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RAPID COMMUNICATION

Neuroprotective effect of aspirin by inhibition of glutamate release after permanent focal cerebral ischaemia in rats

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Abstract

Aspirin reduces the size of infarcts after ischaemic stroke. Although this fact has been attributed to its anti-platelet actions, direct neuroprotective effects have also been reported. We have recently demonstrated that aspirin is neuroprotective by inhibiting glutamate release in '*in vitro*' models of brain ischaemia, via an increase in ATP production. The present study was designed to determine whether the inhibition of glutamate release induced by aspirin might be protective in a whole-animal model of permanent focal brain ischaemia. Focal brain ischaemia was produced in male adult Fischer rats by occluding both the common carotid and middle cerebral arteries. Central and serum glutamate levels were determined at fixed intervals after occlusion. The animals were then killed and infarct volume was measured. Aspirin (30 mg/kg i.p. administered 2 h before the occlusion) produced a significant reduction in infarct volume, an effect that correlated with the inhibition caused by aspirin on ischaemia-induced increase in brain and serum glutamate concentrations after the onset of the ischaemia. Aspirin also inhibited ischaemia-induced decrease in brain ATP levels. Our present findings show a novel mechanism for the neuroprotective effects of aspirin, which takes place at concentrations in the anti-aggregant–analgesic range, useful in the management of patients with risk of ischaemic events.

Keywords: ATP, cerebral infarcts, MCAO, stroke.

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Aspirin is an anti-inflammatory drug with a wide spectrum of pharmacological activities and multiple sites of action. Aspirin may reduce the size of infarcts after ischaemic stroke (Grotta *et al*. 1985; Carolei *et al*. 1986), a fact that has been generally attributed to its anti-platelet actions through the inhibition of the COX-dependent pathway. In contrast, other data have indicated alternative mechanisms for explaining the neuroprotective effects of aspirin, some of which require very high doses of this drug, such as the reduction of oxidative stress (Pekoe *et al*. 1982; Kuhn *et al*. 1995) and the inhibition of the activation of the transcription factor nuclear factor-κB (NF-κB; Kopp and Gosh 1994; Grilli *et al*. 1996).

During brain ischaemia, extracellular glutamate concentration increases, reaching levels that activate the NMDA type of glutamate receptor, thereby causing neuronal death (Choi and Rothman 1990). Using *in vitro* models of cerebral ischaemia, in which rat forebrain slices or cultured cortical neurones are exposed to oxygen–glucose deprivation (OGD), we have found a neuroprotective effect of aspirin concomitant to the inhibition of OGD-induced glutamate release by recovering the fall of ATP levels (Moro *et al*. 2000; De Cristóbal *et al*. 2001).

In '*in vivo*' models of brain ischaemia, a neuroprotective action of aspirin has been also demonstrated; however, the precise mechanism by which aspirin produces this effect has not been elucidated (Khayyam *et al*. 1999).

The present study was designed to determine whether the neuroprotective effect of aspirin is the result of an inhibition of glutamate release by using a rat model of permanent middle cerebral artery occlusion (MCAO).

Materials and methods

Experimental groups

Aspirin was dissolved in distilled water and injected via an i.p. route (injection volume 1 mL/100 g body weight). Two groups were used for determinations of glutamate levels and infarct area: (a) MCAO 2 h after an i.p. injection of saline (MCAO; $n = 8$) and (b) MCAO 2 h after an i.p. injection of 30 mg/kg aspirin (MCAO + aspirin; $n = 8$). In addition, four groups were used in order to determine brain ATP levels (sham-operated animals, SHAM; SHAM + aspirin; MCAO; MCAO + aspirin; with $n = 6$ in each group). The doses and time of administration of aspirin were chosen according to previous data showing a neuroprotective effect of aspirin (at doses of 15 mg/kg and above) when it was injected 2 h or 30 min before occlusion in the same experimental model of focal brain ischaemia (Khayyam *et al*. 1999). The dose most thoroughly studied (30 mg/kg) is in the range of those reported to be anti-thrombotic and analgesic/anti-pyretic in mice and rats (Borchard *et al*. 1992).

