Decrease of Proinflammatory Molecules Correlates With Neuroprotective Effect of the Fluorinated Salicylate Triflusal After Postnatal Excitotoxic Damage

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Background and Purpose—The fluorinated salicylate triflusal has been shown to have a neuroprotective effect after an excitotoxic lesion to the postnatal brain. In this regard, the aim of this study was to elucidate whether neuroprotection was associated with changes in the expression of proinflammatory molecules such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS), or cyclooxygenase-2 (COX-2), well-known mediators of oxidative stress and inflammation, mechanisms underlying secondary damage occurring after excitotoxic/ischemic brain injury.

Methods—Postnatal day 9 rats received an intracortical injection of N-methyl-d-aspartate followed by oral administration of triflusal (30 mg/kg) 8 hours later. Ten or 24 hours after lesion, animals were killed, and brain sections processed for the immunohistochemical demonstration of IL-1β, TNF-α, iNOS, and COX-2.

Results—Besides a reduction in the neurodegenerative area, triflusal strongly decreased iNOS immunolabeling at both survival times analyzed, attenuating iNOS immunoreactivity in astroglial cells and infiltrated neutrophils. Additionally, a moderate reduction in COX-2, IL-1β, and TNF-α was observed. Triflusal decreased neuronal and microglial COX-2 expression at 10 and 24 hours after lesion and microglial and astrogial expression of IL-1β and TNF-α at 24 hours after lesion. TNF-α expression in neuronal cells at 10 hours after lesion was, however, maintained.

Conclusions—This study suggests that triflusal neuroprotection is associated with a decrease of iNOS and other inflammatory mediators and therefore may constitute a good therapeutic agent in pathological situations in which regulation of inflammatory genes constitutes a relevant step in the outcome of the neurodegenerative event. (Stroke. 2002;33:2499-2505.)

Key Words: astrocytes ■ cyclooxygenase-2 ■ inflammation ■ interleukins ■ microglia ■ nitric oxide synthase ■ tumor necrosis factor

We have recently shown that treatment with the fluorinated salicylate triflusal (2-acetoxy-4-trifluoromethylbenzoic acid), an antiplatelet agent related to salicylates,1 induces a 49% reduction of lesion volume after postnatal excitotoxic damage.2 Neuroprotection by triflusal is accompanied by downregulation of nuclear factor-κB (NF-κB) activation and attenuation of the glial response,2 but the exact mechanisms whereby triflusal provides neuroprotection are not fully understood. It is well known that brain injury caused by ischemic/excitotoxic damage is exacerbated by the initiation of oxidative stress pathways and a local inflammatory response that may underlie extension of secondary tissue damage. Accordingly, several studies have suggested a key function of different inflammatory mediators in the progression of secondary neural damage, including the NF-κB–modulated proinflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) and the enzymes inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2).3–7 Proinflammatory cytokines such as IL-1β and TNF-α are low-molecular-weight proteins that play a crucial role in the initiation and propagation of the inflammatory response in the central nervous system7,8 and are upregulated after postnatal brain damage.9–12 Moreover, postinjury inflammation in the immature brain is also characterized by expression of iNOS and COX-2.13–16 The iNOS isoform is thought to be responsible for the prolonged production of relatively large amounts of nitric oxide (NO) that underlie its potential cytotoxic actions,17,18 and the inducible form of cyclooxygenase, COX-2, is the key enzyme in the synthesis of prostaglandins from arachidonic acid under pathological conditions, generating free radical species as a by-product of the reaction,4 being therefore implicated in the generation of free radicals, edema, and neuronal death.4

The aim of the present study was to evaluate whether the neuroprotective effect of triflusal after excitotoxic damage to
the immature rat brain was associated with changes in the expression of IL-1β, TNF-α, iNOS, and COX-2.

