Inhibition of Glutamate Release via Recovery of ATP Levels Accounts for a Neuroprotective Effect of Aspirin in Rat Cortical Neurons Exposed to Oxygen-Glucose Deprivation

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Background and Purpose—Aspirin is preventive against stroke not only because of its antithrombotic properties but also by other direct effects. The aim of this study was to elucidate its direct neuroprotective effects.

Methods—Viability parameters, glutamate release and uptake, and ATP levels were measured in cultured cortical neurons exposed to oxygen-glucose deprivation (OGD). In addition, ATP levels and oxygen consumption were studied in isolated brain mitochondria or submitochondrial particles.

Results—Aspirin inhibited OGD-induced neuronal damage at concentrations lower (0.3 mmol/L) than those reported to act via inhibition of the transcription factor nuclear factor-κB (which are >1 mmol/L), an effect that correlated with the inhibition caused by aspirin on glutamate release. This effect was shared by sodium salicylate but not by indomethacin, thus excluding the involvement of cyclooxygenase. A pharmacological dissection of the components involved indicated that aspirin selectively inhibits the increase in extracellular glutamate concentration that results from reversal of the glutamate transporter, a component of release that is due to ATP depletion. Moreover, aspirin-afforded neuroprotection occurred in parallel with a lesser decrease in ATP levels after OGD. Aspirin elevated ATP levels not only in intact cortical neurons but also in isolated brain mitochondria, an effect concomitant with an increase in NADH-dependent respiration by brain submitochondrial particles.

Conclusions—Taken together, our present findings show a novel mechanism for the neuroprotective effects of aspirin, which takes place at concentrations in the antithrombotic-analgesic range, useful in the management of patients with high risk of ischemic events. (***Stroke***. 2002;33:261-267.)

Key Words: aspirin ∎ cell respiration ∎ glutamates ∎ ischemia ∎ mitochondria ∎ neuronal death ∎ neurons ∎ salicylates ∎ stroke, experimental

Aspirin may reduce the size of infarcts after ischemic stroke,[1–3] and this was generally attributed to its antiplatelet actions through inhibition of the cyclooxygenase (COX)-dependent pathway.[4] Other data have indicated alternative mechanisms explaining the neuroprotective effects of aspirin, some of them requiring very high doses of this drug, such as the reduction of oxidative stress[5,6] and the inhibition of the activation of the transcription factor nuclear factor-κB (NF-κB).[7] Since NF-κB is involved in the transcription of proinflammatory and inflammatory mediators such as inducible nitric oxide synthase and COX-2, its inhibition would translate into anti-inflammatory effects with neuroprotective consequences. In addition, aspirin has been shown to increase tolerance against hypoxia by delaying the intracellular ATP loss.[8]

During brain ischemia, extracellular glutamate concentration ([glu]$_o$) increases, reaching levels that activate the *N*-methyl-D-aspartate (NMDA) type of glutamate receptor, thereby causing neuronal death.[9] Therefore, exogenously added glutamate is a widely used neurotoxicity paradigm when neuroprotective agents are studied. However, this approach does not elucidate the neuroprotection resulting from inhibition of the rise in [glu]$_o$. Using an in vitro model of cerebral ischemia in which rat forebrain slices are exposed to oxygen-glucose deprivation (OGD) to resemble more closely the in vivo situation, we have found a neuroprotective effect of aspirin concomitant with inhibition of OGD-induced glutamate release.[10] Now we have used an alternative model of cerebral ischemia consisting of cultured rat cortical neurons exposed to OGD to further elucidate this neuroprotective effect of aspirin.

Materials and Methods

Primary Culture of Rat Cortical Neurons

Primary cultures of cortical neurons were performed as described.[11] Brains were removed from fetal Wistar rats at embryonic day 18, and

the cortical area was dissected. Neurons were mechanically dissociated in 80% Eagle's minimum essential medium containing 33 mmol/L glucose, 2 mmol/L glutamine, 16 mg/L gentamicin, 10% horse serum, and 10% fetal calf serum (FCS) (growth medium) and plated at 2×10^5 cells per square centimeter in polylysine-precoated 6-, 12-, or 96-multiwell plates. Plates were kept in a 37°C incubator in a humidified atmosphere containing 95% air/5% CO_2. On day 4, medium was changed to fresh growth medium containing cytosine arabinoside (10 μmol/L) and lacking FCS. Medium was replaced 3 days later with fresh growth medium lacking both FCS and cytosine arabinoside.

Cellular identification was assessed by staining the cultures with the use of specific cellular markers for astrocytes (glial fibrillary acidic protein [GFAP]) and microglial cells (biotin conjugate of *Lycopersicon esculentum* agglutinin[12]): a monoclonal anti-GFAP antibody (Chemicon; 1:100) or a fluorescein-labeled agglutinin from *L esculentum* (Sigma; 1:150). Anti-GFAP–stained cells were revealed with Cy 3–labeled anti-mouse IgG (Amersham Pharmacia Biothec; 1:300). Counting was performed under a Nikon Eclipse TE300 fluorescence microscope (Nikon Corporation). Four random fields were used for counting cells for each staining. The average number of neurons (GFAP-negative cells, *L esculentum* agglutinin–negative cells, and birefringent appearance under phase optics), astrocytes (GFAP-positive cells), and microglial cells (*L esculentum* agglutinin–positive cells) ($\pm$SEM) was calculated. Each experiment was performed in duplicate and repeated 3 times. Image acquisition was performed with a laser-scanning confocal imaging system (MRC1024 BioRad).

