane A_2 synthesis by platelets without inhibiting production of PGI_2 by endothelial cells, and this is best achieved with low doses of ASA (for a review, see Patrono, 1994). However, data obtained more recently indicates other possible mechanisms explaining the neuroprotective effects of ASA: (1) The ability of aspirin to inhibit the activation of the transcription factor nuclear factor-κB (NF-κB; Kopp and Gosh, 1994) has been postulated to account for the neuroprotective actions of this drug against glutamate-induced neurotoxicity (Grilli et al., 1996). (2) A further important activity is the reduction of oxidative stress, due to its actions as a free radical scavenger or by other mechanisms (Pekoe et al., 1982; Dowling et al., 1987; Kuhn et al., 1995; Oberle et al., 1998) and (3) prostaglandins (PGs), which are synthesised by cyclooxygenase (COX), potentiate glutamatergic transmission by stimulating the release of glutamate from astrocytes (Bezzi et al., 1998), suggesting that inhibition of the formation of PGs by ASA may protect neuronal cells from excitotoxic damage. In contrast with the antiplatelet effects of ASA, the neuronal actions require higher doses of ASA, therefore

Abbreviations: ASA: acetylsalicylic acid; COX: cyclooxygenase; EAA: excitatory amino acids; EMSA: electrophoretic mobility shift assay; iNOS: inducible NOS; LDH: lactate dehydrogenase; NF-κB: nuclear factor κB; NMDA: N-methyl-D-aspartate; NO: nitric oxide; NOS: nitric oxide synthase; nNOS: neuronal NOS; NSE: neurone-specific enolase; OGD: oxygen and glucose deprivation; PDTC: pyrrolidine dithiocarbamate; TLCK: N-tosyl-L-phenylalanine chloromethyl ketone.

* Corresponding author. Tel.: +34-91-394-1478; fax: +34-91-394-1463.

E-mail address: nlfucm@eucmax.sim.ucm.es (M.A. Moro).

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increasing undesirable adverse effects of this drug such as gastrointestinal and bleeding complications (Patrono, 1994).

In the present study, we decided to investigate whether ASA has any effect on the neuronal death that occurs in an in vitro model of brain ischaemia, using rat forebrain slices exposed to oxygen and glucose deprivation (OGD), in order to elucidate the mechanisms which underlie its neuroprotective actions. Our results show that, in forebrain slices, ASA exhibits a remarkable and specific protection against OGD through mechanisms including the decrease in extracellular excitatory amino acid concentration and the inhibition of the activation of NF-κB.

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 the Committee of Animal Care at the Universidad Complutense of Madrid), the forebrain was removed and coronally cut (2 mm anterior and 6 mm posterior to bregma), and the central portion was sliced (0.4 mm slice thickness) using a Vibroslice (WPI, Stevenage, UK) in cold (12–14°C) modified Krebs–Henseleit solution (preincubation solution) containing (mM): NaCl (120), KCl (2), $CaCl_2$ (0.5), $NaHCO_3$ (26), $MgSO_4$ (10), KH_2PO_4 (1.18), glucose (11) and sucrose (200) (Moro et al., 1998).

Slices were incubated in sucrose-free preincubation solution equilibrated with 95% O_2/ 5% CO_2, in a shaking water bath at 37°C for 45 min. After the preincubation period, slices were 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 20 min further in the same conditions. Slices corresponding to the "ischaemic" experimental group were incubated for 20 min in incubation solution without glucose and equilibrated with 95% N_2/5% CO_2 to mimic an "ischaemic" condition. After these periods of 20 min, the medium was replaced with fresh incubation solution equilibrated with 95% O_2/5% CO_2 to simulate a "reperfusion" period. In a set of experiments, ASA (0.01–1 mM), sodium salicylate (1 mM) or indomethacin (50 μM) were added to the preincubation solution and were present during the whole experiment (preincubation, "ischaemia" and "reperfusion" periods). Slices were taken out at different times: 30 to 180 min after the "ischaemic" period and frozen immediately with liquid nitrogen. Samples of incubation solution were taken every 30 min during 180 min of reperfusion, except for the experiments aimed to NO_x^- determination, in which samples were collected only at 180 min of "reperfusion".

2.2. Lactate dehydrogenase (LDH) activity assay

As a marker of necrotic tissue damage, LDH released from damaged cells in the slices was determined in the incubation solution. Samples of this solution were taken every 30 min during the 180 min reperfusion period. LDH 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 Thermomax microplate reader (Molecular Devices, Sunnyvale, CA, USA). LDH levels remaining in control and OGD-slices at 180 min of reperfusion were very high and not significantly different from each other (8375±69 vs 8512±293 mOD/min in control and OGD-slices, respectively, n=4 $p>0.05$). Therefore, LDH efflux was expressed merely as the LDH activity present in the incubation solution. Data are expressed as mOD/min and reflect the total LDH release after subtracting from it the control LDH release.

