Aspirin Inhibits p44/42 Mitogen-Activated Protein Kinase and Is Protective Against Hypoxia/Reoxygenation Neuronal Damage

Nina Vartiainen, PhD; Gundars Goldsteins, PhD; Velta Keksa-Goldsteine, MSc; Pak H. Chan, PhD; Jari Koistinaho, MD, PhD

Background and Purpose—Acetylsalicylic acid (ASA) is preventive against stroke and protects against focal brain ischemia in rats. We studied the mechanisms of the manner in which ASA provides neuroprotection against hypoxia/reoxygenation (H/R) injury.

Methods—Spinal cord cultures exposed to 20 hours of hypoxia followed by reoxygenation were treated with a vehicle, ASA or inhibitors of inducible nitric oxide synthase (iNOS), mitogen-activated protein kinases p38 MAPK and ERK1/2, or an N-methyl-D-aspartic acid (NMDA) receptor antagonist. Cell viability was assessed by LDH release measurement and cell counts. Prostaglandin production was measured by enzyme immunoassay, MAPK signaling by immunoblotting, and DNA binding of nuclear factor-κB (NF-κB) and activating protein-1 (AP-1) by electrophoretic mobility shift assay.

Results—One to 3 mmol/L ASA inhibited H/R-induced neuronal death when present during H/R but not when administered only for the reoxygenation period. Prostaglandin E₂ production was very low and was not altered by ASA. The AP-1 and NF-κB DNA binding activities increased after H/R. ASA increased the H/R-induced AP-1 binding but had no effect on NF-κB binding. H/R induced a sustained ERK1/2 activation followed by neuronal death, whereas no changes in p38 or c-Jun N-terminal kinase were detected. ASA strongly inhibited this ERK1/2 activation. PD98059, an ERK1/2 inhibitor, was also neuroprotective, prevented H/R-induced ERK1/2 activation, and had no effect on NF-κB binding activity. Inhibition of NMDA receptors, iNOS, or p38 MAPK did not provide neuroprotection.

Conclusions—Inhibition of the sustained activation of ERK1/2 may partially contribute to neuroprotection achieved by ASA against H/R injury. (Stroke. 2003;34:752-757.)

Key Words: cell death ■ cyclooxygenase ■ reactive oxygen species ■ transcription factors
but excitotoxicity through N-methyl-d-aspartate (NMDA) receptors has no major role in the H/R injury, thus corresponding with several findings on global ischemia models in vivo.26–28 Here we demonstrate that ASA provides neuroprotection against H/R by a mechanism that is independent of COX and iNOS activities, NMDA receptor–mediated toxicity, and NF-κB binding. Instead, inhibition of ERKs may mediate the beneficial effect of ASA on neuronal survival.

**Materials and Methods**

**Cell Culture**

The spinal cord mixed culture has been previously established with approximately 70% neurons, 25% astrocytes, and 5% other cells.29 Briefly, spinal cords were dissected from 14-day-old rat embryos (Sprague-Dawley, Charles River), were trypsinized for 15 minutes (0.25% trypsin-EDTA, Gibco Life Sciences), triturated with a fire-polished Pasteur pipette, and plated in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) (Gibco Life Sciences) supplemented with 10% horse serum (Gibco Life Sciences) and 10% fetal bovine serum (Gibco Life Sciences). The medium was replaced the next day with DMEM with 5% both sera. After 4 days in vitro, 5 μmol/L ara-C (cytosine arabinoside, Sigma) was added for 24 hours, and the medium was replaced with DMEM plus 5% horse serum.

**Drug Treatments**

After 1 week in culture, the medium was replaced with DMEM plus 1% horse serum containing 0.1 to 3 mmol/L ASA (Sigma), 5 to 20 μmol/L PD98059 (specific ERK kinase inhibitor; Tocris Cookson), 5 to 20 μmol/L SB203580 (specific p38 MAPK inhibitor; Tocris Cookson), 10 μmol/L MK-801 (an NMDA antagonist; Tocris Cookson), or 100 μmol/L aminoguanidine (an inhibitor of iNOS; Sigma). ASA serum levels up to 3 mmol/L can be reached easily in humans.30 The drugs were dissolved in dimethyl sulfoxide, and the drugs in the medium were administered to the cells 15 to 30 minutes before the induction of hypoxia or at the onset of reoxygenation. PBS and dimethyl sulfoxide controls were used.

**Hypoxia Treatment**

Plates were placed into a humidified chamber (modular incubator chamber, Billups Rothenberg) and flushed for 10 minutes with 95% N₂ and 5% CO₂ to achieve a low-oxygen environment. The sealed chamber was placed into a 37°C incubator for 20 hours. After the hypoxia period, the plates were placed back into a normoxic incubator for 0 to 24 hours. The control plates were kept in normoxic conditions for a corresponding time.