Studies were performed at in vitro days 9 to 10, when the cultures consisted of 95$\pm$5% neurons, 4$\pm$1% astrocytes, and 1$\pm$1% microglial cells (Figure 1).
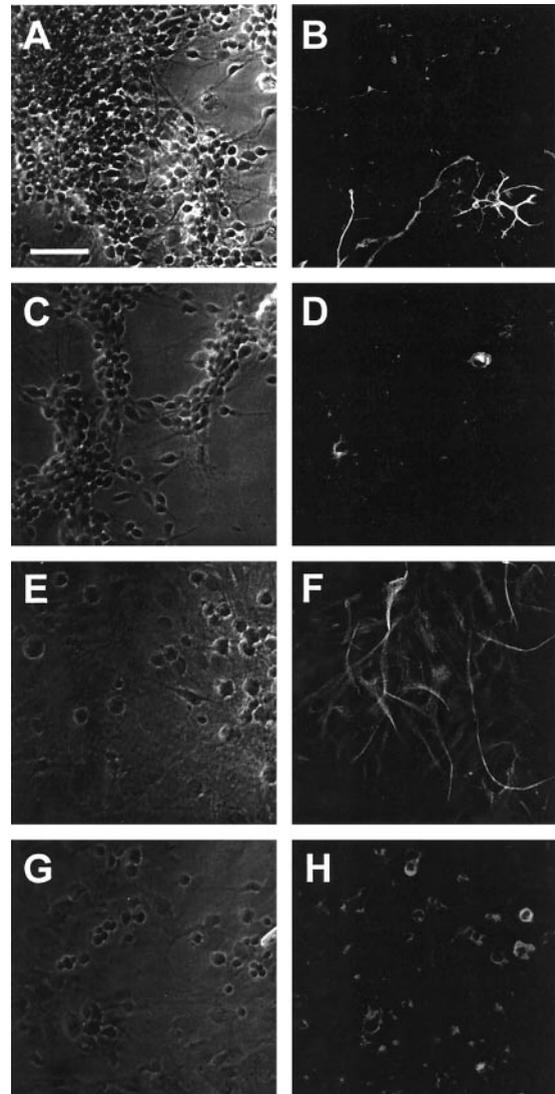
Glia-Enriched Cortical Cultures

To determine the contribution of the few glial cells present in the neuronal cultures, we studied OGD-induced glutamate release in glia-enriched cultures, which were prepared as described above for cortical neurons but at a density of 0.5×10^5 cells per square centimeter and without cytosine arabinoside. Cellular identification was performed as above. Studies were performed at in vitro days 16 to 17, when cultures consisted mostly of glial cells (75$\pm$8% astrocytes, 15$\pm$3% neurons, and 10$\pm$2% microglial cells; Figure 1).

Exposure to OGD or to Glutamate

OGD was performed as described[13] with modifications. Culture medium was replaced by a solution containing (mmol/L): NaCl 130, KCl 5.4, $CaCl_2$ 1.8, $NaHCO_3$ 26, $MgSO_4$ 0.8, NaH_2PO_4 1.18, bubbled with 95% N_2/5% CO_2 for OGD cells (OGD solution). OGD cells were transferred to an anaerobic chamber (Forma Scientific, Hucoa Erloss) containing 95% N_2/5% CO_2 and humidified at 37°C and maintained at 0.15 bar. The time of exposure to OGD was 150 minutes. This time was selected from previous time-response experiments in which we observed that OGD-induced glutamate release from intact cells began abruptly after 140 to 160 minutes of OGD, an effect closely followed (approximately 10 minutes after) by cell lysis, characterized by release of cytosolic components as lactate dehydrogenase (LDH) and cytosolic glutamate (secondary release of glutamate, from broken cells). To study the effect of aspirin on OGD-induced glutamate release from intact cells without interferences due to the secondary release of glutamate from broken cells, we had to select those experiments in which OGD had triggered glutamate release from intact cells but in which there were not yet any lysed cells. It is not always possible to capture this precise time point, and therefore those experiments in which LDH release or staining with propidium iodide immediately after OGD was higher than that from control cultures were excluded.

In some experiments, the water-soluble salt of aspirin, lysine acetylsalicylate (aspirin; 0.03 to 3 mmol/L), sodium salicylate (0.3 to 3 mmol/L), indomethacin (3 to 100 μmol/L), or pyrrolidine dithiocarbamate (PDTC; 100 μmol/L; an inhibitor of NF-κB)[14] was included in the OGD solution during the OGD period. In other experiments, the acetoxymethyl ester of 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*,tetra-acetic

Figure 1. Rat cortical neurons (A through D) and glia-enriched (E through H) cortical cultures. Phase-contrast micrographs (A, C; E, G) of representative cultures and their respective fluorescence micrographs show astrocytes after staining with anti-GFAP (B, F) or microglial cells after staining with FITC-labeled *L esculentum* agglutinin (D, H). Bar=50 μm/L.

