Effects of Triflusal and Aspirin in a Rat Model of Cerebral Ischemia

Shawn N. Whitehead, PhD; Nestor A. Bayona, MSc; Guanliang Cheng, MD; Gary V. Allen, PhD; Vladimir C. Hachinski, MD; David F. Cechetto, PhD

Background and Purpose—Neuroinflammation plays a critical role in the pathogenesis of cerebral ischemia. Triflusal, a selective cyclooxygenase-2, and its active metabolite 3-hydroxy-4-trifluoromethylbenzoic acid may inhibit apoptosis and inflammation after cerebral ischemia. An in vivo model of cerebral ischemia was used to investigate the effects of triflusal and aspirin treatment on infarct volume, and inflammation after cerebral ischemia in the rat.

Methods—Male Wistar rats were subjected to a permanent right-sided middle cerebral artery occlusion. Rats received oral administration of either triflusal or aspirin. After 3 days after surgery, immunostaining was used to detect neuroinflammatory cells and molecules, and infarct volumes were measured.

Results—Both triflusal and aspirin at a dose of 30 mg/kg but not 10 mg/kg significantly reduced infarct volume compared with vehicle treatment. Middle cerebral artery occlusion resulted in increased astrocyte and heat shock protein-27 (Hsp27) immunostaining in the ipsilateral cortex. Triflusal (30 mg/kg) or aspirin treatment (30 mg/kg) did not reduce the levels of GFAP or Hsp27 immunostaining. Triflusal (30 mg/kg) also significantly decreased the protein levels of IL-1β but not nuclear factor kappa B or tumor necrosis factor-α in the cortex ipsilateral to the middle cerebral artery occlusion.

Conclusions—The results suggest that triflusal and aspirin appear to be equally neuroprotective against middle cerebral artery occlusion-induced cerebral ischemia. Therefore, strong rationale exists to continue the neuroprotective examination of triflusal in brain injury. (Stroke. 2007;38:381-387.)

Key Words: astrocytes ■ cerebral ischemia ■ cytokines ■ inflammation ■ reactive microglia

Several studies have reported on the prophylactic use of aspirin in the secondary prevention of stroke. In vivo studies have also indicated the neuroprotective role of aspirin in models of stroke. In one such study, aspirin at 15 or 30 mg/kg given up to 2 hours before ischemia significantly reduced infarct volume in a rat model of focal cortical ischemia. In addition, posthypoxic recovery improved significantly with either in vivo or in vitro aspirin pretreatment in hippocampal slices. Aspirin and its metabolite sodium salicylate were also found to be protective against glutamate neurotoxicity in rat primary neuronal cultures and hippocampal slices. These results suggested that the neuroprotection induced by aspirin was attributable to specific inhibition of glutamate-mediated induction of nuclear factor kappa B (NFκB). Although there appears to be a strong rationale for examining the potential effectiveness of aspirin after cerebral ischemia, few studies have done so.

Triflusal (2-acetoxyl-4-trifluoromethylbenzoic acid), a 4 fluoromethyl derivative of salicylate acid, and its active metabolite 3-hydroxy-4-trifluoromethylbenzoic acid are direct inhibitors of cyclooxygenase-2 (COX-2) and indirect inhibitors of NFκB, whereas aspirin is a COX-1/COX-2 inhibitor. NFκB plays a critical role in the production of multiple genes that are involved in inflammatory and infectious responses of the body. NFκB production leads to the upregulation of tumor necrosis factor-α (TNF-α) and IL-1β, both pro-inflammatory cytokines that contribute to apoptosis in neural cells. Also, triflusal has been purposed to have anti-apoptotic properties attributable to its ability to better inhibit cAMP phosphodiesterase, leading to a reduction in the mobilization of Ca⁺⁺ ions.

Both triflusal and aspirin have properties that may help protect the brain against neuroinflammatory and apoptotic mechanisms in cerebral ischemia; however, triflusal may have additional neuroprotective potential attributable to its direct action on NFκB. In the present investigation, the rat middle cerebral artery occlusion (MCAO) model of cerebral ischemia was used to investigate the neuroprotective effects

© 2007 American Heart Association, Inc.