**Measurement of PO₂ and Glucose**

After the desired hypoxia or H/R period, the medium was collected to air-sealed syringes, and medium PO₂ was determined with a blood gas/pH analyzer (ABL-5, Radiometer Medical A/S). Culture medium kept in a 7.5% CO₂ incubator was used as control to which the values were normalized. The glucose values were determined from the medium with a One Touch Basic analyzer (LifeScan Inc.).

**Assessment of Cell Death**

Cell death was determined by LDH release measurement and by counting the number of bis-benzimide–stained cells. Medium (100 μL) was collected from each 96-well plate well, and the samples were measured with a Sigma LDL-20 kit at least as triplicates at 340 nm by a Labsystems Multiscan plate reader and application software (Labsystems). Cells fixed with 4% paraformaldehyde were stained for 5 minutes with 5 μg/mL Hoechst stain No. 33258 (Sigma) and washed with PBS. Cell numbers were counted with the use of a fluorescence microscope with appropriate filters. Because our initial findings indicated that neuronal protection provided by ASA was reliably observed as early as 4 hours after reoxygenation, 15-minute to 4-hour time points were used for additional mechanism studies.

**Immunoblotting**

Cells grown on 35-mm dishes were washed with PBS and lysed to the following buffer: 320 mmol/L sucrose, 1 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EGTA, 1 mmol/L Na₂VO₄, 5 mmol/L dithiothreitol, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 0.2 mmol/L phenylmethylsulfonyl fluoride, 50 mmol/L NaF, 0.5% NP-40. Laemmli sample buffer (5%) was added, and the samples were boiled for 5 minutes. Electrophoresis was performed with 10-μg protein samples in 10% SDS-PAGE. Separated proteins were transferred onto a Hybond-P membrane (Amersham-Pharmacia Biotech). Anti-phospho-ERK, anti-phospho-p38 and anti-phospho-JNK, and anti-ERK antibodies were purchased from New England Biolabs (dilution 1:1000). The blots were stripped and reprobed with anti-tubulin antibody (Santa Cruz) as loading control.

**Prostaglandin Measurement**

Prostaglandin E₂ (PGE₂) production was measured from media and cell lysates after 1 hour of reoxygenation with the use of an Amersham-Pharmacia Biotech PGE₂ EIA kit; PGE₂ and its metabolites were determined with the use of a Cayman Chemical Bicyclo PGE₂, EIA kit. Briefly, to measure from both the medium and lysate, the cells were washed with PBS and lysed according to the manufacturer’s instructions. Each sample was measured at 405 nm as a duplicate, and the data from 3 different experiments were combined. For the measurement from only the medium, samples from 96-well plates were pooled to a 500-μL aliquot. Sample PGE₂ metabolites and standards were derivatized overnight to bicyclo-PGE₂. The results were measured at 412 nm with a Spectramax plate reader and application program (Molecular Devices).

**Electromobility Gel Shift Assay**

The samples were prepared from 35-mm dishes according to Dignam et al.31 Nuclear proteins were isolated with the use of hypotonic buffer (10 mmol/L HEPES [pH 7.9], 1.5 mmol/L MgCl₂, 10 mmol/L KCl, 1.0 mmol/L dithiothreitol, 1 μg/mL aprotinin and leupeptin), low-salt buffer (20 mmol/L HEPES [pH 7.9], 25% glycerol, 1.5 mmol/L MgCl₂, 20 mmol/L KCl, 0.2 mmol/L EDTA, 1 μg/mL aprotinin and leupeptin), and high-salt buffer (as low-salt buffer except 1.2 mol/L KCl). Consensus oligonucleotides (AP-1, NF-κB; Promega) were labeled with 32P-pYpATP (NEN) with the use of T4 polynucleotide kinase (Promega). The probes were purified with the use of Pharmacia Microspin columns. The binding reactions were conducted with 3 μg nuclear proteins. The reaction solution contained 5× binding buffer (100 mmol/L HEPES [pH 7.9], 7.6 mmol/L MgCl₂, 5 mmol/L EDTA, 50% glycerol), poly-dIdC (1.5 μg/mL, Pharmacia Biotech), 1 mmol/L dithiothreitol, 0.1 mol/L NaCl. The binding specificity was studied with competitive cold oligonucleotides for NF-κB and AP-1. 5′-oligonucleotide (Promega) was used as a nonspecific competitor. The bound and free probes were separated by 6% nondenaturing PAGE. The bound probes were visualized by STORM PhosphorImager (Molecular Dynamics), and the bands were quantified with Image QuaNT software (Molecular Dynamics).