acid (BAPTA-AM) (5 μmol/L) was included before OGD (45 minutes). These concentrations of BAPTA-AM are effective in inhibiting high K^+-evoked neurotransmitter release.[15] In an additional set of experiments, L-*trans*-pyrrolidine-2,4-dicarboxylic acid (tPDC; 300 μmol/L), an inhibitor of glutamate uptake, was included before OGD (45 minutes), in the presence of 1 mmol/L kynurenic acid as described,[16] as control neurons exposed to the same treatment. All the pre-OGD treatments were washed before the start of OGD. OGD was terminated by replacing the exposure medium with oxygenated minimum essential medium containing 0.6% glucose, 0.029% glutamine, 50 IU/mL penicillin, and 50 μg/mL streptomycin (GGPS medium) and returned to the normoxic incubator. Control cultures in a solution identical to OGD solution but containing glucose (33 mmol/L; control solution) were kept in the normoxic incubator for the same time period as the OGD, and then the incubation solution was replaced with GGPS medium and cultures were returned to the normoxic incubator. In other experiments cells were exposed to 10 μmol/L glutamate in control solution lacking Mg^{2+} for 5 minutes, after which this solution was replaced by GGPS medium and cultures were returned to the incubator. Cell viability was

evaluated 0 to 24 hours after the treatment without changing the medium unless indicated.

ATP Levels in Cortical Neurons After OGD

For determination of ATP levels in control and OGD-subjected cortical neurons, cells were collected after exposure to a "submaximal" OGD of 90 minutes, a time at which glutamate release had not yet begun, to avoid loss of ATP due to cell lysis or to consumption by exocytosis, allowing comparisons between intact control and OGD-exposed cortical neurons. The absence of cell lysis was determined by LDH release and propidium iodide staining. ATP concentrations were measured with a firefly luciferin-luciferase assay-based commercial kit (cytotoxicity and cell proliferation kit, Labsystems). Data are expressed as percentage of control levels.

Isolation of Mitochondria and Submitochondrial Particles

Rat brain mitochondria were prepared as described.[17] Protein content was determined with the use of bicinchoninic acid.[18]

Determination of ATP Levels in Isolated Mitochondria

Isolated mitochondria were resuspended in a buffer containing 100 mmol/L KCl, 75 mmol/L mannitol, 25 mmol/L sucrose, 10 μmol/L EGTA, and 5 mmol/L potassium phosphate, pH 7.4. Mitochondria (0.5 mg protein per milliliter) were incubated with glutamate/malate (2.5 mmol/L) or succinate (5 mmol/L) plus ADP (25 mmol/L) in the absence or presence of aspirin (0.03 to 0.3 mmol/L), sodium salicylate (0.03 to 0.3 mmol/L), or indomethacin (3 to 10 μmol/L) for 7 minutes. ATP levels were determined with the commercial kit described above.

Determination of Oxygen Consumption by Submitochondrial Particles

Oxygen consumption by submitochondrial particles was studied as described.[17] NADH (50 μmol/L), succinate (5 mmol/L), or ascorbate (5 mmol/L) plus N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD; 0.5 mmol/L) was used to quantify complex I-, II-, III-, or IV-dependent respiration, respectively. Rat brain submitochondrial particles were exposed to aspirin (0.03 to 1 mmol/L), sodium salicylate (0.3 mmol/L), and indomethacin (3 μmol/L) in 2 mL of buffer. Submitochondrial particle respiration was measured in an oxygen electrode (Digital Model 10, Rank Brothers Ltd).

Assay of LDH Activity From Cultured Cortical Neurons

As a marker of cellular lysis, LDH released from damaged cells was determined 24 hours after the OGD period. LDH activity was measured spectrophotometrically as described[19] with a Beckman DU7500 spectrophotometer (Beckman Instruments). LDH release is expressed as percentage of total cell LDH and is plotted as percentage of net LDH release induced by OGD. Basal LDH release was 6±1% (n=12).

MTT Assay on Cultured Cortical Neurons

Cell viability was monitored by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described.[20] For the assays, 8 wells were used for each condition, and each experiment was repeated at least 3 times. Results from MTT assay in this preparation correlate well with other methods of quantifying cell viability.[11] Viability of control neuronal cultures as assessed with this method was unaffected during the duration of the experiment.

Propidium Iodide Staining of Cultured Cortical Neurons

Viability was also assessed with propidium iodide (excitation/emission maxima ≈535/617 nm when bound to DNA), which stains dead cells.[21] After OGD, cells were kept for 30 minutes in GGPS medium or as indicated in other sections. Then cells were washed in cold PBS and subsequently incubated with 1 μg/mL propidium iodide in PBS for 30 minutes on ice. Cells were visualized with a Nikon Eclipse TE300 fluorescence microscope. For each condition, 2 random fields from duplicate wells were counted, and each experiment was repeated at least 4 times. Data are expressed as percentage of stained cells.

[³H]Glutamate Uptake by Cortical Neurons

Cultures were washed and incubated in control solution containing 3 μmol/L glutamate and 8 μCi/mL of [³H]glutamate for 90 seconds, a time in which glutamate uptake was found to proceed linearly with time. In a parallel set of experiments, cultures were incubated during the same time in the same solution, but Na⁺ was equiosmotically substituted by choline. At the end of the incubation, solution was collected, and cells were lysed with perchloric acid (0.3 mol/L). [³H]Glutamate uptake was calculated by subtracting uptake in the absence of Na⁺ from uptake in its presence and expressed as percentage of total [³H]glutamate.

Determination of Glutamate Concentration by High-Performance Liquid Chromatography

Samples of incubation solution were collected at the end of the OGD period. Analysis of glutamate in each sample was performed by reverse-phase high-performance liquid chromatography with fluorometric detection as described.[10] Glutamate release is expressed as percentage of net release induced by OGD. Basal release was below the detection limit of our assay (0.17 nmol/10⁶ cells).