Materials and Methods

Excitotoxic Lesions

Experimental animal work with the use of Long-Evans black-hooded rats was conducted according to the ethical commission of the Autonomous University of Barcelona and Spanish and European Union directives. As previously described, 9,19,20 9-day-old pups were placed in a stereotaxic frame (Kopf Instruments) under ether anesthesia. The skull was opened with a surgical blade, and 37 nmol of N-methyl-D-aspartate (NMDA) (Sigma, M-3262) in 0.15 μL of saline solution (0.9% NaCl, pH 7.4) was injected into the right sensorimotor cortex with the use of a Hamilton microsyringe. Saline control animals received an injection of 0.15 μL of saline solution. After suture, pups were placed on a thermal pad and maintained at normothermia for 2 hours before being returned to their mothers.

Triflusal Treatment

Rat pups received an oral administration of triflusal, supplied by Uriach & Cia, with the use of a gastric probe. Triflusal was administered in a single dose of 30 mg/kg (3 mg/mL solution) 8 hours after NMDA or saline injection. Nine-day-old rat pups that did not receive triflusal were injected with NMDA or saline and used as controls. Four animals injected with NMDA, 6 animals injected with NMDA and posttreated with triflusal, 2 saline-injected animals, and 3 saline-injected animals posttreated with triflusal were used for each survival time.

Immunohistochemistry for iNOS, COX-2, IL-1β, and TNF-α

At 10 or 24 hours after NMDA or saline injection, rats were anesthetized by ether inhalation and perfused intracardially with 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4). Brains were postfixed for 4 hours and cryoprotected with 30% solution before being frozen with dry CO2. Coronal sections (30 μm thick) obtained in a Leitz cryostat were processed free floating.

After endogenous peroxidase blocking, sections were treated with blocking buffer (10% fetal calf serum in Tris-buffered saline [pH 7.4]) for 30 minutes and incubated overnight in the following primary antibodies: (1) rabbit anti-iNOS (AB5382, Chemicon) (1:6000); (2) mouse anti-COX-2 (C22420, Transduction Laboratories) (1:100); (3) goat anti-IL-1β (sc-1252, Santa Cruz Biotechnology) (1:200); or (4) goat anti–TNF-α (AB-410-NA, R&D Systems) (1:50). After they were washed, sections were incubated for 1 hour with biotinylated anti-rabbit antibody (RPN-1004, Amersham-Pharmacia) (1:200), biotinylated anti-mouse antibody (RPN-1001, Amersham-Pharmacia) (1:200), or biotinylated anti-goat antibody (305-065-003, Jackson Immunoresearch) (1:250). After they were rinsed, sections were incubated for 1 hour with avidin-peroxidase (P0364, Dakopatts) (1:400). Reaction product was visualized with Tris buffer containing 0.5 mg/mL 3′-diaminobenzidine and 0.33 μL/mL H2O2. For each immunomarker, an optimal developing duration was chosen, and sections were incubated during that selected amount of time. As controls, primary antibodies were omitted.

Quantitative Analysis of Immunocytochemical Staining

Density of immunoreactivity within the lesioned hemisphere was quantified in sections processed for iNOS, COX-2, IL-1β, and TNF-α as previously described. 9,19,30 Briefly, sections were digitized by a video camera mounted on a Leitz microscope and interfaced to a Macintosh computer. National Institutes of Health Image software was used. The quantification measure, referred to as the reactivity grade, was defined as the ratio between density values of an area of 0.25 mm2 in the neurodegenerating area versus the same area in the contralateral control hemisphere. A reactivity grade was obtained for each marker and for each animal (mean of 3 sections). The researcher was blinded to sample identity. Statistical analysis was performed with Statview 4.5 software with the use of ANOVA and Fisher’s protected least significant difference post hoc comparisons.