**Results**

**Oxygen and Glucose Levels**

The PO₂ of the culture medium decreased to 54.7±8.4% within 60 minutes after a 10-minute flush with 5% CO₂/N₂. At 3 hours the oxygen level was 47.2±9.4% and at 20 hours 36.2±8.9% of the control value. Ten minutes after reoxygenation the PO₂ value had recovered to 63.1±19.0%, and at 1 hour oxygen levels reached 83.9±15.6% of the control value. The glucose levels did not change during hypoxia or reoxygenation periods (not shown).
Drug Treatments and Neuronal Survival
After 20- or 24-hour hypoxia, no significant increase in LDH release was detected. Some cell death was measurable after 2-hour reoxygenation, and a significant increase \( (P<0.01, \text{ANOVA}) \) was seen after 4 hours (Figure 1A). After 24-hour reoxygenation the LDH release was further doubled (Figure 1C). The ASA administration 15 to 30 minutes before induction of hypoxia reduced LDH release dose dependently, and the protection was significant at 1 to 3 mmol/L concentrations \( (P<0.01; \text{ANOVA}) \). The neuroprotection was evident after both 4 and 24 hours of reoxygenation (Figure 1A and 1C). ASA (3 mmol/L) was not neuroprotective when administered at the time of reoxygenation (not shown). Cell counts from bis-benzamide–stained cultures confirmed the LDH release data (Figure 1B). Administration of 10 \( \mu \text{mol/L} \) MK-801 before H/R or before reoxygenation did not reduce LDH release measured 4 hours after reoxygenation \( (\text{increase in LDH release without treatment } 142\pm3.1\% \text{ and with MK801 treatment } 200\pm35\% \text{ when present during hypoxia and } 135\pm24.5\% \text{ when administered at the onset of reoxygenation}) \). Five, 10, and 20 \( \mu \text{mol/L} \) PD98059 protected against H/R neuronal injury, but the effect was less dramatic than with 3 mmol/L ASA. Coadministration of ASA and PD98059 did not demonstrate a synergistic effect (Figure 1D). The p38 MAPK inhibitor SB203580 at 5 to 20 \( \mu \text{mol/L} \) concentrations or 100 \( \mu \text{mol/L} \) aminoguanidine did not have a protective effect (data not shown).

Prostaglandin Measurements
PGE\(_2\) was used as a marker of COX activity because PGE\(_2\) is one of the most readily detectable prostaglandins produced in the spinal cord.\(^{32}\) No PGE\(_2\) was detected in medium even after hypoxia, and no changes in PGE\(_2\) values in the cell lysates were detected between untreated and ASA-treated samples (data not shown). Because the half-life of PGE\(_2\) is much shorter than the H/R period, PGE\(_2\) catabolites were also measured as stable bicyclo-PGE\(_2\). Some bicyclo-PGE\(_2\) form was detected in normoxia, and a significant increase after 20 hours of hypoxia and 1 hour of reoxygenation was measured \( (\text{mean} \pm \text{SD, from } 10.75\pm0.50 \text{ to } 20.75\pm1.26 \text{ pg/mL;} \ P<0.05, \text{ANOVA}) \). However, ASA treatment had no effect on this increase \( (\text{from } 11.00\pm0.41 \text{ to } 20.75\pm2.06 \text{ pg/mL;} \ P<0.05, \text{ANOVA}) \).

NF-\(\kappa\)B and AP-1 Binding Activity
NF-\(\kappa\)B binding increased after hypoxia and after hypoxia/1-hour reoxygenation (Figure 2). ASA or PD98059 did not reduce NF-\(\kappa\)B binding in normoxia and did not inhibit hypoxia or H/R-induced NF-\(\kappa\)B binding compared with untreated samples. AP-1 binding was not influenced by hypoxia alone but was increased after H/R. Treatment with ASA or PD98059 increased AP-1 binding after hypoxia and after 1-hour reoxygenation \( (P<0.05, \text{ANOVA}) \).

ERK Activation
Reoxygenation increased the phosphorylated ERK/ERK ratio as early as 15 minutes after the onset of reoxygenation, and at 60 minutes the increase was 5- to 6-fold compared with control values (Figure 3A). Pretreatment with 3 mmol/L ASA
as well as PD98059 prevented the reoxygenation-induced increase of phosphorylated ERK (Figure 3B and 3C). Hypoxia, H/R, and ASA did not induce detectable changes in phosphorylated JNK immunoreactivity, and no phosphorylated p38 MAPK immunoreactivity was seen in control cultures after hypoxia or after ASA treatment (not shown).