Chemicals and Statistical Analyses

Unless otherwise stated, chemicals were from Sigma. The IC_{50} values were calculated with the use of MicroCal Origin 5.0 (Micro-Cal Software, Inc). Results are expressed as mean±SEM of the indicated number of experiments; statistical comparisons were made with Newman-Keuls test, and $P < 0.05$ was considered statistically significant.
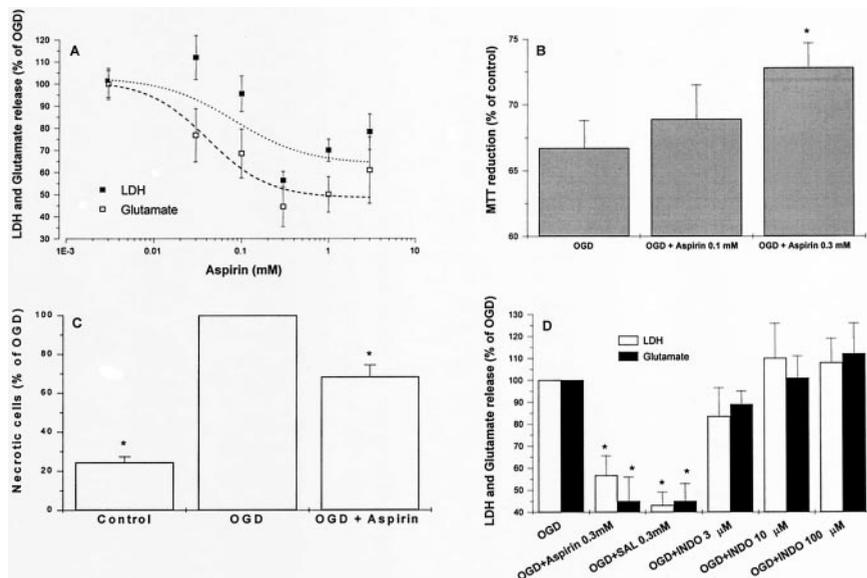
Results

Effect of Aspirin on OGD-Induced Neuronal Death

OGD caused neuronal death, as shown by an increase in LDH efflux to the medium (Figure 2A and 2D), a decrease in MTT reduction (Figure 2B), and an increase in the number of propidium iodide–stained cells (Figure 2C) compared with control cells. Aspirin inhibited OGD-induced neuronal death, as shown by a reduction in OGD-induced LDH release (IC_{50}=0.115±0.015 mmol/L; Figure 2A and 2D), an increase in MTT reduction (Figure 2B), and a decrease in propidium iodide staining (Figure 2C).

Effect of Sodium Salicylate and Indomethacin on OGD-Induced Neuronal Death

Sodium salicylate (Figure 2D) inhibited neuronal death, measured as OGD-induced LDH release. To examine whether aspirin- and sodium salicylate–induced decreases in LDH release were due to inhibition of COX activity, we studied the effects of indomethacin, a nonsalicylate NSAID that shows a COX-2/COX-1 ratio—referred to as the inhibition of COX isoforms—similar to that of aspirin.[22] Indomethacin (Figure 2D) did not affect neuronal viability. Higher concentrations of indomethacin could not be tested because they increased LDH values in control neurons (6±1% of total LDH in control neurons versus 33±2% of total LDH in control neurons in the presence of 300 μmol/L indomethacin; n=6 to 12; $P > 0.05$).

Figure 2. Neuroprotective effect of aspirin and sodium salicylate in cortical neurons. A, Inhibition by aspirin of OGD-induced LDH and glutamate release. Cell death was assessed by measuring the release of the cytosolic marker LDH and is expressed as percentage of net LDH release induced by OGD ($67\pm6\%$ of total LDH; n=12). Glutamate release is expressed as percentage of net release induced by OGD (1.22 ± 0.23 nmol/10^6 cells; n=12). B, Inhibition by aspirin (0.1 to 0.3 mmol/L) of OGD-induced cell death assessed by the MTT assay. C, Inhibition by aspirin (0.3 mmol/L) of OGD-induced cell death in cortical cultures detected by propidium iodide staining. OGD increased the propidium iodide staining compared with control cells. Aspirin (0.3 mmol/L; OGD+Aspirin) decreased OGD-induced propidium iodide staining. Results shown are representative of 4 similar experiments. D, Effect of aspirin (0.3 mmol/L), sodium salicylate (SAL) (0.3 mmol/L), and indomethacin (INDO) (3 to 100 μmol/L) on OGD-induced LDH and glutamate release. Data are mean$\pm$SEM; n=4 to 12; *$P<0.05$ vs OGD.

Effect of Aspirin, Sodium Salicylate, and Indomethacin on OGD-Induced Glutamate Release From Cortical Neurons

OGD caused glutamate release from rat cortical neurons compared with the control group (below detection limit; see Materials and Methods; Figure 2A and 2D). The incubation of cells with aspirin (0.03 to 3 mmol/L) during the OGD period caused an inhibition of OGD-induced glutamate release (Figure 2A; IC$_{50}$=0.042$\pm$0.009 mmol/L).

The incubation of cells with sodium salicylate but not with indomethacin during the OGD period inhibited OGD-induced glutamate release (Figure 2D).