Stroke is available at http://www.strokeaha.org

DOI: 10.1161/01.STR.0000254464.05561.72

381
of aspirin and triflusal. Direct measures of infarct volume, as well as the levels of inflammatory markers (NFkB, TNF-α, IL-1β) activated microglia and astrocytes, and stress molecule heat shock protein-27 (Hsp27) were measured using immunohistochemical methods 3 days after a right-sided MCAO.

Materials and Methods

Surgical Procedures

All experimental procedures were performed according to the animal care guidelines of the University of Western Ontario. Male Wistar rats were randomly divided into treatment groups. Before and after surgery rats were housed in single cages (12/12 hour light/dark cycle) and were fed ad libitum. Before surgery, during the light cycle, all rats were anesthetized with a single dose of sodium pentobarbital (40 mg/kg intraperitoneally) and placed in a David Kopf stereotaxic apparatus. Rectal temperature was monitored and maintained constant at 37°C by a heating pad while the rats were under anesthesia. The right MCA was exposed as previously described. The MCA was permanently occluded at 2 points, 1 above and 1 below the inferior cerebral vein. Immediately after surgery, rats were administered 0, 10, or 30 mg/kg triflusal, aspirin or vehicle by oral gavage (n = 5 for each treatment group). Each group received the same drug treatment for 2 additional days. Details on the pharmacokinetics of triflusal can be found in Ramis et al. Benzathine penicillin G was supplied as prophylactic antibiotic. After wound suture, all rats received 40 mg/kg of buprenorphine intramuscularly and were allowed to recover from surgery for 3 days.

Brain Temperature Measurements

A brain temperature probe holder consisting of a short segment of polyethylene tubing (internal diameter 8 mm) was cemented to the rat’s skull over the occipital cortex contralateral to MCAO using dental acrylic. A brain temperature probe (XM-FH-BP; MiniMitter Co) was inserted into this holder with the tip of the probe 6 mm into the occipital cortex and brain temperature sampling was initiated. The animals were then subjected to the MCAO procedure as described, followed by triflusal (30 mg/kg) or vehicle treatment. Brain temperature sampling continued for an additional 3 hours after the conclusion of the MCAO procedure in the unrestrained rats using a telemetry system (Data Sciences). Brain temperature was also monitored for 1 hour at 24, 48, and 72 hours after MCAO. After recovery, all rats had complete mobility within their cage. Brain temperature values were analyzed using the Data Sciences, Datasquest LabPRO software package.

Tissue Processing

Three days after surgery, all animals were euthanized via pentobarbital overdose and perfused transaortically first with saline followed by 4% formaldehyde (pH 7.4). The brains were removed and cryoprotected in 30% sucrose for 36 hours at 4°C. Coronal sections (30 μm) were cut using a cryostat.

Infarct Volume Assessment

The sections were then examined under a light microscope (Leitz Diaplan; Leica Canada, Willowdale Ontario, Canada), and the areas of infarcted tissue were measured (SigmaScan Pro 5.0; SPSS Inc). In addition, the hemispheric areas of each tissue section were measured to account for any brain swelling that might have occurred after cerebral ischemia. Infarct size for each section was calculated as the ratio of the contralateral to ipsilateral hemisphere multiplied by the area of the infarct. The volume of the infarct was calculated in mm³ by integrating the infarct sizes for each of the tissue sections that contained infarcted tissue.