Double Immunohistochemistry Using Specific Cellular Markers

We used double-staining techniques for the simultaneous visualization of iNOS, COX-2, IL-1β, or TNF-α and microglial cells (by tomato lectin, histochemistry), astroglial cells (by glial fibrillary acidic protein [GFAP], immunohistochemistry), neuronal cells (by the demonstration of the anti-neuronal nuclei [NeuN]), and neutrophils (by the demonstration of myeloperoxidase [MPO]).

Sections were immunoreacted for proinflammatory molecules as reported above but using Cy3-conjugated anti-rabbit antibody (PA-43004, Amersham-Pharma) (1:1000) or Cy3-conjugated antimouse antibody (PA-43004, Amersham-Pharma) (1:1000). Sections were then incubated in rabbit anti-GFAP (Z-0334, Dakopatts) (1:1000), mouse anti-NeuN (MAB377, Chemicon) (1:1000), or rabbit anti-MPO (A0398, Dakopatts) (1:400). Finally, Cy2-conjugated anti-rabbit antibody (PA-42004, Amersham-Pharma) (1:1000) or Cy2-conjugated anti-mouse antibody (PA-42002, Amersham-Pharma) (1:1000) was used to visualize labeling.

Sections for double staining with tomato lectin were incubated with the biotinylated lectin from Lycopersicon esculentum (Sigma, L-9389) (6 μg/mL) and Cy2-conjugated streptavidin (PA-42000, DAKO)).

Figure 1. Expression of iNOS at 10 hours after NMDA (A, C) and NMDA + triflusal (NMDA + T) (B, D). Immunolabeling for iNOS is observed throughout the degenerating area (A) and is strongly reduced after triflusal treatment (B). After the NMDA lesion, iNOS is mainly found in ramified cells (C) within the parenchyma and in association with blood vessels. After triflusal treatment, iNOS-positive cells are scarce (D) and mainly observed in a perivascular location. The quantification graph presents iNOS immunoreactivity in cortical areas after NMDA injection or NMDA + triflusal. Data are mean ± SEM values of reactivity grades in comparison with NMDA-injected littermates of each survival time (P < 0.0001). Strong decrease of iNOS immunolabeling is observed in triflusal-treated animals at 10 and 24 hours after lesion, showing a stronger reduction at 10 hours. Bar = 200 μm in A and B; bar = 50 μm in C and D.
Results

Injection of saline solution into the cortex caused no significant neuronal degeneration and a very local tissue damage around the needle track, where a few microglial and astroglial cells were noted.\textsuperscript{19,20} The NMDA injection into the right sensorimotor neocortex of 9-day-old rats caused a lesion involving neuronal loss and glial response across the entire thickness of the cortex at the level of the injection site.\textsuperscript{19,20} As has been previously described in detail,\textsuperscript{12,16} neuronal degeneration and the glial response were accompanied by expression of the proinflammatory cytokines IL-1\textsuperscript{\beta} and TNF-\alpha and the enzymes iNOS and COX-2.

Triflusal treatment 8 hours after lesion caused an important decrease of the microglial and astroglial response and a 49\% reduction in the lesion size.\textsuperscript{2} The expression of iNOS, COX-2, IL-1\textsuperscript{\beta}, and TNF-\alpha was restricted to the area of neurodegeneration, and therefore the area of expression of these inflammatory factors was strongly reduced in triflusal-treated animals. Moreover, the density of immunolabeling measured within the neurodegenerating area (representing the intensity of labeling as well as the number of positive cells) and referred to as the reactivity grade in Figures 1, 3, 4, and 5 also showed a general reduction, as described in detail for each marker.

iNOS Expression

In saline-injected animals that were not administered triflusal, faint iNOS labeling was observed in specific neuronal populations and in scattered cortical blood vessels in some animals. In addition, surrounding the needle track of saline-injected animals, some iNOS-positive blood infiltrates were observed. No differences were noticed when saline-injected animals were treated with triflusal. In NMDA-injected animals, iNOS immunoreactivity achieved maximal levels of expression at 10 hours after lesion, although high levels of immunoreactivity were also seen at 24 hours (Figure 1). Labeling for iNOS was observed throughout the neurodegenerating area (Figure 1A) in highly ramified hairy astrocytes (Figures 1C and 2A) related to blood vessels and in neutrophils (Figure 2B) located in the parenchyma but often observed in the vicinity of blood vessels. Moreover, scattered iNOS-positive neuronal cells were found in the lesion borders.