As an additional control, we determined whether inhibition of glutamate receptors is protective in this model by using the NMDA antagonist dizocilpine (MK-801). Dizocilpine (100 nmol/L) reduced OGD-induced increase in LDH values up to $37\pm4\%$ of OGD values (n=12).

Effect of Aspirin on Glutamate-Induced Damage to Cortical Neurons

The exposure of cortical cultures to glutamate (10 μmol/L) caused cell death, which was not affected in the presence of 0.3 mmol/L aspirin (LDH release=$32.9\pm1.5\%$ in the absence of aspirin and $30.0\pm2.0\%$ of total LDH in the presence of aspirin; n=4; $P<0.05$).
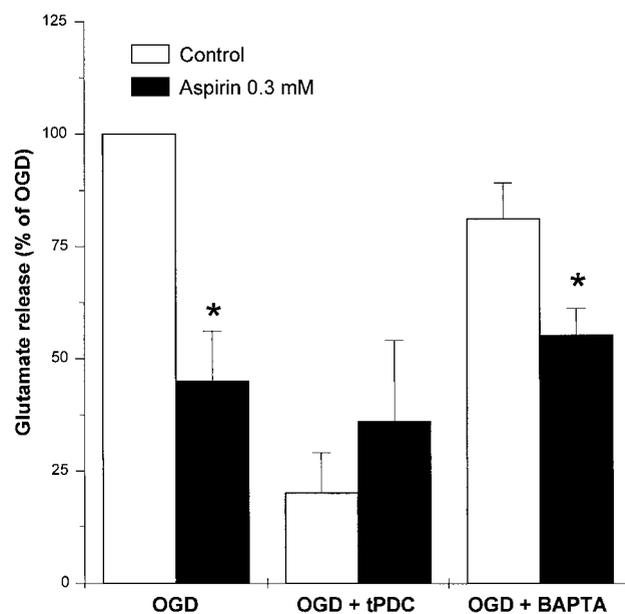
Effect of tPDC and BAPTA on OGD-Induced Glutamate Release From Cortical Neurons: Effect of Aspirin

OGD-induced glutamate release results mainly from reversed operation of neuronal glutamate transporters,[16,23] but other mechanisms may also contribute, such as a Ca^{2+}-dependent exocytotic release. Therefore, the inhibitor of glutamate uptake tPDC (300 μmol/L) and the intracellular Ca^{2+} chelator BAPTA-AM (5 μmol/L) were used in the presence of aspirin to dissect the component inhibited by aspirin.

Both tPDC and BAPTA partially inhibited OGD-induced glutamate release (Figure 3). When coincubated with these agents, aspirin (0.3 mmol/L) did not further inhibit OGD-induced glutamate release beyond that seen with tPDC alone. Aspirin further reduced glutamate release caused by BAPTA when the 2 drugs were used together (Figure 3).

Since neuroprotection due to salicylates has been suggested to result from NF-κB inhibition,[7] we tested the effect of PDTC (100 μmol/L). This compound failed to inhibit

Figure 3. Characterization of OGD-induced glutamate release. Effect of aspirin (0.3 mmol/L) on OGD-induced glutamate release, in the absence or presence of the glutamate uptake inhibitor tPDC (300 μmol/L) or of the intracellular calcium chelator BAPTA-AM (BAPTA) (5 μmol/L), is shown. Data are mean$\pm$SEM; n=12; *$P<0.05$ vs control.

OGD-induced glutamate release (1.20 ± 0.12 nmol/10^6 cells; n=4; $P>0.05$).

Effect of Aspirin on OGD-Induced Glutamate Release in Glia-Enriched Cultures

When glial cells were exposed to OGD, aspirin (0.3 mmol/L) did not affect OGD-induced glutamate release (1.29 ± 0.13 versus 1.12 ± 0.11 nmol glutamate per milliliter in the absence and presence of 0.3 mmol/L aspirin, respectively; n=6; $P>0.05$).

Effect of Aspirin on Glutamate Uptake by Cortical Neurons

Glutamate uptake ($19.1\pm0.5\%$; n=4) was not affected in the presence of 0.3 mmol/L aspirin ($20.5\pm0.8\%$; n=4; $P>0.05$) but was significantly inhibited in the presence of the glutamate transporter inhibitor tPDC (300 μmol/L) ($8.2\pm0.8\%$; n=4; $P<0.05$).

