The Novel Neuroprotective Action of Sulfasalazine through Blockade of NMDA Receptors

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ABSTRACT

Sulfasalazine is widely used to treat inflammatory diseases. Besides anti-inflammatory actions such as blockade of nuclear factor-κB and cyclooxygenases, we found that 30 to 1000 μM sulfasalazine dose dependently blocked N-methyl-d-aspartate receptor-mediated excitotoxicity without intervening kainate or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid neurotoxicity. The neuroprotective effects of sulfasalazine were attributable to prevention of Ca2+ influx and accumulation through N-methyl-d-aspartate receptors as a low-affinity antagonist. The systemic administration of sulfasalazine reduced neuronal death following transient cerebral and retinal ischemia in adult rat. The present findings suggest that the neuroprotective action of sulfasalazine can be therapeutically applied to halt devastating neuronal death following hypoxic ischemia, trauma, and neurodegenerative diseases.

Accumulating evidence suggests that inflammatory processes play a role in degeneration of neuronal cells in acute and chronic neurodegenerative diseases. For example, the inducible enzyme cyclooxygenase-2 (COX-2) is up-regulated in ischemic brain areas following focal cerebral ischemia and global forebrain ischemia (Planas et al., 1995; Nakayama et al., 1998), which converts arachidonic acid into the proinflammatory mediators such as prostaglandins. Selective inhibitors and genetic knockout of COX-2 reduce ischemic neuronal death (Sasaki et al., 1998; Iadecola et al., 2001). Increased expression of COX-2 is observed in Alzheimer’s disease and traumatized brain and spinal cord and probably contributes to progress of diseases (Oka and Takashima, 1997; Resnick et al., 1998; Dash et al., 2000).

The transcription factor nuclear factor-κB (NF-κB) regulates expression of proinflammatory cytokines such as tumor necrosis factor and interleukins, cell adhesion molecules, and the inducible enzymes such as nitric oxide synthase, COXs, and manganese superoxide dismutase (Baueerle and Baltimore, 1996; O’Neill and Kaltshmidt, 1997) and modulates degeneration of neurons and non-neuronal cells (Beg and Baltimore, 1996; Scatena et al., 1998). Activation of NF-κB is observed in basal forebrain cholinergic neurons of patients with Alzheimer’s disease and in vulnerable brain areas after ischemic injury (Boissiere et al., 1997; Clemens et al., 1997; Stephenson et al., 2000). Activation of NF-κB mediates N-methyl-d-aspartate (NMDA) receptor-mediated neuronal death but can protect neurons from oxidative stress and apoptosis (Mattson et al., 1997; Taglialetela et al., 1997; Qin et al., 1998; Won et al., 1999).

Acetysalicylate (aspirin), an inhibitor of COXs and NF-κB, holds multiple therapeutic effects, including anti-inflammatory, analgesic, and antipyretic effects (Kopp and Ghosh, 1994; Vane and Botting, 1998). Aspirin reduces platelet aggregation and the risk of recurrent stroke (Diez-Tejedor et al., 1995). Acetyl salicylate also attenuates ischemic neuronal death, cognitive deficiency in Alzheimer’s disease, and

ABBREVIATIONS: COX-2, cyclooxygenase-2; NF-κB, nuclear factor-κB; NMDA, N-methyl-d-aspartate; BSO, L-buthionine-(S,R)-sulfoximine; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; SKF38393, 2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzazepine; DC-DHF, dichlorodihydrofluorescein diacetate; DIV, days in vitro; HCSS buffer, HEPES controlled salt solution; LDH, lactate dehydrogenase; PGE2, prostaglandin E2; [ROS], intracellular reactive oxygen species; MK-801, dizocilpine maleate; PDTC, 1-pyrrolidinecarboxodithioic acid; NS398, N-[2-(cyclohexyloxyl)-4-nitrophenyl]-methanesulfonamide; MCAO, occlusion of middle cerebral artery; ANOVA, analysis of variance; MnTBAP, meso-tetrakis(4-benzoic acid)porphyrin.
motor deficits in the transgenic mouse of amyotrophic lateral sclerosis (Rich et al., 1995; Barneoud and Curet, 1999; Khayyam et al., 1999). Aspirin appears to exert its neuroprotective effects by preventing activation of NF-κB and c-jun N-terminal kinase, voltage-gated Ca\(^{2+}\) channels, and free radical production (Grilli et al., 1996; Aubin et al., 1998; Ko et al., 1998; Kim et al., 2001). Aspirin prevents NMDA neurotoxicity without preventing NMDA-induced accumulation of intracellular Ca\(^{2+}\) (Grilli et al., 1996). Nevertheless, the neuroprotective effects of aspirin at higher doses (>3 mM) limit its therapeutic potential to prevent neuronal death in brain diseases.