2.3. Neuronal specific enolase (NSE) assay

As a marker of specific neuronal damage (Marangos et al., 1979), NSE released from damaged neurones into the incubation solution was determined by radioimmunoassay (Påhlman et al., 1984). Data are expressed as μg/L min and reflect the total NSE release after subtracting from it the control NSE release.

2.4. Preparation of cytosolic and nuclear extracts

A modified procedure based on the method of Schreiber et al. (1989) was used. Slices were homogenised with 100 μl of buffer A (10 mM HEPES, pH 7.9; 1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF, 2 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml TLCK, 5 mM NaF, 1 mM $NaVO_4$, 0.5 M sucrose and 10 mM Na_2MO_4). After 10 min at 4°C Nonidet P-40 was added to reach a 0.5% concentration. The tubes were gently vortexed for 15 s and nuclei were collected by centrifugation at 8000***g*** for 15 min. The supernatants were stored at −80°C (cytosolic extracts) and the pellets were resuspended in 50 μl of buffer A supplemented with 20% glycerol, 0.4 M KCl, and gently shaken for 30 min at 4°C. Nuclear protein extracts were obtained by centrifugation at 13 000***g*** for 15 min, and aliquots of the supernatant were stored at −80°C. All of the steps of the fractionation were carried out at 4°C. To dephosphorylate proteins, extracts were treated for 1 h at 40°C with 1 unit per μg of protein of agarose-immobilized

alkaline phosphatase. Appropriate controls of heat-inactivated alkaline phosphatase were used to ensure the specificity of the reaction.

2.5. Electrophoretic mobility shift assay (EMSA) for NF-κB

Oligonucleotides were synthesised in a Pharmacia oligonucleotide synthesiser (Pharmacia, Uppsala, Sweden). The oligonucleotide sequence corresponding to the consensus NF-κB binding site (nucleotides −978 to −952) of the murine iNOS promoter, was 5′TGCTAGGGGGATTTTCCCTCTCTCTGT3′ (Xie et al., 1994). Oligonucleotides were annealed with their complementary sequence by incubation for 5 min at 85°C in 10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT. Aliquots of 50 ng of these annealed oligonucleotides were end-labeled with Klenow enzyme fragment in the presence of 50 μCi of [α-^{32}P]dCTP and the other unlabeled dNTPs in a final volume of 50 μl. 5×10^4 dpm of the DNA probe were used for each binding assay of nuclear extracts as follows: 3 μg of protein were incubated for 15 min at 4°C with the DNA and 2 μg of poly(dl:dC), 5% glycerol, 1 mM EDTA, 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 10 mM Tris-HCl, pH 7.8, in a final volume of 20 μl. The DNA protein complexes were separated on native 6% polyacrylamide gels in 0.5% Tris borate-EDTA buffer (Díaz-Guerra et al., 1996). Supershift assays were carried out after incubation of the nuclear extract with the antibody (0.5 μg) for 1 h at 4°C, followed by EMSA. Cycloheximide (10 μM) was used as a control of NF-κB translocation to the nucleus.

2.6. NOS activity assay

NO synthase activity was determined after sonication of the forebrain slice (Labsonic 2000, Barcelona, Spain) 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 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), FAD (3 μM) and 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 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).

2.7. NO_x^- (NO_2^- and NO_3^-) assay

NO release was estimated from the amounts of nitrite (NO_2^-) and nitrate (NO_3^-) in the incubation solution. NO_3^- was calculated by first reducing NO_3^- into NO_2^- in the presence of Cd (Cortas and Wakid, 1990) and NO_2^- was determined by a colourimetric assay based on the Griess reaction (Green et al., 1982).

2.8. Western blot analyses

For iNOS and nNOS characterisation, 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% SDS-polyacrilamide gel electrophoresis (50 mA). The gels were blotted onto a PVDF membrane (Millipore, Bedford, MA, USA) and incubated either with a specific polyclonal iNOS antibody (Transduction Laboratories, Lexington, KY, USA; 1:1000 dilution) or with a specific polyclonal nNOS antibody (kind gift of Dr. J. Rodrigo; 1:1000 dilution). For p50, p65, IκBα and IκBβ, samples were boiled in 250 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol and 2% β-mercaptoethanol. Proteins (15 μg) were size-separated in minigels (7 cm) of 10% SDS-PAGE. When a higher resolution was required, proteins were separated in a 15 cm gel, and allowing the 36.5 kDa band of the prestained MW markers to reach the border of the gel. As before, gels were blotted onto a PVDF membrane, incubated with anti-p50, anti-p65, anti-IκBα and anti-IκBβ (murine; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and processed as recommended by the supplier. Proteins recognised by the antibodies were revealed by a ECL™-kit following the manufacturers instructions (Amersham lberica, Madrid, Spain).