Effect of OGD on ATP Levels in Cortical Neurons: Effect of Aspirin

The exposure of cortical neurons to a "submaximal" OGD caused a decrease in ATP levels compared with control cells (Figure 4A). Aspirin and sodium salicylate but not indomethacin ($41\pm3\%$ at 3 μmol/L and $37\pm6\%$ at 100 μmol/L of control ATP levels; n=6; $P>0.05$ versus OGD) caused a partial recovery in ATP levels after OGD (Figure 4A).
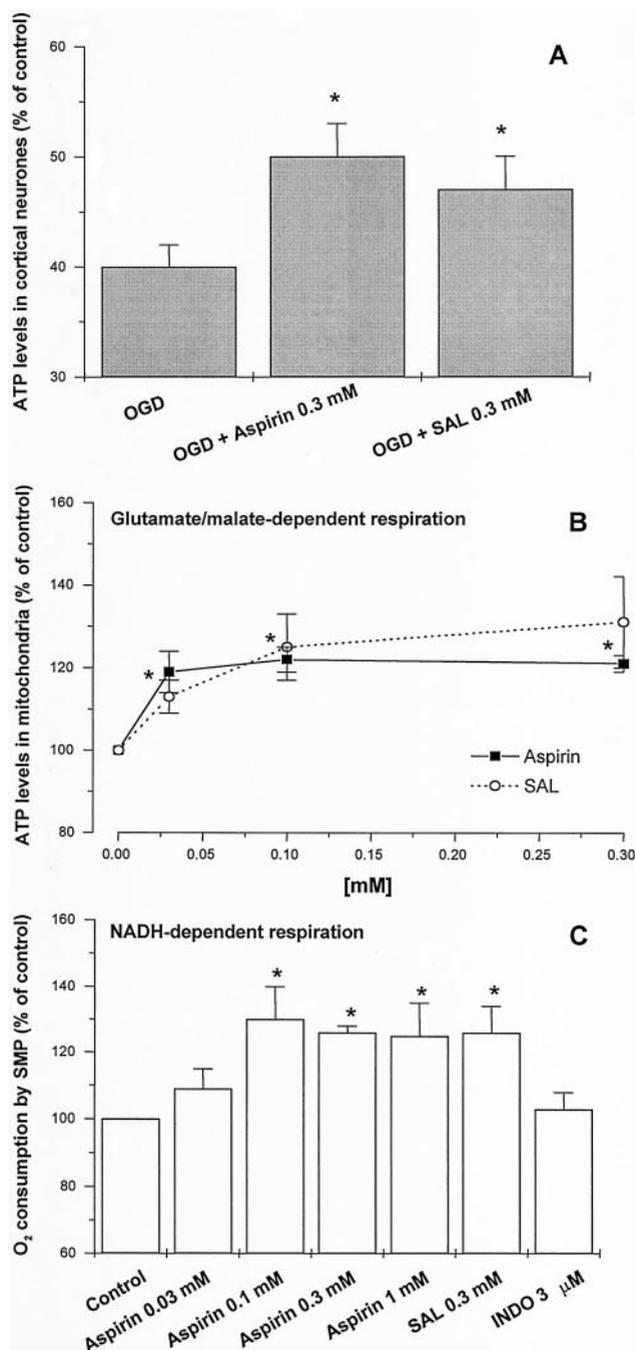
Effect of Aspirin on ATP Levels in Isolated Mitochondria and in Oxygen Consumption by Submitochondrial Particles

The production of ATP by isolated rat mitochondria incubated with glutamate/malate (complex I-, III-, and IV-dependent respiration) was increased by aspirin and sodium salicylate (Figure 4B) but not by indomethacin ($100\pm4\%$, $99\pm8\%$, and $90\pm10\%$ of control ATP levels, respectively, for indomethacin 3, 10, and 100 μmol/L; n=6; $P>0.05$). Aspirin (0.3 mmol/L) did not modify the production of ATP by mitochondria incubated with succinate (complex II-, III-, and IV-dependent respiration; 399 ± 31 versus 380 ± 40 nmol/mg of mitochondrial protein in the absence and presence of aspirin; n=6; $P>0.05$).

The exposure of submitochondrial particles to aspirin and sodium salicylate produced an increase of NADH-dependent respiration but not the exposure to indomethacin 3 μmol/L (Figure 4C) or 100 μmol/L ($105\pm4\%$ of control; n=6 to 12; $P>0.05$). Succinate and TMPD/ascorbate-dependent respiration (control values: 9.5 ± 0.9 and 19.1 ± 1.9 μmol/L O_2 per minute, respectively) was not affected by aspirin (10.2 ± 0.8 and 17.3 ± 1.4 μmol/L O_2 per minute, respectively; n=8; $P>0.05$).

Discussion

We have recently reported a neuroprotective effect of aspirin concomitant with inhibition of glutamate release in an in vitro model of brain ischemia using rat forebrain slices exposed to OGD.[10] We have now used another model of cerebral ischemia consisting of cultured rat cortical neurons exposed to OGD to elucidate further this neuroprotective effect. Our

Figure 4. Effect of aspirin on ATP levels and mitochondrial respiration. A, ATP levels in cortical neurons after OGD (control value=23.2 ± 3.7 nmol/mg protein; n=8). Effect of aspirin (0.3 mmol/L) and sodium salicylate (SAL) (0.3 mmol/L) is shown. B, Effect of aspirin and sodium salicylate (0.03 to 0.3 mmol/L) on ATP production by glutamate/malate-dependent respiration (control value=929 ± 77 nmol/mg of mitochondrial protein) in isolated mitochondria. C, Effect of aspirin (0.03 to 1 mmol/L), sodium salicylate (0.3 mmol/L), and indomethacin (INDO) (3 μmol/L) on NADH-dependent respiration (control value=11.2 ± 0.9 μmol/L O_2 per minute) by submitochondrial particles (SMP). Data are mean$\pm$SEM; n=12; *$P<0.05$ vs control.

results show that, in cortical neurons, aspirin causes specific protection that is due to inhibition of OGD-induced release of glutamate, by the inhibition of the fall in ATP responsible for the reversal of glutamate uptake systems in cerebral ischemia.

OGD causes cell damage to cortical neurons, as deduced by the release of LDH, the inhibition of MTT reduction, and the staining with propidium iodide. In addition, aspirin inhibits neuronal death induced by OGD, as demonstrated with all 3 of these viability parameters.