Sulfasalazine, a conjugate of 5-aminosalicylic acid and sulfapyridine, inhibits activity of COXs and NF-κB and has been widely used as an anti-inflammatory drug to treat rheumatoid arthritis and inflammatory bowel disease (Swartz, 1942; Wahl et al., 1998). Interestingly, we found that sulfasalazine prevented NMDA-induced neuronal death at the therapeutic doses needed to treat inflammatory diseases. Complete blockade of NMDA-induced neuronal death by sulfasalazine raises the possibility that sulfasalazine protects neurons through a novel mechanism irrespective of anti-inflammatory effects. We set out experiments to delineate how sulfasalazine prevents NMDA-induced neuronal death and to examine if sulfasalazine prevents hypoxic ischemic brain injury in animal models.

**Materials and Methods**

**Materials**

1-Buthionine-(S,R)-sulfoximine (BSO), ferrous chloride, cytosine arabinofuranoside, and 2,3,5-triphyltetrazolium chloride were purchased from Sigma-Aldrich (St. Louis, MO). α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate, NMDA, and SKF83893 were obtained from Sigma/RBI (Natick, MA). Trolox was obtained from Aldrich Chemical (Milwaukee, WI). Dichlorodihydrofluorescein diacetate (DCFH), dihydroethidium, Fura-2 AM, and Pluronic F-127 were purchased from Molecular Probes (Eugene, OR). MnTBAP was obtained from Calbiochem (La Jolla, CA).

**Methods**

**Mouse Cortical Cell Cultures.** Cortical cells were obtained from brains of fetal ICR mice at a 14- to 15-day gestation and plated on a 24-well plate (approximately 10^6 cells/well) precoated with 100 μg/ml poly-d-lysine and 4 μg/ml laminin in a plating medium containing Eagle’s minimal essential medium (with Earle’s salts) supplemented with 21 mM glucose, 5% fetal bovine serum, 5% horse serum, and 2 mM glutamine (Gwag et al., 1995). Animal care and treatment were in compliance with a protocol approved by our institutional animal care committee. For cocultures of neurons and glia, 10 μM cytosine arabinofuranoside was included to cultures at 7 to 9 days in vitro (DIV), when glial cells were confluent underneath neurons. Cultures were fed with plating medium without fetal bovine serum twice a week. Cultures were maintained at 37°C in a humidified 5% CO\(_2\) atmosphere.

**Neurotoxicity Experiment.** Experiments were performed in cortical cell cultures (DIV 12 to 14). For NMDA receptor-mediated excitotoxicity, cultures were exposed to NMDA for 10 min in HEPES controlled salt solution (HCCS buffer) containing 120 mM NaCl, 5 mM KCl, 1.6 mM MgCl\(_2\), 2.3 mM CaCl\(_2\), 15 mM glucose, 20 mM HEPES, and 10 mM NaOH. For non-NMDA receptor-mediated excitotoxity or free radical injury, cultures were continuously exposed to AMPA, kainate, Fe\(^{3+}\), or BSO in minimal essential medium supplemented with 21 mM glucose. Neuronal death was analyzed 24 h later by measuring levels of LDH released into bathing medium and scaled to the mean LDH value released 24 h after continuous exposure to 500 μM NMDA (±100%) or a sham wash (±0%).