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

2.9. HPLC determination of excitatory amino acid (EAA) concentration

Samples of incubation solution were collected after 10 min of OGD, since LDH efflux after this time of OGD was not significantly different from control values, thus allowing for the study of EAA release caused by OGD and excluding EAA efflux due to damaged membranes. In addition, shorter times of collection were methodologically very difficult to obtain at reproducible time periods. Analysis of the EAA aspartate and glutamate in

each sample was performed by HPLC with fluorimetric detection (Perkin Elmer Binary LC Pump 250 and Fluorescence Detector LC 240) following pre-column derivatisation with the o-phtalaldialdehyde 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 pH 6.5), 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. The limit of detection in these conditions was 10 ng/ml.

2.10. Chemicals and statistical analyses

L-[U-^{14}C]arginine was obtained from ICN Biochemicals (Costa Mesa, CA, USA), BH_4 [(6R)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride] was obtained from RBI (Research Biochemicals International, Natick, MA, USA), NSE RIA kit was from Pharmacia and Upjohn (Uppsala, Sweden) and, unless otherwise stated, the other chemicals were from Sigma (Madrid, Spain). Results are expressed as mean ± SEM of the indicated number of experiments; statistical comparisons were made using a Newman-Keuls test and $p<0.05$ was considered as statistically significant.

3. Results

3.1. Effects of ASA, sodium salicylate and indomethacin on LDH and NSE released during reperfusion period

Oxygen and glucose deprivation (OGD) caused the release of both LDH and NSE into the incubation medium when compared with control slices (Figs. 1 and 2). ASA (0.1–1 mM) inhibited both LDH and NSE effluxes from rat forebrain slices after OGD (Figs. 1 and 2). ASA (1 mM) did not have any effect on LDH and NSE efflux from control slices (data not shown). Sodium salicylate did not inhibit LDH efflux at the concentration tested (1 mM; n=4, $p>0.05$). Finally, to examine whether ASA decreases the release of LDH and NSE inhibiting COX activity, we studied the effects of the non-salicylate NSAID indomethacin. lndomethacin (50 μM) did not have any effect on OGD-induced LDH efflux at any time studied (data not shown, n=4, $p>0.05$).

3.2. Effects of ASA on OGD-induced NF-κB activation

OGD caused the translocation of NF-κB to the nucleus when measured 0–2 hours after OGD (Fig. 3). These results were corroborated by an increase in the nuclear levels of NF-κB subunits p50 and p65 and a decrease in the cytosolic levels of IκBβ (Fig. 4) as determined by Western blot. The levels of IκBα were very low in our samples and, therefore, it was difficult to evaluate its changes. ASA (1 mM) inhibited NF-κB translocation to the nucleus in slices at both times studied (Fig. 3). ASA (1 mM) also inhibited the increase in the nuclear levels of p50 and p65 (Fig. 4). Moreover, ASA (1 mM) increased the levels of IκBβ when compared with ischaemic values (Fig. 4). In order to study the concentration-dependent effect of ASA on NF-κB translocation, slices were collected immediately after OGD. ASA (0.1–10 mM) inhibited NF-κB translocation in a concentration-dependent manner. This inhibitory effect was significant even at 0.5 mM ASA (Fig. 5).

3.3. Effect of ASA and PDTC on excitatory amino acid (EAA) release caused by OGD

OGD for 10 min caused the release of the EAA aspartate and glutamate from rat forebrain slices. No release was detected from control slices (Fig. 6). The incubation of slices with ASA (0.5–1 mM) during the OGD period caused a significant inhibition of the levels of EAA released by OGD (Fig. 6). On the other hand, the incubation of slices with the inhibitor of NF-κB activation PDTC (pyrrolidine dithiocarbamate, 100 μM; Schreck et al., 1992a), did not affect OGD-induced EAA release (n=4, $p>0.05$).

3.4. Effects of ASA, sodium salicylate and indomethacin on iNOS activity and protein expression during the reperfusion period

OGD for 20 min caused the expression of an inducible NOS (iNOS) as shown by the appearance of a calcium-independent NOS activity (Fig. 7A) and the detection of iNOS protein (Fig. 8) in samples collected 180 min after the OGD period. The incubation of rat forebrain slices with ASA (0.01–1 mM) during OGD and the subsequent "reperfusion" period inhibited the appearance of the calcium-independent NOS activity (Fig. 7A) and the expression of iNOS protein (Fig. 8) in a concentration-dependent manner. In contrast to ASA, sodium salicylate did not inhibit the appearance of the calcium-independent NOS activity at the concentration tested (1 mM; n=4, $p>0.05$). Similarly, indomethacin (50 μM) did not have any effect on OGD-induced iNOS expression at any times studied (data not shown, n=4, $p>0.05$).