The next question we approached concerned the mechanism involved. Although aspirin possesses a wide spectrum of pharmacological actions, our previous findings pointed to glutamate release as the target of this neuroprotective effect. In this context, the neurotoxic actions of glutamate are well known to play a predominant role in the pathogenesis of brain injury after cerebral ischemia.[9] We have shown that glutamate is released after OGD in this preparation and that aspirin inhibits this release in a way that parallels its neuroprotective effect.

Several mechanisms, alone or combined, may be responsible for OGD-induced glutamate release, such as a Ca^{2+}-dependent, prostaglandin-stimulated release in astrocytes,[24] a Ca^{2+}-dependent exocytosis of its vesicular pool,[25] or the reversal of the electrogenic uptake transport systems.[16,23] We therefore searched for the target of the neuroprotective effect of aspirin by pharmacological dissection of the components implicated. First, an important piece of evidence resulted from the fact that the inhibitory effect of aspirin on glutamate release was shared by sodium salicylate but not by indomethacin, a nonsalicylate NSAID that shows IC_{50} and IC_{80} ratios— referred to as the inhibition of COX isoforms—similar to those of aspirin (IC_{50} ratio=4.4 for aspirin and 10 for indomethacin; IC_{80} ratio=3.8 for aspirin and 4.3 for indomethacin).[22] Thus, this was the first indication of the lack of involvement of COX- and prostaglandin-mediated release[24] in the phenomenon described in this work. Additional information was obtained from glia-enriched cultures, in which the effect of aspirin was not observed, therefore excluding glutamate release mediated by astrocytes.[24] We had previously reported that sodium salicylate did not share the neuroprotective effect of aspirin on rat forebrain slices. This apparent discrepancy results very likely from a restricted diffusion of salicylate, less lipophilic than aspirin, into the slice.

Second, BAPTA, an intracellular Ca^{2+} chelator, was used as a tool to investigate Ca^{2+}-dependent, exocytotic glutamate release, regardless of whether the source of Ca^{2+} is extracellular or from intracellular stores. Aspirin still inhibited the remaining component of OGD-induced glutamate release after the cells were loaded with this compound, indicating that this NSAID was acting at a step different from vesicular exocytosis, and again excluding the involvement of astroglial mechanisms.

Third, it has recently been shown that glutamate release induced by severe ischemia is largely due to reversed operation of neuronal glutamate transporters.[16,23] Therefore, we investigated OGD-induced glutamate release after inhibiting glutamate transporters with tPDC.[26] In these conditions, aspirin was unable to inhibit any further OGD-induced glutamate release, strongly indicating that release resulting from reversed operation of glutamate transporters is the component affected by aspirin.

On the other hand, neuroprotection due to salicylates[7] has been suggested to result from NF-κB inhibition after glutamate receptor stimulation, an effect that occurs at high concentrations of these compounds (>1 mmol/L); we therefore tested the effect of maximally neuroprotective concentrations of aspirin on the neuronal death caused by exogenously added glutamate. In these conditions, no inhibition of aspirin on glutamate-induced neuronal death was found. In addition, the inhibitor of NF-κB activation PDTC[14] did not affect OGD-induced glutamate release. These findings exclude the involvement of NF-κB in the neuroprotective effect of these concentrations of aspirin.

The direct approach was then the study of glutamate uptake by cortical neurons. Surprisingly, no effect of aspirin was observed on [^{3}H]glutamate uptake, indicating that aspirin was not acting directly but rather at some point upstream of the reversal of the glutamate transporter. It has been demonstrated that release of the cytosolic neuronal pools of glutamate by reversed operation of glutamate transporters results from severe ATP depletions.[27] In addition, it has been described that aspirin increases tolerance against hypoxia concomitant with a delay of intracellular ATP content.[8] Indeed, we have found that OGD-induced ATP loss was decreased by aspirin and salicylate in cortical neurons. Moreover, aspirin increased ATP production by isolated mitochondria and respiration by submitochondrial particles stimulated only by complex I-III substrates. All these data indicate that aspirin targets mitochondrial respiratory chain complex I-III, resulting in an increased ATP production that inhibits OGD-induced ATP loss and subsequent reversal of glutamate transporters. In addition, experiments performed on isolated mitochondria and submitochondrial particles obtained from whole brain suggest that aspirin and salicylates may affect not only neuronal ATP formation but also ATP production in other cells such as glial cells, with implications that remain to be elucidated. Our data indicate that neuroprotective concentrations of aspirin increase oxygen consumption by brain mitochondria, leading to an elevation in intracellular ATP levels. These data indicate that this effect of aspirin must be exerted before OGD onset, when oxygen is still present, thus implying that this action of aspirin should be considered at a preventive level. To our knowledge, this is the first report of this action of aspirin and salicylates, with very important implications not only as a mechanism to prevent glutamate release after cerebral ischemia but also in other pathophysiological conditions that arise from a depletion of cellular energetic stores, such as ischemic heart disease. Our findings open a line of research directed toward the elucidation of other roles of aspirin and salicylates by affecting ATP levels in glial cells, which may have important implications after ischemia at the level of glutamate metabolism and uptake.

In summary, our present findings show that aspirin exerts direct neuroprotective actions at concentrations corresponding to antithrombotic-analgesic doses, with therapeutic implications in the management of patients at risk of ischemic events and devoid of undesirable effects of the high, anti-inflammatory doses of this drug.

Acknowledgments

References

1. Grotta JC, Lemak NA, Gary H, Fields WS, Vital D. Does platelet antiaggregant therapy lessen the severity of stroke? *Neurology*. 1985;35:632–636.