**Electrophoretic Mobility Shift Assay.** Cells were harvested, resuspended in a hypotonic buffer, incubated with 0.5% Nonidet P-40, and centrifuged at 13,000 rpm for 15 min (ko et al., 1998). Crude nuclear proteins were reacted with a double-stranded oligonucleotides (Genosys, The Wood-lands, TX) containing the NFB binding sequence from the murine B- immunoglobulin light-chain gene enhancer, 5′-GGGAGTTGAGGAGACTTCCCCAGG-3′ end-labeled with ^32P using a Klenow fragment in a buffer containing 8.5 mM EDTA, 8.5 mM EGTA, 8% glycerol, 50 μg/ml polyid-l-dc), 1 mM diithiothreitol, 0.3 mg/ml bovine serum albumin, and 6 mM MgCl\(_2\). The reaction mixture was resolved on a 6% nondenaturing polyacrylamide gel. The DNA binding activity of NF-κB was detected by exposing the gel to the X-ray film.

**Measurement of Prostaglandin E2 (PGE\(_2\)).** Activity of COXs was analyzed by measuring levels of PGE\(_2\) according to the manufacturer’s manual (Cayman Chemicals, Ann Arbor, MI). In brief, cortical cell cultures were pretreated with 30 μM arachidonic acid for 1 h and exposed to target drugs. Supernatants were collected immediately after drug treatment and used to determine the amount of accumulated PGE\(_2\) by enzyme-linked immunoassay.

**Measurement of Intracellular Reactive Oxygen Species ([ROS]) and [Ca\(^{2+}\)].** For analysis of [ROS], cortical cell cultures grown on a glass bottom dish were incubated in 2% Pluronic F-127 plus 10 μM 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, DCFH, or 5 μM dihydroethidium in HCSS buffer for 20 min at 37°C. For analysis of [Ca\(^{2+}\)], cortical cell cultures were incubated in 2% Pluronic F-127 plus 5 μM Fura-2 AM in HCSS buffer for 30 min at room temperature (Seo et al., 1999). The fluorescence signal of 6-carboxy-2′,7′-dichlorofluorescin diacetate (Ex = 490 nm; Em = 510: the oxidation product of DCFH by reactive oxygen species), ethidium (Ex = 518 nm; Em = 605 nm: the oxidation product of dihydroethidium by superoxide), and Fura-2 (Ex = 340/380 nm; Em = 510 nm) was acquired with a Nikon Diaphot inverted microscope equipped with a 100-W Xenon lamp and a Nikon 20×, 0.4 N.A. objective. The fluorescence signals were analyzed using a QuantiCell 700 system (Applied Imaging, Newcastle, England).

**Uptake of ^45Ca\(^{2+}\).** Uptake of ^45Ca\(^{2+}\) was performed as described before (Hartley et al., 1993). Cortical cell cultures were treated with drugs in HCSS buffer containing 1 μM/ml ^45Ca\(^{2+}\) for 10 min. Cultures were then washed three times with HCSS and dissolved in 400 μl of 0.2% SDS. Radioactivity of ^45Ca\(^{2+}\) was read in the scintillation counter (Beckman Coulter, Inc., Fullerton, CA). Electrode seal resistances were monitored from 2 to 3 MΩ when filled with the internal solution consisting of 135 mM CsCl, 10 mM HEPES, 1.2 mM MgCl\(_2\), 4 mM ATP-Na\(_2\), 0.5 mM CaCl\(_2\), and 11 mM EGTA (pH 7.3). The external solution contained 140 mM NaCl, 2 mM KCl, 2 mM CaCl\(_2\), 25 mM d-glucose, 10 mM HEPES, and 0.01 mM glycine (pH 7.4). Leak and capacitive currents were subtracted on-line from active responses using a P4 protocol (Bezanilla and Armstrong, 1977). Drugs were applied by a gravity-driven rapid perfusion system (Yellen, 1982).