3.5. Effects of ASA on nNOS activity and protein and NO_x^- levels during reperfusion period

OGD for 20 min caused a decrease in calcium-dependent NOS activity and neuronal NOS (nNOS) expression

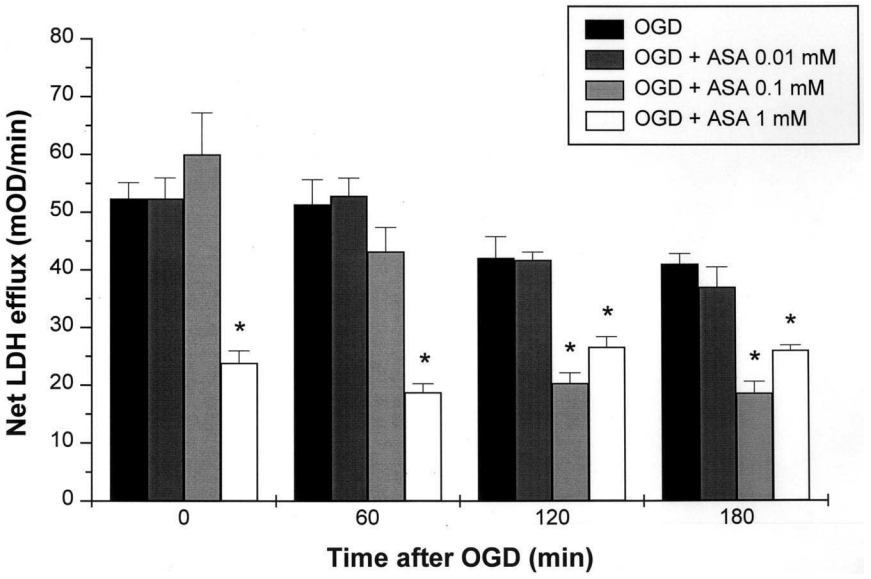

Fig. 1. Effect of ASA on OGD-induced net LDH release. LDH levels were measured by monitoring the oxidation of NADH in the presence of pyruvate (see Materials and methods). The data represent the means ± S.E.M. of 12 independent experiments and reflect the total LDH release after subtracting from it the control LDH release. *$p<0.05$ vs OGD (Newman–Keuls test). Control LDH release ranged from 35.0±2.3 mOD/min at 0 min up to 14.3±1.1 mOD/min at 180 min.

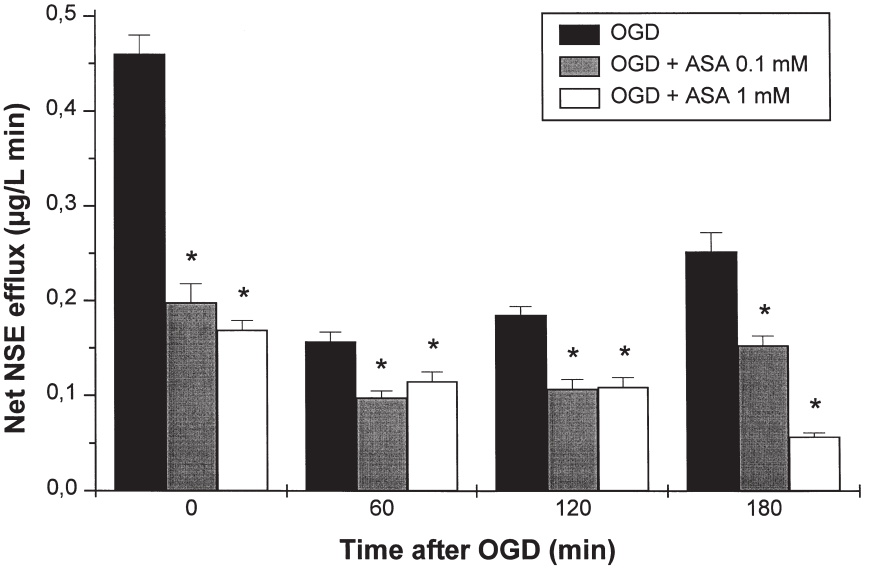

Fig. 2. Effect of ASA on OGD-induced net NSE efflux. NSE levels were determined by radioimmunoassay (see Materials and methods). The data represent the means ± S.E.M. of 6–8 independent experiments and reflect the total NSE release after subtracting from it the control NSE release. *$p<0.05$ vs OGD (Newman–Keuls test). Control NSE release ranged from 0.44±0.04 µg/L at 0 min up to 0.16±0.03 µg/L at 180 min.

when compared with control slices (Figs. 7B and 8). The incubation of rat forebrain slices with ASA (0.01–1 mM) during both OGD and "reperfusion" periods caused a significant recovery in nNOS activity and protein expression (Figs. 7B and 8).