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Abbreviations used: CCA, common carotid artery; iNOS, inducible nitric oxide synthase; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; NF-κB, nuclear factor-κB; OGD, oxygen–glucose deprivation.

Experimental model

Experiments were performed on male Fischer rats weighing 225–275 g. Rats were anaesthetized with 2.5% halothane in a mixture of 70% nitrogen/30% oxygen and secured in a Kopf stereotaxic frame with the tooth bar at −3.3 mm below the interaural zero. The dialysis probe (3.5 mm × 240 μm; Cuprophan) was implanted in the left striatum according to the following coordinates: + 1.0 mm anteroposterior, + 3.0 mm mediolateral to the bregma and −8.0 mm dorsoventral from the surface of the brain (Paxinos and Watson 1986). Probes were secured to the skull as described by Baldwin *et al*. (1994). The correct placement of the probes was verified by dye perfusion on test animals prior to proceeding with experimental groups. The day after probe implantation, probes were perfused with artificial CSF (KCl, 2.5 mM; NaCl, 125 mM; $MgCl_2$ $6H_2O$, 1.18 mM; $CaCl_2$ $2H_2O$, 1.26 mM) at a rate of 1 μL/min. After a 60-min resting period samples were collected every 30 min. Three basal samples were taken prior to MCAO to achieve steady baseline concentration of glutamate. These samples were averaged and all subsequent values were expressed as a percentage of these basal pre-ischaemic levels. The *in vitro* probe recovery of a solution containing 1 ng/μL of glutamate was 20.5 ± 1.5% ($n = 6$). Permanent focal cerebral ischaemia was induced by ligature of the left common carotid artery (CCA) and occlusion of the ipsilateral distal middle cerebral artery (MCA) as described previously (Puig *et al*. 2000). Briefly, for the CCA ligature, a midline ventral cervical incision was made, and the CCA was isolated and permanently occluded with a silk ligature. For the MCA occlusion, a 1-cm incision perpendicular to the line connecting the lateral canthus of the left eye and the external auditory canal was made to expose and retract the temporalis muscle. A 2-mm burr hole was drilled and the MCA was exposed by cutting and retracting the dura. The MCA was elevated and cauterized. Rats in which the MCA was exposed but not occluded served as sham-operated animals. Following surgery, subjects were returned to their cages and allowed free access to water and food. The body temperature of animals was monitored throughout the experiment and was maintained at 37.5 ± 0.5°C using a heating pad. All procedures conformed to the Committee of Animal Care at the Universidad Complutense of Madrid according with EU rules (DC86/609/CEE).

Glutamate determination

Dialysate samples were collected at 30-min intervals over a period of 8 h, and stored at −40°C until glutamate determination. Blood samples were obtained from the tail at 2 h (basal 1; B1) and 15 min (basal 2; B2) before permanent MCAO and 2, 4, 6, 8, 24 and 48 h after MCAO in the presence or absence of aspirin. The corresponding serum was obtained by centrifugation, and stored at −40°C until glutamate determination. Analysis of glutamate in each sample was performed by HPLC with fluorimetric detection as described previously (Moro *et al*. 2000). Glutamate levels are expressed in μmol/L.

Infarct area determination

The brains were removed 48 h after MCAO, and a series of 2-mm coronal brain slices were obtained (Brain Matrix; WPI) and stained in 1% 2,3,5-triphenyl-tetrazolium chloride (TTC; Merck) in 0.1 M phosphate buffer. The infarcted area, which is not stained, was quantified by image analysis (VISILOG 5.0; Noesis).