Discussion

Our results show that in this spinal cord culture model ASA protects neurons against H/R injury by a mechanism that does not involve COX or iNOS and in which NMDA-mediated excitotoxicity and NF-κB transcription factor have no major role. In addition, no neuroprotection was seen when ASA was
administered only for the reoxygenation period, indicating that the neuroprotective effect cannot be attributed to scavenging of reactive oxygen species. Instead, we found that from the MAPK superfamily ERK1/2 is specifically induced in neuronal cultures on reoxygenation and that ASA strongly inhibits ERK1/2 activation. When it is considered that the ERK1/2 pathway has been found to mediate neuronal death in various models of neuronal injury, and that selective inhibition of ERK1/2 increased neuronal survival in the present study, it is likely that ASA partially provides neuroprotection against H/R injury by preventing sustained ERK1/2 activation. However, the possibility that other mechanisms are also involved in the neuroprotection cannot be excluded because ASA was a more potent protectant than PD98059, a selective MEK inhibitor.

One of the main targets of ASA is thought to be the COX enzyme pathway. However, in these cultures the PGE2 levels were virtually undetectable even after H/R, and slight accumulation of PGE2 catabolites was unaffected by ASA. We have previously demonstrated that these spinal cord cultures do not express significant amounts of either COX-1 or COX-2 enzymes, further supporting our conclusion that COX activity plays no role in the ASA neuroprotection in this study.

While there are no reports on the effect of ASA on NF-κB in the brain in vivo, ASA has been shown to block phosphorylation of I-κB component in the cytoplasmic NF-κB complex and thereby inhibit NF-κB activation in vitro. Even though the total binding activities of NF-κB and AP-1 were increased after H/R, ASA did not reduce the binding activity of NF-κB. This is in agreement with a recent study by De Cristobal et al., who by using an oxygen-glucose deprivation model of cortical neurons found ASA protection that was not blocked by pyrrolidine dithiocarbamate, an inhibitor of NF-κB. It is likely that both the injury pathways and mechanisms of ASA protection depend on the cell types and injury model used. Interestingly, hypoxia as well as ASA specifically increased the binding of the lower NF-κB band. The composition and function of this binding complex remain to be studied, because no such difference was seen in PD98059-treated samples.

Both ASA and selective inhibition of ERK1/2 with PD98059 increased AP-1 binding activity after H/R, which has not been previously demonstrated in any other neuronal model. Instead, several studies have shown that inhibition of the ERK pathway either reduces or has no effect on AP-1 binding. Because ERK activation may downregulate p38 MAPK activity and thereby inhibit p38 MAPK–induced AP-1 binding, it may be possible that ASA-inhibited ERK activation and increased AP-1 binding go through p38 MAPK. Whatever the mechanism of ASA-induced AP-1 binding may be, the possibility that ASA provides neuroprotection by enhancing AP-1 binding cannot be excluded.

Both in vivo evidence and in vitro evidence indicate that ASA may inhibit iNOS, a proinflammatory NO-releasing enzyme involved in ischemic brain damage. However, iNOS does not mediate neuronal cell death in H/R damage because aminoguanidine did not protect neurons in the present study. The reason that aminoguanidine is protective in focal brain ischemia but not in our H/R model may be that in focal brain ischemia iNOS expression is restricted to infiltrating leukocytes, which are not present in brain cell cultures.

ASA has been shown to delay intracellular ATP loss during hypoxia and most recently to elevate ATP levels, thereby possibly inhibiting reversal of glutamate receptors and excitotoxicity during oxygen-glucose deprivation. While this is evidently a potential mechanism of the manner in which ASA provides protection after brief oxygen-glucose deprivation, our study on the model of long-term hypoxia followed by reoxygenation demonstrates that prevention of ERK1/2 activation is still another mechanism of ASA neuroprotection. The effectors of ERK1/2 include a diverse set of targets, such as cytoskeletal proteins (neurofilaments and Tau), transcription factors (c-Myc, c-Jun, C-fos, Elk-1, CREB, and ATF-2), and cell-signaling proteins (phospholipase-A2 and ribosomal S6 kinase). In addition, synapsin I, a presynaptic phosphoprotein regulating glutamate release, is phosphorylated by ERK1/2, raising the possibility that inhibition of ERK1/2-mediated glutamate release contributes also to ASA neuroprotection observed in the models, which involves NMDA excitotoxicity. Further studies are needed to identify the ERK pathway inhibited by ASA.

In conclusion, ASA is neuroprotective against H/R damage partially by inhibiting the ERK signaling pathway. When one considers previous reports on the neuroprotective mechanisms of ASA, the combination of multiple pharmacological activities and sites of action may explain the beneficial effects of ASA on patients with high stroke risk.

Acknowledgments

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References


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