**Transient Focal Cerebral Ischemia.** Male Sprague-Dawley rats (250–300 g) were anesthetized with chloral hydrate (i.p., 400 mg/kg). The rectal temperature was recorded and maintained at 37°C with a homeothermic blanket. The right middle cerebral artery was exposed, ligated, and both common carotid arteries then occluded using aneurysm clips (Tamura et al., 1981). The ischemic injury was terminated by removing the aneurysm clips. Immediately after occlusion of common carotid arteries, animals received sulfasalazine solved in phosphate-buffered saline at high doses (60 mg/kg).
mg/kg) for the first 2 h and low doses (240 mg/kg) for the next 22 h into femoral vein through infusion pump (Harvard Apparatus, Inc., South Natick, MA). To analyze infarct volume, rats were euthanized by chloral hydrate 24 h after reperfusion, sectioned coronally into six slices (2 mm thick) in a rodent brain matrix (Harvard Instruments, Inc.), and placed in 2% 2,3,5-triphenyltetrazolium chloride at 37°C for 30 min. The images of brain sections were captured by an image analyzing system (BioRad, Hercules, CA), and hemispheric infarct volume was determined by summation of infarct volumes measured in each brain slice using TINA 2.0 (KAIST, Daejeon, Korea).

**Retinal Ischemia.** Male rats (Sprague-Dawley, 180–200 g) were anesthetized with chloral hydrate. The intraocular pressure was increased to 160 to 180 mm Hg for 90 min by inserting a 30.5-gauge needle into the anterior chamber. Sulfasalazine or vehicle was injected into the eye through a Hamilton syringe inserted into the vitreous chamber 15 min before the ischemic injury. Rats were euthanized 24 h after reperfusion, sectioned coronally into six slices (2 mm thick) in a rodent brain matrix (Harvard Instruments, Inc.), and placed in 2% 2,3,5-triphenyltetrazolium chloride at 37°C for 30 min. The images of brain sections were captured by an image analyzing system (BioRad, Hercules, CA), and hemispheric infarct volume was determined by summation of infarct volumes measured in each brain slice using TINA 2.0 (KAIST, Daejeon, Korea).

**Results**

**Neuroprotective Effects of Sulfasalazine against NMDA Receptor-Mediated Excitotoxicity in Cortical Cell Cultures.** We first examined effects of sulfasalazine against excitotoxicity in primary cortical cell cultures. Cortical neurons exposed to 300 μM NMDA revealed marked swelling of cell body within 30 min. Administration of 1 mM sulfasalazine completely blocked the NMDA-induced morphological change (Fig. 1A). The neuroprotective effect of 300 μM to 1 mM sulfasalazine was not reversed by increasing doses of NMDA up to 1 mM, suggesting a noncompetitive mode of inhibition (Fig. 1B). A 10-min exposure to 300 μM NMDA produced approximately 80% neuronal death 24 h later that was completely blocked by concurrent treatment with 1 mM sulfasalazine as well as 3 μM MK-801 (Fig. 1C). The neuroprotective effects of sulfasalazine against 300 μM NMDA were apparent at concentrations as low as 300 μM, whereas aspirin prevented NMDA neurotoxicity at a dose of 10 mM (Fig. 1D). The neuroprotective concentrations of sulfasalazine were comparable to the amounts in serum after oral ingestion of 4- to 12-g dose in humans (Schroder and Campbell, 1972; Reynolds, 1996). We performed additional experiments to determine whether sulfasalazine would interfere with non-NMDA receptor-mediated excitotoxicity. Continuous exposure to 50 μM kainate or 20 μM AMPA produced 50 to 60% neuronal death 24 h later (Fig. 1E). Concurrent administration of sulfasalazine did not reduce kainate- or AMPA-induced neuronal death.