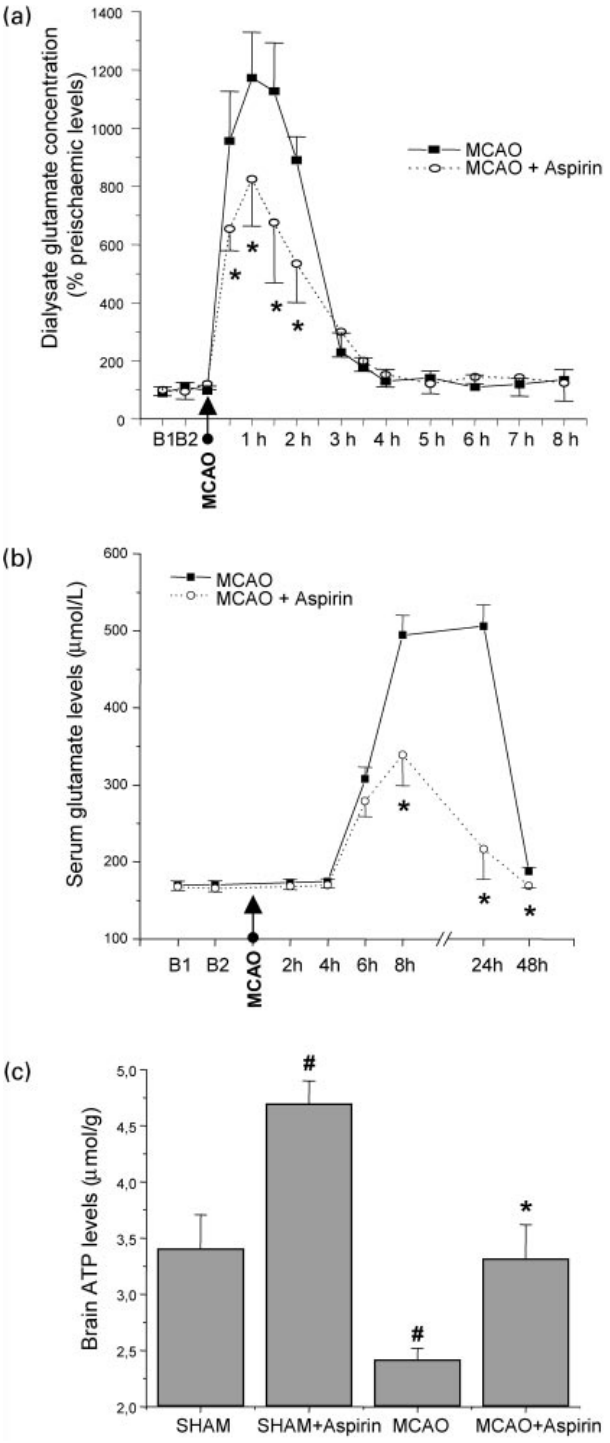

Fig. 1 (a) Effect of aspirin on brain glutamate levels after MCAO. (b) Effect of aspirin on serum glutamate levels after MCAO. Data are mean ± SEM; $n = 8$; ■, MCAO; ○, MCAO + aspirin. (c) Effect of aspirin on brain ATP levels after MCAO. Data are mean ± SEM, $n = 6$. (See Materials and methods for details). *$p < 0.01$ vs. MCAO, #$p < 0.01$ vs. SHAM.

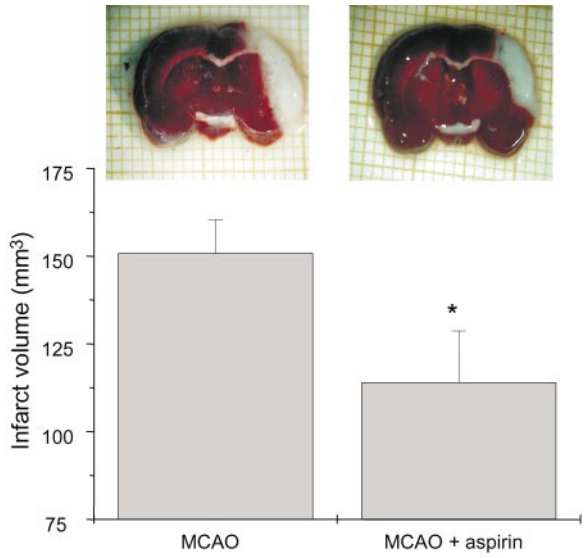

Fig. 2 Effect of aspirin (30 mg/kg) on infarct volume after MCAO. Photomicrographs of representative animals. A smaller cortical infarct is seen after administration of aspirin.