After triflusal treatment, iNOS immunoreactivity within the neurodegenerating area was decreased at 10 and 24 hours after lesion, achieving maximal reduction at the first survival time (Figure 1). Triflusal-treated animal showed a reduction of iNOS immunoreactivity within the nervous parenchyma, displaying iNOS labeling exclusively in perivascular locations (Figure 1B). Both a decrease in the number of iNOS-positive highly ramified astrocytes and scattered neutrophils in close apposition with blood vessels could be seen (Figure 1D).

COX-2 Expression

In saline-injected animals, no immunoreactivity for COX-2 was observed, except for the needle track, where COX-2–positive neuronal cells and blood vessels were observed. No differences were noted in saline-injected animals treated with triflusal. In NMDA-injected animals, COX-2 expression was observed throughout the neurodegenerating area and in the adjacent corpus callosum at 10 and 24 hours after lesion, showing comparable levels of immunoreactivity between these times (Figure 3). COX-2 expression was evident in microglia/macrophages located in the deep cortical layers and the adjacent corpus callosum (Figures 2D and 3C) as well as in cortical neuronal cells (Figures 2C and 3A) located within the neurodegenerating area.
After triflusal treatment, COX-2 immunoreactivity within the neurodegenerating area was decreased at 10 and 24 hours after lesion, achieving maximal reduction at the first survival time (Figure 3). Furthermore, triflusal-treated animals showed reduced COX-2 labeling in microglial cells, displaying a decrease in staining as well as a lower number of labeled cells (Figure 3D). In addition, neuronal COX-2 immunoreactivity was also clearly reduced, both in neuronal cells located within the lesion site at 10 hours after lesion (Figure 3B) and bordering the degenerative area at 24 hours.

**IL-1β Expression**

In saline-injected animals, homogeneous IL-1β labeling was observed in association with endothelial cells and perivascular cells of some blood vessels. In addition, surrounding the needle track of saline-injected animals, blood infiltrates and scattered primary ramified microglial cells expressing IL-1β were observed. No differences were noticed in saline-injected animals treated with triflusal. In NMDA-injected animals, a rapid increase in IL-1β within the lesioned cortex was also induced, reaching maximal levels of expression at 10 hours after lesion, which were still maintained at 24 hours after lesion (Figure 4). IL-1β-labeled cells were mostly pseudopodic and primary ramified microglial cells in the degenerating area and the adjacent corpus callosum at 10 hours after lesion (Figure 4A). At 24 hours after lesion, most IL-1β-labeled cells were reactive pseudopodic/amoeboid microglial cells or astrocytes (Figures 2E, 2F, and 4C).

After triflusal treatment, immunolabeling for IL-1β was not quantitatively decreased at 10 hours after lesion (Figure 4B), but it was reduced at 24 hours after lesion (Figure 4), when an important decrease in IL-1β-positive microglial and astrogial cells concentrating in deep cortical layers and adjacent corpus callosum (Figure 4D) could be observed.