![Image](https://example.com/image.png)

**Fig. 1.** Sulfasalazine protects cortical neurons from NMDA neurotoxicity. A, phase contrast photomicrographs of cortical cell cultures 30 min after 10-min exposure to 300 μM NMDA, alone (a1) or with 1 mM sulfasalazine (a2). Bar denotes 30 μm. Arrows indicate the swollen neurons. B, cortical cell cultures were exposed to the indicated doses of NMDA for 10 min, alone (filled circles) or in the presence of 300 μM (filled triangles) or 1 mM (open circles) sulfasalazine. Neuronal death was analyzed 24 h later that was completely blocked by concurrent treatment with 1 mM sulfasalazine as well as 3 μM MK-801 (Fig. 1C). The neuroprotective effects of sulfasalazine against 300 μM NMDA were apparent at concentrations as low as 300 μM, whereas aspirin prevented NMDA neurotoxicity at a dose of 10 mM (Fig. 1D). The neuroprotective concentrations of sulfasalazine were comparable to the amounts in serum after oral ingestion of 4- to 12-g dose in humans (Schroder and Campbell, 1972; Reynolds, 1996). We performed additional experiments to determine whether sulfasalazine would interfere with non-NMDA receptor-mediated excitotoxicity. Continuous exposure to 50 μM kainate or 20 μM AMPA produced 50 to 60% neuronal death 24 h later (Fig. 1E). Concurrent administration of sulfasalazine did not reduce kainate- or AMPA-induced neuronal death.

![Image](https://example.com/image.png)
Neither NF-κB nor COXs Mediates the Neuroprotective Effect of Sulfasalazine. We examined the possibility that NF-κB would mediate the neuroprotective actions of sulfasalazine against NMDA. Nuclear extracts from sham-operated cortical cell cultures showed slight NF-κB DNA binding activity. Administration of NMDA increased the DNA binding activity of NF-κB within 10 min, as previously reported (Ko et al., 1998). Inclusion of 300 μM sulfasalazine or 1-pyridolinecarboxaldehyde (PDTC), a selective inhibitor of NF-κB, blocked NMDA-induced activation of NF-κB (Fig. 2A). In contrast to the protective effect of sulfasalazine, PDTC did not reduce NMDA-induced neuronal death (Fig. 2B). Thus, sulfasalazine appears to prevent NMDA neurotoxicity through NF-κB-independent mechanisms.

We next investigated COXs as a putative target for the neuroprotective effects of sulfasalazine against NMDA toxicity. As previously reported (Hewett et al., 2000), a brief exposure of cortical cell cultures to 300 μM NMDA increased activity of COXs within 2 h (Fig. 3A). Inclusion of 300 μM to 1 mM sulfasalazine or 100 μM NS398, a selective COX-2 inhibitor, prevented NMDA-induced activation of COXs. Nevertheless, none of COX inhibitors except sulfasalazine prevented NMDA-induced neuronal death (Fig. 3B). Thus, sulfasalazine appears to prevent NMDA neurotoxicity irrespective of its anti-inflammatory actions, inhibition of COX-2 as well as NF-κB.

The Antioxidant Property of Sulfasalazine Does Not Mediate the Neuroprotective Effect against NMDA. Sulfasalazine can act as a free radical scavenger (Aruoma et al., 1987), which can contribute to prevention of free radical production and neuronal death following activation of NMDA receptors. Cortical cell cultures exposed to free radical-inducing agents, Fe^{2+} or BSO, underwent widespread neuronal death that was sensitive to antioxidants such as vitamin E (Gwag et al., 1995; Ryu et al., 1999). Administration of 30 to 100 μM sulfasalazine prevented the oxidative neuronal death (Fig. 4A). Neither aspirin nor salicylate, however, prevented oxidative neuronal death following exposure to Fe^{2+} or BSO (data not shown), suggesting that the neuroprotective effects against oxidative stress are unique to sulfasalazine compared with other salicylates. Sulfasalazine reduced production of [ROS], possibly as a direct antioxidant (Fig. 4B).