Immunohistochemistry

Free-floating brain sections were initially treated with 0.03% peroxidase to inhibit endogenous peroxidase activity. Brain sections to be used for Hsp27 immunostaining were blocked with 3 mL of rabbit serum; brain sections to be used for glial fibrillary acidic protein (GFAP) or OX-6 (MHC class II antigen) were blocked with 3 mL of horse serum. All sections were treated with 3 mL of the following primary antibodies diluted in phosphate-buffered saline at 4°C for 24 hours: Hsp27 (1:6000; StressGen Biotechnologies Corp), GFAP (1:1000; Chemicon), or OX-6 (1:1000; Sigma). Sections were washed in phosphate-buffered saline and incubated with the following biotinylated (Hsp27 and GFAP) or fluorescent Cy-3 tagged (OX-6) secondary antibodies diluted in phosphate-buffered saline: Hsp27 (anti-rabbit IgG 1:500; Vector Laboratory; Burlington, Ontario, Canada), GFAP (anti-mouse IgG 1:500; Vector Laboratory), or OX-6 (anti-mouse IgG, 1:500; Sigma; Oakville, Ontario, Canada). Hsp-27 and GFAP immunostained sections were then washed in phosphate-buffered saline, and then incubated in 3 mL of 0.05% diaminobenzidine and 1% H₂O₂ for 15 minutes. Sections were then washed, air-dried, cleared in xylene, and coverslipped using DePeX mounting medium. Brains to be compared were processed at the same time using the same solutions to reduce variability in immunostaining caused by separate processing.

Densitometry of GFAP and HSP27

All brain sections were studied using identical illumination parameters and images were digitized using a SPOT Cooled Color Digital camera and compiled and organized using Adobe Photoshop software (v.7.0). The images were exported to Scion-Image software and a standardized procedure was used to distinguish background staining from immunolabeled structures in matched sections of the cerebral cortex that were processed and reacted in the same incubation solutions. The mean density of Hsp27 immunolabeled cells in the ipsilateral cerebral cortex (bregma −2.3 to −3.0; 5 sections per case) was determined using standard thresholding. The mean density of GFAP-immunolabeled cells in the cerebral cortex (2×2 mm² box at the level of the rhinal fissure, bregma −2.3 to −3.0) was determined using standard thresholding.

Western Blotting

Frozen coronal sections were cut 2.7 mm anterior to bregma. Bilateral micropunches were taken from the ipsilateral and contralateral cortices at the level of the infarct from each section. Tissue was homogenized in RIPA lysis buffer (150 nm Tris-HCL, 150 nm NaCl, 1% deoxycholate, 0.1% SDS, 2 nm EDTA) with protease inhibitors (Sigma). Protein extracts were quantified by the Pierce bicinchoninic acid protein assay (Pierce, Rockford, IL). Equal amounts of protein were separated by electrophoresis on 10% SDS-polyacrylamide gels, transferred to a nitrocellulose membrane (Bio-Rad Laboratories), and stained Ponceau red to verify equal loading. Immunoblotting for NFkB (1:50), TNF-α (1:50), and IL-1β (1:50) was performed using SuperSignal-enhanced chemiluminescent substrate (Pierce) and developed on hyperfilm (Amersham Biosciences Inc).

Data Analysis

Results are expressed as mean±SEM of each measure. The volumes of the infarcts and the density of immunolabeling for GFAP and Hsp27 for each of the groups were subjected to an analysis of variance (ANOVA) and the Dunnett’s multiple comparison test to determine differences between treatments (P<0.05).

Results

Infarct Volume

The unilateral occlusion of the right MCAO resulted in circumscribed infarcts restricted to the right cortex and striatum. No injury was observed in the contralateral hemisphere. The infarct volume in rats receiving vehicle treatment immediately after MCAO was 22.3±2.5 mm³. The infarct volume in the animals treated with triflusal (30 mg/kg) or aspirin (30 mg/kg) after MCAO were 12.5±3.2 and 11.7±2.9 mm³, respectively. Infarct volume in both the triflusal-treated (30 mg/kg) and aspirin-treated (30 mg/kg)
animals were significantly decreased compared with vehicle-treated animals (Figure 1A). When rats were treated with a lower dose (10 mg/kg) of triflusal or aspirin the resulting infarct volumes (19.7±3.3 and 24.8±6.2 mm³, respectively) were not significantly different from vehicle controls.

Brain Temperature
Brain temperature recordings were made in the occipital cortex contralateral to the site of the MCAO immediately after surgery. Brain temperature measurements were made for the next 3 days as well. There were no significant changes in brain temperature recordings between any of the groups immediately after MCAO or sham surgery or in the next 3 days of survival (Figure 1B).