2. Carolei A, Prencipe M, Fiorelli M, Fieschi C. Severity of stroke and aspirin. *Neurology*. 1986;36:1010–1011.

3. Joseph R, Han E, Tsering C, Grunfeld S, Welch KM. Platelet activity and stroke severity. *J Neurol Sci*. 1992;108:1–6.

4. Vane JR. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat New Biol*. 1971;231:232–235.

5. Kuhn W, Müller T, Büttner T, Gerlach M. Aspirin as a free radical scavenger: consequences for therapy of cerebrovascular ischemia. *Stroke*. 1995;26:1959–1960.

6. Oberle S, Polte T, Abate A, Podhaisky HP, Schroder H. Aspirin increases ferritin synthesis in endothelial cells: a novel antioxidant pathway. *Circ Res*. 1998;82:1016–1020.

7. Grilli M, Pizzi M, Memo M, Spano F. Neuroprotection by aspirin and sodium salicylate through blockade of NF-κB activation. *Science*. 1996;274:1383–1385.

8. Riepe MW, Phys D, Kasischke K, Raupach A. Acetylsalicylic acid increases tolerance against hypoxic and chemical hypoxia. *Stroke*. 1997;28:2006–2011.

9. Choi DW, Rothman SM. The role of glutamate neurotoxicity in hypoxic-ischaemic neuronal death. *Annu Rev Neurosci*. 1990;13:171–182.

10. Moro MA, De Alba J, Cárdenas A, De Cristóbal J, Leza JC, Lizasoain I, Díaz-Guerra MJM, Boscá L, Lorenzo P. Mechanisms of the neuroprotective effect of aspirin after oxygen and glucose deprivation in rat forebrain slices. *Neuropharmacology*. 2000;39:1309–1318.

11. Moro MA, Fernández-Tomé P, Leza JC, Lorenzo P, Lizasoain I. Neuronal death induced by SIN-1 in the presence of superoxide dismutase: protection by cyclic GMP. *Neuropharmacology*. 1998;37:1071–1079.

12. Acarin L, Vela JM, González B, Castellano B. Demonstration of poly-N-acetyl lactosamine residues in ameboid and ramified microglial cells in rat brain by tomato lectin binding. *J Histochem Cytochem*. 1994;42:1033–1041.

13. Goldberg MP, Choi DW. Combined oxygen and glucose deprivation in cortical cell culture: calcium-dependent and calcium-independent mechanisms of neuronal injury. *J Neurosci*. 1993;13:3510–3524.

14. Schreck R, Meier B, Männel DN, Dröge W, Baeuerle PA. Dithiocarbamates as potent inhibitors of NFκB activation in intact cells. *J Exp Med*. 1992;175:1181–1194.

15. Moro MA, Leza JC, Lorenzo P, Lizasoain I. Peroxynitrite causes aspartate release from dissociated rat cerebellar granule neurones. *Free Radic Res*. 1998;28:193–204.

16. Rossi DJ, Oshima T, Attwell D. Glutamate release in severe brain ischaemia is mainly by reversed uptake. *Nature*. 2000;403:316–321.

17. Lizasoain I, Moro MA, Knowles RG, Darley-Usmar V, Moncada S. Nitric oxide and peroxynitrite exert distinct effects on respiration by brain mitochondria which are differentially blocked by glutathione or glucose. *Biochem J*. 1996;314:877–880.

18. Hill HD, Straka JG. Protein determination using bicinchoninic acid in the presence of sulfhydryl reagents. *Anal Biochem*. 1988;170:203–208.

19. Koh JY, Choi DW. Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. *J Neurosci Methods*. 1987;20:83–90.

20. Hansen MB, Nielsen SE, Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J Immunol Methods*. 1989;119:203–210.

21. Tanke HJ, van der Linden PW, Langerak J. Alternative fluorochromes to ethidium bromide for automated read out of cytotoxicity tests. *J Immunol Methods*. 1982;52:91–96.

22. Warner TD, Giuliano F, Vojnovic I, Bukasa A, Mitchell JA, Vane JR. Nonsteroid drug selectivities for cyclo-oxygenase-1 rather than cyclo-oxygenase-2 are associated with human gastrointestinal toxicity: a full *in vitro* analysis. *Proc Natl Acad Sci U S A*. 1999;96:7563–7568.

23. Jabaudon D, Scanziani M, Gähwiler BH, Gerber U. Acute decrease in net glutamate uptake during energy deprivation. *Proc Natl Acad Sci U S A*. 2000;97:5610–5615.

24. Bezzi P, Carmignoto G, Pasti L, Vesce S, Rossi D, Rizzini BL, Pozzan T, Volterra A. Prostaglandins stimulate calcium-dependent glutamate release in astrocytes. *Nature*. 1998;391:281–285.

25. Drejer J, Benveniste H, Diemer NH, Schousboe A. Cellular origin of ischemia-induced glutamate release from brain tissue in vivo and in vitro. *J Neurochem*. 1985;45:145–151.

26. Bridges RJ, Stanley MS, Anderson MW, Cotman CW, Chamberlin AR. Conformationally defined neurotransmitter analogues: selective inhibition of glutamate uptake by one pyrrolidine-2,4-dicarboxylate diastereomer. *J Med Chem*. 1991;34:717–725.

27. Madl JE, Burgesser K. Adenosine triphosphate depletion reverses sodium-dependent, neuronal uptake of glutamate in rat hippocampal slices. *J Neurosci*. 1993;13:4429–4444.