**TNF-α Expression**

Constitutive TNF-α neuronal labeling was seen throughout the brain, and mildly TNF-α–stained astrocytes were located in the corpus callosum. No differences were noted in saline-injected animals treated with triflusal. In NMDA-injected animals, TNF-α immunoreactivity in the lesioned cortex was increased at 10 hours after lesion and was maintained at 24 hours after lesion (Figure 5). TNF-α immunoreactivity was mostly observed in neuronal cells at 10 hours after lesion (Figures 2G and 5A), mainly within the degenerating area and in its immediate vicinity. At 24 hours, when massive neuronal death occurred, TNF-α–positive neuronal cells could only be observed in the lesion border (Figure 5C), and most TNF-α–positive cells were astrocytes (Figures 2H and 5E).
After triflusal treatment, immunoreactivity for TNF-α was significantly decreased at 24 hours after lesion (Figure 5). Changes in TNF-α immunoreactivity density within the neurodegenerating area were not observed in triflusal-treated animals analyzed 10 hours after lesion (Figure 5); similar to untreated littermates, these animals mainly showed TNF-α immunoreactivity in neuronal cells (Figure 5B). However, at 24 hours after lesion, a decrease in TNF-α immunoreactivity was evident both in neuronal cells located in the lesion border (Figure 5D) and in astroglial cells (Figure 5F), diminishing the number of labeled cells.

**Discussion**

In this study we have demonstrated that the neuroprotective effects of triflusal treatment after excitotoxic damage are accompanied by a strong reduction in iNOS expression and a moderate decrease in other inflammatory mediators such as COX-2 and the cytokines IL-1β and TNF-α. However, whether the decrease of these inflammatory factors is a direct effect of triflusal administration or is indirectly caused by a reduction in cell death and glial response remains to be clarified.

**Strong Reduction in iNOS Expression**

After NMDA-induced excitotoxicity in the postnatal brain, iNOS was mainly observed in astrocytes and neutrophils during the first 24 hours after injury. Triflusal treatment caused an important reduction in iNOS immunolabeling, showing a reduction in the number of iNOS-positive cells within the degenerating area. This is in agreement with a previous in vitro study showing a decrease in iNOS-dependent nitrite production by triflusal. This reduction was mediated by the inhibition of NF-κB activation, an important modulator of iNOS gene expression in astrocytes, which is also diminished after triflusal treatment.

The potent iNOS reduction by triflusal may imply that the neuroprotective mechanism of triflusal is dependent on iNOS expression, more than in the other inflammatory products analyzed. In this sense, both after central nervous system injury and in other inflammatory states, iNOS-derived NO, produced in high amounts and for prolonged periods of time, is associated with NO cytotoxicity. Although its exact mechanism of toxicity is not fully elucidated, it is now understood that iNOS-derived NO is one of the major sources of toxic free radicals in the brain, since its reaction with superoxide anion leads to the formation of peroxynitrite, an extremely potent oxidizing agent that is thought to play a key role in secondary tissue damage after brain injury. Specifically, astrocytic iNOS-derived NO potentiates NMDA excitotoxicity and could explain the neuroprotective effect of triflusal involving reduced iNOS expression. Moreover, neutrophil iNOS-derived NO has also been reported to play an important role in the production of NO-derived oxidants. In agreement, several studies have demonstrated the role of iNOS in neural damage exacerbation by using transgenic mice or pharmacological agents: aminoguanidine, a selective iNOS inhibitor, reduces NMDA-induced cell death and decreases posts ischemic iNOS activity and infarct size. Similarly, iNOS-deficient mice have smaller infarcts than wild-type mice, suggesting that iNOS expression may be an important factor contributing to the expansion of secondary brain damage after ischemic/excitotoxic lesions.