Immunostaining of Activated Microglia
Fluorescence microscopy revealed an increase in activated microglia (OX-6) immunostaining in the peri-infarct region compared with the rest of the brain for all rats 3 days after MCAO. Positive OX-6 staining appeared scarce and random in the remainder of the infarcted hemisphere and in the whole of the contralateral hemisphere (not shown). There was a reduction in the amount of OX-6 immunostaining in the animals treated with triflusal (30 mg/kg) compared with the control vehicle treatment (Figure 2). The levels OX-6 staining in the peri-infarct region decreased as the concentration of triflusal treatment increased (not shown). The positive OX-6 staining that appeared in the rest of the brain seemed to be unaffected by the dosage of triflusal (not shown).

Immunoblotting
Triflusal (30 mg/kg) treatment significantly reduced IL-1β protein expression to (55.3±2.2% of vehicle) in the ipsilateral cortex (Figure 3A, 3B). No such decrease was observed in the contralateral hemisphere. We also examined the effects of triflusal treatment on NFκB and TNF-α protein levels.
There were no significant differences in either NFκB or TNF-α protein levels between triflusal-treated (30 mg/kg) and vehicle animals.

**Hsp27 and GFAP Immunostaining**

After unilateral occlusion of middle cerebral artery, there was a dense induction of Hsp27 in the ipsilateral cortex, especially in the ventral cerebral cortex at the level of the insular cortex (Figure 4A). After treatment with aspirin (30 mg/kg) there was a moderate attenuation of the Hsp27 expression (Figure 4B). Triflusal treatment (30 mg/kg) after MCAO almost completely attenuated the Hsp27 induction in the insular cortex and adjacent ventral cortical region (Figure 4C). Quantitative results using optical densitometry of Hsp27 (Figure 4D) and GFAP (Figure 4E) showed that rats subjected to a MCAO regardless of subsequent treatment (ie, vehicle, aspirin, or triflusal) significantly increased the levels of Hsp27 and GFAP compared with sham treatment. How-
ever, there were no significant differences in the expression of either Hsp27 or GFAP between vehicle, aspirin, or triflusal groups.

Discussion

Infarct Volume
The results indicate that both triflusal and aspirin at a dose of 30 mg/kg when administered concurrent with ischemia significantly decrease the size of the infarct compared with vehicle controls when assessed 3 days after MCAO. The decrease in infarct volume is indicative of the neuroprotective effects of triflusal and aspirin in stroke. Several studies have demonstrated that aspirin can reduce infarct volume when administered 2 hours before MCAO.1,2,11 Here we extend these findings by demonstrating that aspirin (30 mg/kg) can reduce infarct volume when administered immediately after a MCAO. Another study reported that triflusal decreased the number of microthrombi after MCAO compared with untreated animals,12 supporting its proposed anti-aggregatory worth in stroke. However, this study did not directly assess infarct volume. Our findings demonstrate that triflusal (30 mg/kg) treatment immediately after a MCAO can directly decrease the size of the resulting infarct 3 days later compared with vehicle-treated animals.

Brain Temperature
Ischemia can evoke an increase in temperature, and even a modest change in temperature can significantly alter the outcome of the infarct.13–15 Therefore, brain temperature was monitored during ischemia to ensure that drug treatments did not influence brain temperature and thus the infarct volume. Although it is important to note that brain temperatures were recorded for only 1 hour per day, our results showed that there was no significant difference in brain temperature between any of the treatment groups, at the time of the MCAO or in the 3-day survival period. Thus the neuroprotection observed in the triflusal groups is not related to changes in brain temperature. Although triflusal had no effect on brain temperature, it is possible that aspirin did. Therefore, future studies need to examine the time course of the effects of aspirin treatment on brain temperature after MCAO.

Inflammatory Response
We have shown that there is an increase in positive staining for the activated microglia marker (OX-6) in the peri-infarct area 3 days after MCAO. Microglia actively participate in the progression of ischemic injury. Our data show a reduction in the amount of OX-6 immunohistochemical staining in the region of the penumbra in the animals treated with triflusal (30 mg/kg) compared with vehicle-treated controls, suggest-
ing that triflusal is able to decrease the activation of microglia and thus inflammation after MCAO. The concomitant decrease in infarct volume in the triflusal group suggests that a decrease in the amount of inflammation might play a significant role in reducing the resulting insult on the brain after stroke. It is also important to note that the level of inflammation changes over time after injury.