As previously reported (Dugan et al., 1995; Sengpiel et al., 1998), treatment with 300 μM NMDA caused production of superoxide in cortical neurons as determined by oxidation of dihydroethidium to ethidium (Fig. 4, C–D). Addition of Trolox or sulfasalazine blocked NMDA-induced production of superoxide. Except sulfasalazine, neither Trolox nor other antioxidants (N-acetylcyestein, SKF38393, or MnTBAP, a cell-permeable superoxide dismutase mimetic) attenuated NMDA neurotoxicity (Fig. 4E). This implies that sulfasalazine should prevent NMDA neurotoxicity via a novel mech-
anism that differs from anti-inflammatory and antioxidant actions. In support of this, a combination of indomethacin, PDTC, and Trolox slightly reduced NMDA neurotoxicity (Fig. 4F). This means that the antagonistic actions of sulfasalazine against NMDA receptors are attributable primarily to blockade of NMDA neurotoxicity.

**Sulfasalazine Prevents Ca\(^{2+}\) Overload following Activation of NMDA Receptors.** Excess activation of NMDA receptors results in massive influx of Ca\(^{2+}\) that causes delayed neuronal death (Choi, 1987). We reasoned that the neuroprotective effect of sulfasalazine against NMDA would involve buffering intracellular Ca\(^{2+}\) overload. Neuronal...
[Ca$^{2+}$]), was elevated to a peak level within 20 s after exposure of cortical cell cultures to 300 μM NMDA (Fig. 5A). Inclusion of sulfasalazine prevented NMDA-induced elevation of [Ca$^{2+}$]. Sulfasalazine also prevented $^{48}$Ca$^{2+}$ uptake subsequent to activation of NMDA receptors (Fig. 5B). Taken together, the neuroprotective effect of sulfasalazine against NMDA likely stems from prevention of Ca$^{2+}$ influx and accumulation.

We performed whole-cell recordings to study whether sulfasalazine would reduce NMDA-induced inward currents. Bath application of 300 μM sulfasalazine immediately depressed NMDA-evoked currents in cortical neurons (Fig. 5C). NMDA currents were rapidly recovered after removal of sulfasalazine. Sulfasalazine inhibited NMDA currents in a dose-dependent manner. The IC$_{50}$ value of sulfasalazine for NMDA currents was 294 ± 1.21 μM, and the Hill coefficient was 1.19 (Fig. 5D). Sulfasalazine alone did not influence the holding current, and pretreatment with sulfasalazine did not block the inward current evolving after a subsequent application of NMDA (Fig. 5E). Thus, sulfasalazine appears to act as an open-channel blocker that blocks agonist-activated NMDA receptors. In support of this, a competition binding assay showed that sulfasalazine inhibited binding of $[^{3}H]N$-[1-(2-thienyl)cyclohexyl]piperidine, a selective ligand of phencyclidine binding sites, without significant effects on NMDA and glycine binding sites in rat cortical homogenates (for inhibition of N-[1-(2-thienyl)cyclohexyl]piperidine: IC$_{50}$ = 48 μM and $K_I$ = 32.5 μM). It is possible, however, that sulfasalazine may antagonize NMDA receptors through allosteric modulation. Application of NMDA elicited the outward current when cortical neurons were held at 50 mV. Administration of sulfasalazine prevented NMDA-induced outward current (Fig. 5F). Thus, sulfasalazine appears to block NMDA currents in a voltage-independent manner. The voltage-independence and open-channel blockade of NMDA receptors underlies the pharmacological action of MK-801, a typical open-channel blocker (Halliwell et al., 1989).