**Moderate Decrease of COX-2 and Cytokine Expression**

Administration of triflusal after the excitotoxic lesion also caused a reduced expression of COX-2 expression both in neurons and in microglia/macrophages. These results are not
surprising because triflusal is a pharmacological agent related to salicylates with the ability to inhibit, at therapeutic concentrations, COX-2 activity in blood cells and the activation of newly synthesized COX-2 protein by blocking NF-kB activation.\textsuperscript{33} However, the reduction of COX-2 expression in neuronal cells at 10 hours after lesion was not accompanied by a reduction in neuronal NF-kB,\textsuperscript{2} suggesting that, within the first hours after administration, triflusal could modulate COX-2 in the brain by a direct mechanism. In regard to the importance of COX-2 decrease in the neuroprotective effects of triflusal, several studies have correlated reduced COX-2 expression and neuroprotection after ischemic or excitotoxic damage, using several COX-2-specific inhibitory drugs\textsuperscript{34,35} as well as COX-2 transgenic animals.\textsuperscript{36,37} Although the mechanism by which COX contributes to neuronal vulnerability is unknown, a number of studies have implicated COX-2 activity in excitotoxic-dependent neuronal death and inflammatory processes.\textsuperscript{38,39} These effects are mainly induced by oxidative stress originated by the COX-dependent production of superoxide anion, including their reaction with NO to generate highly reactive peroxynitrite species.\textsuperscript{77} In addition, COX-2 enzyme activity in microglia has been involved in the production of inflammatory prostanoids, implicated in the generation of free radicals, edema, and neuronal cell death.\textsuperscript{4,5}

Administration of triflusal also diminished expression of IL-1β and TNF-α. After an excitotoxic lesion to the postnatal brain, the expression of these proinflammatory cytokines is induced in neurons and glial cells within 24 hours after lesion,\textsuperscript{9–12} when they are thought to play an important role in triggering the inflammatory response, mainly through activation of transcription factor NF-kB and its target genes in neurons and endothelial and glial cells.\textsuperscript{40}

In this regard, neuronal expression of TNF-α observed at 10 hours after lesion correlates with the activation of NF-kB in neuronal cells located in the degenerating area. Neither the neuronal TNF-α or NF-kB immunolabeling was reduced by triflusal (see Results),\textsuperscript{2} in agreement with the putative protective role of neuronal TNF-α. Recent studies have suggested that at lower doses neuronal TNF-α may have neuroprotective actions, preventing excitotoxic cell death.\textsuperscript{41} This TNF-α–mediated protection may be attributed to the increase in several NF-kB–regulated genes such as manganese superoxide dismutase (providing antioxidant protection), calbindin protein (which controls intracellular calcium levels), and the antiapoptotic gene bcl-2.\textsuperscript{4,5}

In contrast, triflusal diminishes expression of TNF-α and IL-1β in glial cells at 24 hours after lesion, correlating with the blockade of glial NF-kB activation and an attenuation of the astroglial and microglial response.\textsuperscript{2} This is not surprising because glial expressions of IL-1β and TNF-α have been attributed to the potentiation of the inflammatory response, playing an important role in triggering glial cell proliferation and scar formation and regulating the expression of several inflammation-related genes, including TNF-α and IL-1β, major histocompatibility complex, cell adhesion molecule expression, and chemokine production; promoting leukocyte extravasation; and potentiating the inflammatory reaction and oxidative stress by raising the production of NO and free radicals.\textsuperscript{7,8,42} Moreover, a direct implication of IL-1β expression in exacerbating neuronal damage comes from reports correlating neuroprotection with the inhibition of IL-1β function, either by blocking the activity of IL-1β activating IL-β–converting enzyme or by blocking IL-1 receptors with the endogenous antagonist IL-1ra.\textsuperscript{4,5}

In conclusion, triflusal treatment after an excitotoxic lesion in the postnatal brain induces a strong reduction of iNOS expression and a moderate decrease in COX-2 and the proinflammatory cytokines IL-1β and TNF-α, suggesting that triflusal may constitute a good therapeutic agent in pathological situations in which regulation of inflammatory gene expression is a relevant step in the outcome of the neurodegenerative event.

Acknowledgments

This work was supported by Dirección General de Enseñanza Superior project PB98-0892, Uriach & Cia, and “la Caixa” project 000074-00. We would like to thank Miguel A. Martí for technical help.

References

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Stroke. 2002;33:2499-2505
doi: 10.1161/01.STR.0000028184.80776.58
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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