The amounts of NO_x^- in the incubation solution after 20 min of OGD and 180 min of "reperfusion" (1.1±0.2 µM, n=4) were significantly decreased by 1 mM ASA (0.5±0.2 µM, n=6, $p<0.05$).

4. Discussion

Our results demonstrate that (1) OGD causes neuronal damage in rat forebrain slices together with translocation of the transcription factor NF-κB to the nucleus, release of EAA and expression of iNOS and that (2) OGD-induced neurotoxicity is attenuated by ASA, which inhibits OGD-induced NF-κB translocation to the nucleus, EAA release and expression of iNOS in this

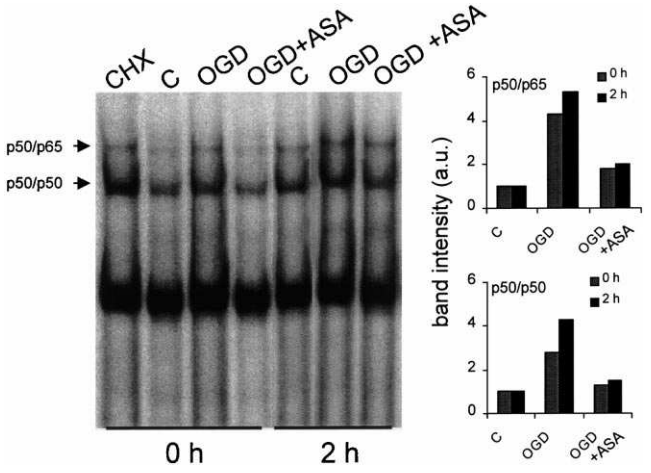

Fig. 3. NF-κB translocation to the nucleus caused by OGD and its inhibition by ASA (1 mM). NF-κB translocation was measured by electrophoretic mobility shift assay (EMSA; see Materials and methods) in control (C), OGD and OGD + ASA (1 mM) brain slices at 0 and 2 h after OGD. CHX: Cycloheximide, control for translocation of NF-κB to the nucleus. Right panel: laser densitometric analysis of p50/p65 (top) and p50/p50 (bottom) dimers. Results are representative of 3 individual experiments.

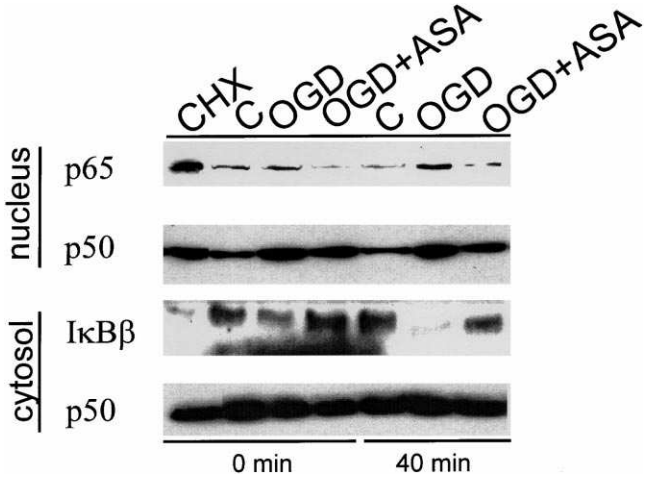

Fig. 4. Effect of ASA (1 mM) on nuclear levels of p50 and p65 and cytosolic levels of IκBβ. Detection of the translocation of p50 and p65 subunits of NF-κB from cytosol to the nucleus was determined by Western blot (see Materials and methods) in control (C), OGD and OGD + ASA (1 mM) brain slices 0 and 40 min after OGD. CHX: Cycloheximide, control for translocation of NF-κB to the nucleus. Results are representative of 3 individual experiments.

particular model. Moreover, we show that ASA exerts these effects at concentrations lower than those reported previously (≥1 mM) in other models.

OGD causes cell damage in forebrain slices as deduced by the release of LDH and NSE. The mechanisms which underlie ischaemic brain damage are complex and numerous. The neurotoxic actions of the EAA have been implicated in the pathogenesis of brain injury after cerebral ischaemia (Choi and Rothman, 1990). Indeed, both aspartate and glutamate are released during

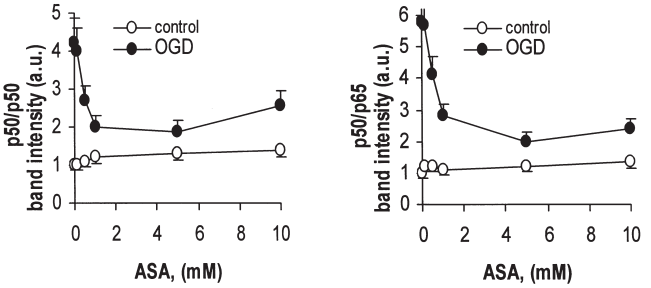

Fig. 5. Concentration-dependent effect of ASA (0.1–10 mM) on NF-κB translocation to the nucleus. Laser densitometric analysis of p50/p50 (right panel) and p50/p65 (left panel) dimers in control and OGD-slices in the presence or absence of ASA immediately after OGD. NF-κB was determined by EMSA (see Materials and methods) and data are means ± S.E.M. of 4 independent experiments.