Brain ATP levels

For determination of ATP levels from SHAM, SHAM + aspirin, MCAO and MCAO + aspirin groups, the ipsilateral side of the forebrains were collected 15 min after MCA occlusion. Cortices were dissected and homogenized using a Teflon-glass homogeniser in a medium containing 0.3% (w/v) trichloracetic acid and 1 mM EDTA. The homogenate was centrifuged at 10 000 *g* for 3 min at 4°C. The supernatant was mixed 1 : 1 with Tris-acetate buffer solution (pH 7.75). After that, luciferin-luciferase was added (at a final concentration of 2 mg/mL), and ATP production was measured in a Fluoroskan Ascent FL microplate reader (Labsystems). ATP levels are expressed in μmol/g.

Chemicals and statistical analyses

Unless otherwise stated, chemicals were from Sigma. Results are expressed as mean ± SEM of the indicated number of experiments; statistical analysis involved one-way analysis of variance (ANOVA, or the Kruskal–Wallis test when the data were not normally distributed) followed by individual comparisons of means (Student Newman–Keuls test, or Dunn's method when the data were not normally distributed).

Results

Effect of aspirin on brain and serum glutamate concentrations after MCAO

Basal extracellular concentration of glutamate in the dialysis perfusate collected from the striatum was stable during the 60-min pre-ischaemic period (1.4 ± 0.1 μmol/L; $n = 8$). Permanent MCAO produced a significant elevation in extracellular concentration of glutamate (Fig. 1a). This increase was observed immediately following occlusion and reached a maximum value 1 h after MCAO (1173 ± 156% of pre-ischaemic concentration; Fig. 1a). Glutamate concentration returned to basal levels 3–4 h after the occlusion. Previous treatment with 30 mg/kg (i.p.) aspirin caused a significant inhibition of the increase in glutamate concentration betweeen 30 min and 2 h after MCAO (Fig. 1a).

As we have previously shown (Puig *et al.* 2000), permanent MCAO caused a three-fold increase in serum glutamate concentration. The onset of glutamate increase began 4–6 h after occlusion, reached peak values at 8–24 h after occlusion and returned to control values by 48 h after occlusion (Fig. 1b). Previous treatment with aspirin also produced an inhibition of the increase in serum glutamate levels by 8–48 h after the ischaemic insult (Fig. 1b).

Effect of aspirin on infarct volume after MCAO

Infarct volume measured at 48 h after permanent MCAO showed a 24% reduction in the group treated with 30 mg/kg of aspirin (114.0 ± 14.7 mm^3, $n = 8$) in comparison with the non-treated group (150.9 ± 9.5 mm^3, $n = 8$; $p < 0.05$; Fig. 2). Other doses of aspirin (10 and 100 mg/kg) did not produce any significant change in the infarct volume (140.8 ± 16.0 and 125.2 ± 15.3 mm^3, $n = 8$, respectively; $p > 0.05$).

Effect of aspirin on brain ATP levels after MCAO

Occlusion of MCA for 15 min caused a reduction of ATP levels by 30% when compared with the sham-operated animals group (Fig. 1c). Previous treatment with aspirin blocked this reduction induced by the ischaemic insult. Aspirin administered to sham operated animals caused an increase in ATP levels by itself (Fig. 1c).

Discussion

We have recently reported a neuroprotective effect of aspirin, associated with an inhibition of glutamate release and an increase in brain ATP in *in vitro* models of brain ischaemia using rat forebrain slices (Moro *et al.* 2000), and cultured cortical neurones exposed to OGD (De Cristóbal *et al.* 2001). We have now used an *in vivo* model of permanent cerebral ischaemia to demonstrate this neuroprotective effect of aspirin. Rectal temperature did not vary during the experiment, therefore excluding possible neuroprotective actions of aspirin as a result of its anti-pyretic effects. Our results show that 30 mg/kg of aspirin administered 2 h before the occlusion exhibits a specific protection that occurs concomitantly with an inhibition of both the ischaemia-induced increase in glutamate and the decrease in brain ATP levels.