Moreover, the possibility exists for a differential effect of the drug given on recovery of cerebral blood flow from ischemia after permanent occlusion. Therefore, future studies need to examine the time course of the potential neuroprotective effects of triflusal along with a time line for the recovery of cerebral blood flow after MCAO in rats.

In an attempt to elucidate the neuroprotective mechanisms of triflusal, we examined the expression of NFκB, IL-1β, and TNF-α protein levels using Western blot analysis. Immunoblotting showed no significant changes in the expression of NFκB and TNF-α between treatment groups. However, immunoblotting revealed that ipsilateral IL-1β protein expression was significantly downregulated in the triflusal-treated group (30 mg/kg) compared with vehicle treatment 3 days after MCAO (Figure 3). The data suggest that at this dose, a single treatment with triflusal following MCAO does not directly alter the expression of NFκB. Further analysis needs to be performed on the effects of long-term triflusal treatment on the activation of NFκB. However, a reduction in IL-1β suggests that triflusal may decrease the expression of downstream pro-inflammatory cytokine products of the NFκB pathway, and thereby indirectly decrease the spread of inflammation and the resultant ischemic injury after stroke. Several reports have indicated that IL-1β is markedly increased during global and focal cerebral ischemia and during postischemic reperfusion.16–18 Reductions in ischemic brain injury have been reported through direct inhibition of IL-1β. Inhibition of IL-1β through exogenous administration or over expression of the IL-1 receptor antagonist (IL-1ra) has been shown to decrease ischemic induced injury.19–21 Results from our study suggest that triflusal may decrease the spread of inflammation and thus diminish the resulting ischemic injury by inhibiting the typical ischemic-induced elevations in IL-1β through similar mechanisms.

In the present study, MCAO in rats resulted in marked induction of GFAP and Hsp27 in the ipsilateral cerebral cortex. GFAP and Hsp27 immunostaining was mostly confined to the region of the infarct and was not reduced by treatment of either aspirin or triflusal. Moreover, it should also be noted that treatment with triflusal seemed to have a more dramatic effect on stroke induced Hsp27 production compared with treatment with aspirin. Although previous studies have reported induction of Hsp27 primarily in astrocytes after ischemic injury,22,23 little is known regarding the function of Hsp27 in the central nervous system; however, it has been suggested that ischemia-induced expression of Hsp27 in astrocytes might protect the brain against subsequent injury resulting in conserved astrocytic functions such as anti-oxidative activity and glutamate uptake.24

Summary
In summary this study shows that aspirin (30 mg/kg) and triflusal (30 mg/kg) can both significantly reduce the infarct volume when administered immediately after MCAO compared with vehicle-treated rats. We have also shown that triflusal treatment (30 mg/kg) after MCAO decreases the progression of activated microglia in the penumbra and the protein expression of IL-1β in the ipsilateral cortex compared with vehicle treatment. Both of these factors are established markers of inflammation. This evidence suggests that triflusal is neuroprotective in our MCAO model of ischemia and that it likely mediates this neuroprotection through a reduction in inflammatory mechanisms leading to an overall reduction in the resulting size of the infarct.

Acknowledgments
We gratefully thank Lisa Tichenoff for their technical assistance. D.F.C is a Career Investigator with Heart and Stroke Foundation of Canada. S.N.W is a National Science and Engineering Research Council Scholar.

Sources of Funding
This study was partially funded by Grupo Uriach, Poligono Industrial Riera de Caldes, Avinguda Camí Reial 51-57, 08184 Palau-solita i Plegamans (Barcelona), Spain.

Disclosures
None.

References


Effects of Triflusal and Aspirin in a Rat Model of Cerebral Ischemia
Shawn N. Whitehead, Nestor A. Bayona, Guanliang Cheng, Gary V. Allen, Vladimir C. Hachinski and David F. Cechetto

Stroke. 2007;38:381-387; originally published online December 28, 2006;
doi: 10.1161/01.STR.0000254464.05561.72

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/38/2/381

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/