OGD in our preparation, and this is linked to cell damage as we have previously shown that dizocilpine, an antagonist of the N-methyl-D-aspartate subtype of glutamate receptor, remarkably inhibits LDH release in this model (De Alba et al., 1999a). In addition, our data show that OGD leads to a remarkable activation of NF-κB in rat forebrain slices, consistently with previous findings in the rat ischaemic cortex (Salminen et al., 1995). In this context, cytokines or excitatory amino acids released during and after the OGD period may cause cell damage by several mechanisms including activation of NF-κB, as it has been suggested previously when cell death was induced with glutamate or NMDA (Grilli et al., 1996). We propose that our model, using forebrain slices, offers important advantages over cell cultures for this study since it is specific as far as cell composition and local effector release are concerned.

Furthermore, we have also shown that iNOS is expressed after OGD in rat forebrain slices, in agreement with our previous results (Moro et al., 1998). Since iNOS is the NOS isoform which has most often been implicated in cytotoxicity (Moncada et al., 1991; Gross and Wolin, 1995), its expression might be one of the mechanisms by which OGD leads to neuronal damage. Indeed, it has been shown that pharmacological inhibition of iNOS reduces ischaemic brain injury and that iNOS null mice have a reduction in ischaemic damage (for a review, see Dirnagl et al., 1999). Additionally, our group has demonstrated that 1400W, a selective iNOS inhibitor, is neuroprotective in the model used in this study (Cárdenas et al., 1998). However, this neuroprotection was observed only at the latest times of "reperfusion", as expected from the time course of iNOS expression in our preparation.

More interestingly, our results demonstrate that ASA remarkably reduces neuronal death caused by OGD in rat forebrain slices, as shown by the inhibition of both LDH and NSE efflux. ASA is a drug with a wide spectrum of pharmacological activities and multiple sites of action. In order to check whether inhibition of COX was

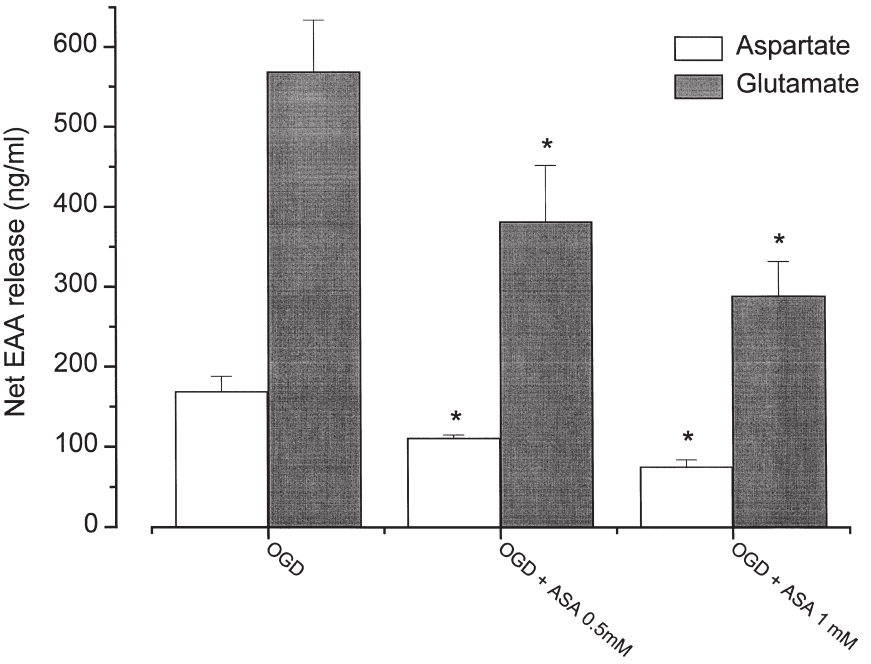

Fig. 6. Effect of ASA on OGD-induced EAA release. EAA release was measured by HPLC with fluorimetric detection (see Materials and methods) in the incubation solution of slices after 10 min of OGD. Data are mean ± S.E.M. of 3-5 experiments. *$p<0.05$ vs OGD (Newman–Keuls test).