**Sulfasalazine Reduces Ischemic Damage in Vivo.** We examined the neuroprotective effect of sulfasalazine in animal models of hypoxic ischemia that would cause neuronal death primarily through excess activation of NMDA receptors (Choi and Rothman, 1990). Approximately 300 mm$^3$ of cerebral infarct was observed 24 h following occlusion of middle cerebral artery (MCAO) for 60 min. When sulfasalazine was continuously injected into the femoral vein at the beginning of occlusion, the infarct volume was significantly reduced (Fig. 6A). Sulfasalazine-treated animals did not show significant change in physiologic variables such as mean arterial blood pressure, Pa$_{O_2}$, Pa$_{CO_2}$, pH, and blood glucose during and after ischemia (Table 1). We finally studied the protective effect of sulfasalazine against hypoxic ischemic injury in retina. As previously reported (Joo et al., 1999), increasing intraocular pressure to 160 to 180 mm Hg for 90 min produced retinal ischemia and subsequent neuronal death in the ganglion cell layer and inner nuclear cell layer 24 h later (Fig. 6B–C). The neuronal death in the retina was significantly reduced by the vitreous injections of sulfasalazine 15 min before ischemic insults, which was comparable to the neuroprotective effects of the NMDA receptor antagonist MK-801 (Joo et al., 1999). Sulfasalazine was more potent than aspirin in reducing ischemic neuronal death in retina (Fig. 6D). Dose-response experiments and assays of inflammatory responses and oxidative stress, however, will be needed for quantitative and qualitative comparison of aspirin and sulfasalazine against ischemic injury.

**Discussion**

We have found that the anti-inflammatory drug sulfasalazine noncompetitively prevents NMDA receptor-mediated neuronal death at doses of 30 μM to 1 mM irrespective of blockade of NF-κB and COXs and antioxidative effect. The neuroprotective actions of sulfasalazine are mediated through blocking NMDA receptors. The novel neuroprotective actions of sulfasalazine are verified in animal models of focal cerebral ischemia and retinal ischemia.

As sulfasalazine contains the acetylsalicylate moiety that prevents NMDA neurotoxicity possibly through blockade of the inflammatory mediators NF-κB and COXs, it is conceivable to deduce that the anti-inflammatory action of sulfasalazine is essential for blocking NMDA neurotoxicity. The present study excluded this possibility, however. First, PDTC, the selective inhibitor of NF-κB, blocked NMDA-induced NF-κB activation but did not protect against NMDA neurotoxicity. Second, administering an inhibitor of COXs resulted in slight or no protection against NMDA neurotoxicity, although they completely blocked NMDA-induced activation of COXs (Hewett et al., 2000; Iadecola et al., 2001). In contrast to an inhibitor of COXs, sulfasalazine completely blocked NMDA neurotoxicity (Fig. 2). This suggests that blockade of NMDA neurotoxicity by sulfasalazine should involve other mechanisms besides COXs and NF-κB.

As previously reported (Dugan et al., 1995; Sengpiel et al., 1998), treatment with 300 μM NMDA caused production of superoxide in cortical neurons, as determined by oxidation of dihydroethidium to ethidium (Fig. 4, C–D). Activation of NMDA receptors results in massive influx and accumulation of ions and accumulation of ROS. We examined the antioxidant property of sulfasalazine as a possible mechanism underlying the neuroprotective effect against NMDA. We observed that sulfasalazine as well as antioxidants prevented pro-oxidant-induced neuronal death and NMDA-induced production of ROS in cortical cell cultures. Antioxidants did not, however, reduce NMDA-induced neuronal death, suggesting that the antioxidant property of sulfasalazine does not mediate the neuroprotective effect against NMDA. Moreover, a combination of indomethacin and PDTC as well as Trolox slightly reduced NMDA neurotoxicity that was completely blocked by sulfasalazine.