It is well known that glutamate plays a predominant role in the pathogenesis of ischaemic brain injury (Choi and Rothman 1990; Castillo *et al.* 1996, 1997). We and others have previously shown that glutamate increases in serum and brain after cerebral ischaemia in this permanent MCAO model (Baker *et al.* 1995; Puig *et al.* 2000). The present results show that aspirin inhibits MCAO-induced glutamate release, in a way that parallels its neuroprotective effect. Several mechanisms, alone or combined, are responsible for ischaemia-induced glutamate release, such as the Ca^{2+}-dependent exocytosis of its vesicular pool or the reversal of the electrogenic uptake transport systems (for a review see Szatkowski and Attwell 1994). Among all of these mechanisms, it has recently been shown that glutamate release induced by severe ischaemia is largely caused by the reversed operation of neuronal glutamate transporters (Jabaudon *et al.* 2000; Rossi *et al.* 2000). Regarding the mechanism of this neuroprotective effect of aspirin, it has been demonstrated that glutamate release from reversed operation of its cellular transporters results from the depletion in ATP levels caused by ischaemia (Madl and Burgesser 1993). In this context, we have recently demonstrated

that, in cortical neurones, aspirin exhibits a remarkable and specific protection that is caused by a decrease in the OGD-induced release of glutamate, by the inhibition of the fall in ATP responsible for the reversal of glutamate uptake systems in cerebral ischaemia (De Cristóbal *et al*. 2001). Moreover, we have shown that this effect is a result of the fact that aspirin targets mitochondrial respiratory chain complex I–III, resulting in an increased ATP production (De Cristóbal *et al*. 2001). Indeed this mechanism also occurs in *in vivo* models, as when we determine the ATP levels in brain we find that the ATP loss induced by ischaemia is inhibited by the previous administration of aspirin, and that this drug is able to increase the levels of ATP by itself when it is administered to sham-operated animals. These findings are in agreement with other data showing that aspirin increases tolerance against hypoxia concomitantly with a delay in the decrease of intracellular ATP content (Riepe *et al*. 1997). Moreover, other strategies aimed to prevent ATP loss (Kass and Lipton 1982; Galeffi *et al*. 2000) have been shown to be neuroprotective and to have an inhibitory effect on glutamate release in the ischaemic brain (Cárdenas *et al*. 2000a).

Apart from the previously mentioned effect, which takes place in the early stages after the ischaemic insult, there are other mechanisms that can contribute to the neuroprotective effects of aspirin. We have previously shown that activation of NMDA receptors by glutamate released after an ischaemic insult is involved in the expression of enzymes such as inducible nitric oxide synthase (iNOS; Cárdenas *et al*. 2000b); therefore, an inhibition of glutamate release may have additional neuroprotective effects by inhibiting the delayed expression of iNOS or other inflammatory enzymes such as cyclooxygenase type II (COX-2). Other actions of aspirin, such as the inhibition of oxidative stress or of NF-κB, have been suggested to be responsible for some of the neuroprotective actions of aspirin (Kuhn *et al*. 1995; Grilli *et al*. 1996); however, these effects only occur at very high concentrations of this drug, correlating with anti-inflammatory dosage.

The present findings show that aspirin exerts direct neuroprotective actions at concentrations corresponding to low doses, in the range of those reported to be anti-thrombotic, analgesic and antipyretic in mice and rats (Borchard *et al*. 1992). Moreover, we show the neuroprotective effect of aspirin is a result of the inhibition of glutamate release and the prevention of ATP loss, an effect that may possess important therapeutic implications in the management of patients at risk of ischaemic events, as we demonstrated that early neurological progression of patients with acute ischaemic stroke is associated with high concentrations of glutamate in the blood and cerebrospinal fluid (Castillo *et al*. 1997). However, future clinical studies will be required to confirm this neuroprotective effect of aspirin.

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