mediating the neuroprotective effects found, we tested indomethacin, another NSAID which shows a ratio COX2/COX1 — referred to the inhibition of COX isoforms — similar to that of ASA (Vane and Botting, 1996). COX inhibition did not seem to be implicated in the neuroprotective actions of ASA as indomethacin failed to inhibit OGD-induced LDH efflux. On the other hand, it is well known that increased oxidative stress may activate the cytoplasmic NF-κB leading to its translocation to the nucleus (Schreck et al., 1992b). Therefore, in order to study other mechanisms of action of ASA we have also assayed sodium salicylate, the metabolic derivative of ASA, which shares the antioxidant and NF-κB-inhibitory properties of ASA (Kopp and Gosh, 1994). We have found that sodium salicylate is also ineffective in neuroprotection at concentrations at which ASA confers the maximal neuroprotection.

The neuroprotection exerted by ASA was concomitant with the inhibitory effect of this molecule on the translocation of NF-κB to the nucleus. Indeed, phosphorylation of subunits of IκB is a prerequisite for its degradation and subsequent liberation of transcriptionally active NF-κB, and ASA has been shown to interfere with the mechanism leading to phosphorylation of IκB (Kopp and Gosh, 1994), via specific inhibition of IKK-β (Yin et al., 1998). In this context, the inhibition of NF-κB activation by ASA has been postulated to account for the neuroprotective actions of this drug against glutamate-induced neurotoxicity (Grilli et al., 1996), although a causal relationship has not been shown. Interestingly, we have found neuroprotection and inhibition of NF-κB activation at concentrations of ASA (500 μM) which have been previously shown to lack any inhibitory effect on NF-κB activation in other models (Kopp and Gosh, 1994; Grilli et al., 1996). This might suggest that ASA, at this concentration, is inhibiting some additional mechanisms induced by OGD and which lead in a distal step to the translocation of NF-κB to the nucleus. The use of OGD to cause neuronal damage instead of EAA-induced neurotoxicity is likely to be the reason why the neuroprotective effects of ASA could be observed at concentrations lower than those previously reported (Grilli et al., 1996), using a model in which either NMDA or glutamate was applied directly. Instead, in our model, EAA are released due to the OGD process and this might be an additional target for the actions of ASA. Therefore, we decided to measure EAA release and we found that OGD-induced EAA release was inhibited by ASA at this concentration. This is the likely explanation for (1) the neuroprotection at early time points, since excitotoxicity is responsible of a high percentage of cell death in our model (De Alba et al., 1999a), (2) the preferential effect of ASA on the neuronal marker NSE as compared with the non-specific LDH, since neurones are the most sensitive cells to excitotoxic insults (Choi et al., 1987); and also (3) for the inhibition of NF-κB translocation at the low concentrations of ASA, in agreement with previous reports showing that EAA can activate NF-κB (Guerrini et al., 1995; Kaltschmidt et al., 1995). This novel effect of ASA explains most of the neuroprotection of this drug in our model and seems to be complementary to its actions on NF-κB, since PDTC, a very effective inhibitor of the translocation of this transcription factor, did not affect EAA release caused by OGD. The mechanisms of