As sulfasalazine blocked downstream events such as activation of COXs and NF-κB, production of ROS, and swelling of neuronal cell body subsequent to activation of NMDA receptors, we reasoned that NMDA receptors could be the pharmacological target of sulfasalazine. In fact, sulfasalazine prevented NMDA-induced accumulation and influx of intracellular Ca$^{2+}$. Electrophysiological study demonstrates that sulfasalazine blocks the open state of NMDA receptors in a voltage-independent mode as open channel blockers such as MK-801 and 1-(1-phenylethylcyclohexyl)piperidine (phencyclidine) do. Nevertheless, the rapid dissociation from NMDA blockade of NF-κB and c-jun N-terminal kinase without re-
Fig. 5. Sulfasalazine blocks NMDA currents. A, cortical cell cultures were exposed to 300 μM NMDA, alone (filled circles) or with inclusion of 300 μM sulfasalazine (open circles) or 1 mM sulfasalazine (filled triangles). [Ca^{2+}]_i in cortical neurons was analyzed using Fura-2 at indicated points of time, mean ± S.E.M. (n = 55–94 randomly chosen neurons from four to six dishes/condition), scaled to mean neuronal [Ca^{2+}]_i after the sham control (100%). [Ca^{2+}]_i in cortical neurons treated with 300 μM to 1 mM sulfasalazine is significantly different from relevant control (NMDA alone) at p < 0.05 using ANOVA and Student-Neuman-Keuls test. B, cortical cell cultures were exposed to a sham wash or 300 μM NMDA, alone or in the presence of 30 μM to 1 mM sulfasalazine (SUL). Ca^{2+} influx was analyzed 10 min later by measuring influx of 45Ca^{2+} into cortical cells, mean ± S.E.M. (n = 12 cultures/each condition). * significant difference from relevant control (NMDA alone) at p < 0.05 using ANOVA and Student-Neuman-Keuls test. C–E, whole-cell currents were elicited from cortical neurons at a holding potential of −60 mV after exposure to 300 μM NMDA. In C, neurons were applied with 300 μM SUL for the indicated times and NMDA-induced peak currents were analyzed, mean ± S.E.M. (n = 7 neurons/each condition). * significant difference from NMDA currents at p < 0.003 using student’s t test (paired). In D, NMDA-induced peak currents were analyzed with inclusion of different doses of SUL, mean ± S.E.M. (n = 10 neurons/each condition). In E, 300 μM SUL was applied before administration of 300 μM NMDA. F, whole-cell currents were elicited from cortical neurons exposed to 300 μM NMDA, alone or with inclusion of 300 μM SUL for the indicated times, at a holding potential of +50 mV as well as −60 mV. The inhibitory effect of sulfasalazine was normalized to NMDA-induced currents (100%), mean ± S.E.M. (n = 5 neurons/each condition). No significant difference between the inhibitory effects of sulfasalazine at +50 mV and −60 mV using student’s t test (paired).
ducing NMDA-induced accumulation of intracellular Ca\(^{2+}\) (Grilli et al., 1996; Ko et al., 1998).

Transient and excess activation of NMDA receptors causes fulminant neuronal death and plays a primary role in neuronal death following hypoxic ischemic brain injury. Several NMDA receptor antagonists have been developed and applied to reduce neuronal death in patients with ischemic stroke (Muir and Lees, 1995). The therapeutic efficacy of NMDA receptor antagonists has not been verified in the clinical trials of ischemic patients (Davis et al., 1997; Lees, 1997), however. Systemic administration of NMDA receptor antagonists impairs normal brain function and can cause widespread neuronal damage in adult rat brain (Olney et al., 1989). The neuropsychopathological side effects are produced by high-affinity NMDA receptor antagonists and appear to be avoided with channel-blocking NMDA receptor antagonists with low-affinity and rapid-kinetic response (Rogawski, 2000). Sulfasalazine antagonizes NMDA receptors as a low-affinity and rapidly dissociating open channel blocker and can be applied to securely block NMDA receptor-mediated neuronal death following hypoxic ischemic injury. In support of this, administration of sulfasalazine into femoral vein or vitreous does not produce neuronal damage in normal rat brain and retina, whereas it significantly reduces degenera-
tion of cortical and retinal neurons following transient ischemic insults. The neuroprotective effects of sulfasalazine against retinal ischemia were more portent than aspirin.

The antithrombotic action of aspirin is widely used to treat acute ischemic stroke and to reduce the incidence of transient ischemic attack (Hennekens et al., 1988). In addition to the antithrombotic action and antioxidant, sulfasalazine can block NMDA receptors, the major routes of ischemic neuronal death, within the range of therapeutic doses to treat inflammatory bowel disease and rheumatoid arthritis. The antithrombotic and multiple neuroprotective actions of sulfasalazine hold a promise for the primary and secondary prevention of acute ischemic stroke.

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