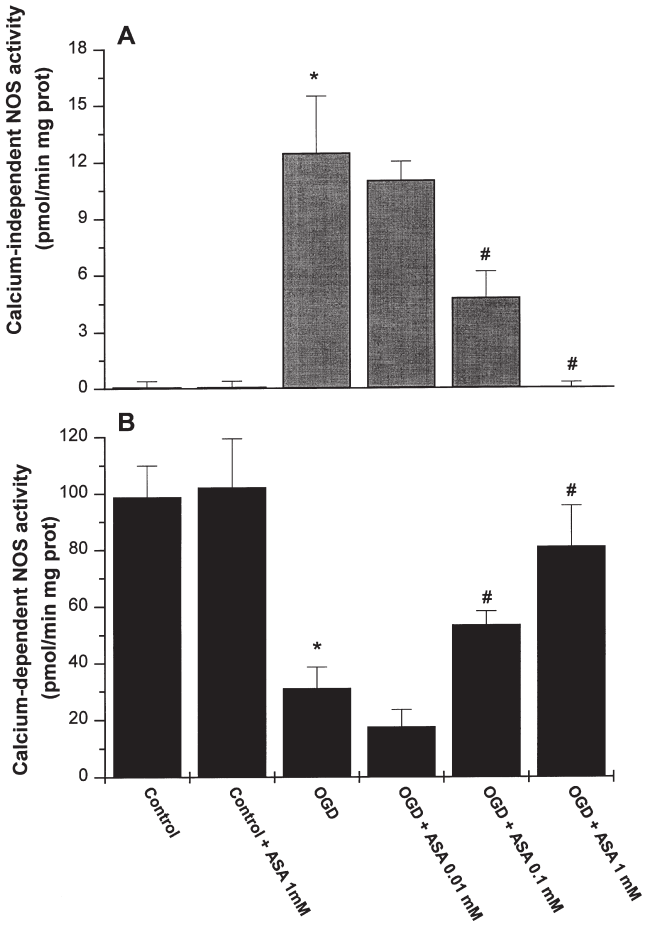

Fig. 7. Effect of ASA (0.01–1 mM) on nitric oxide synthase (NOS) activities from control and OGD-exposed rat forebrain slices. NOS activity was measured by monitoring the conversion of L-[U-^{14}C]arginine into [U-^{14}C]citrulline (see Materials and methods). (A) Calcium-independent NOS activity. (B) Calcium-dependent NOS activity. The data represent the means ± S.E.M. of 12 independent experiments. *$p<0.05$ OGD vs control; #$p<0.05$ OGD + ASA vs OGD (Newman–Keuls test).

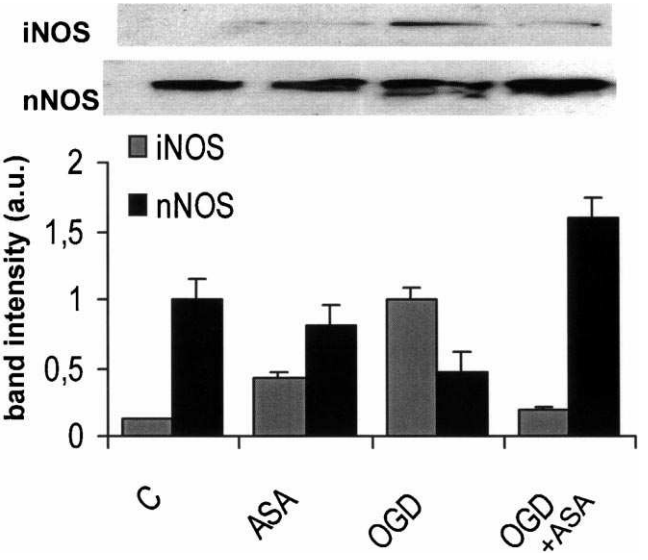

Fig. 8. Detection of inducible nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (nNOS) by Western blot (see Materials and methods) in control (C), OGD and OGD + ASA (1 mM) brain slices. Bottom: Laser densitometric analysis of NOS proteins 180 min after OGD. The data represent the means ± S.E.M. of 4–8 independent experiments.

this effect remain to be established and may perhaps be due to the ability of this molecule to cause acetylation.

The concentration-dependent effects of ASA on the prevention of neuronal death correlated with those on inhibition of iNOS expression in this model (Moro et al., 1998), therefore suggesting the concurrence of additional protective mechanisms triggered by ASA. Indomethacin and sodium salicylate were ineffective as inhibitors of iNOS expression, thus excluding COX inhibition in this effect. Although sodium salicylate inhibits NF-κB translocation, which is essential for expression of the iNOS gene, the lack of effect of this drug on iNOS expression is consistent with previous studies reporting IC_{50} values of 6 mM for the inhibition of NF-κB translocation to the nucleus (Grilli et al., 1996). The inhibition of NF-κB translocation caused by ASA, via a decrease in OGD-induced EAA release, is therefore the most likely explanation for the effects of this drug on iNOS expression. Moreover, the inhibition by ASA of OGD-induced iNOS expression may be an additional mechanism by which this molecule causes neuroprotection, but it is expected to contribute only at the latest times of "reperfusion". In addition, we have recently demonstrated that OGD causes a down-regulation of the neuronal isoform of NOS (nNOS) present in control tissues which is due to NO overproduction resulting from iNOS expression (De Alba et al., 1999b). As expected, we have now shown that ASA-induced inhibition of iNOS expression after OGD causes the recovery of nNOS activity and protein, which is therefore an additional marker of ASA-induced neuroprotection.

The maximal effectiveness of antiplatelet therapy with ASA in the treatment or the prophylaxis of cerebral ischaemia appears to depend upon selective blockade of thromboxane A_2 synthesis by platelets without preventing production of prostacyclin by endothelial cells (Patrono, 1994) and selective antiplatelet action appears to be best achieved when the dose of ASA is 40–325 mg per day. Our present findings show that ASA exerts direct neuroprotective actions not only at very high doses (corresponding to plasma concentrations of 1–3 mM) which might increase undesirable effects of this drug but even at lower doses, close to those recommended for its antithrombotic-analgesic effects (corresponding to plasma concentrations up to 600 μM; Payan and Katzung, 1996). Low doses of ASA can be useful in the treatment and prophylaxis of cerebral ischaemia not only by its antithrombotic properties but also due to a direct neuroprotective effect.

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