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Ischemic stroke triggers lipid peroxidation and neuronal injury. Docosahexaenoic acid released from membrane phospholipids during brain ischemia is a major source of lipid peroxides. Leukocyte infiltration and pro-inflammatory gene expression also contribute to stroke damage. In this study using lipidomic analysis, we have identified stereospecific messengers from docosahexaenoate-oxygenation pathways in a mouse stroke model. Aspirin, widely used to prevent cerebrovascular disease, activates an additional pathway, which includes the 17*R*-resolvins. The newly discovered brain messenger 10,17*S*-docosatriene potently inhibited leukocyte infiltration, NFκB, and cyclooxygenase-2 induction in experimental stroke and elicited neuroprotection. In addition, in neural cells in culture, this lipid messenger also inhibited both interleukin 1-β-induced NFκB activation and cyclooxygenase-2 expression. Thus, the specific novel bioactive docosanoids generated *in vivo* counteract leukocyte-mediated injury as well as pro-inflammatory gene induction. These results challenge the view that docosahexaenoate only participates in brain damage and demonstrate that this fatty acid is also the endogenous precursor to a neuroprotective signaling response to ischemia-reperfusion.

Brain ischemia-reperfusion triggers lipid peroxidation that participates in neural injury (1, 2). Docosahexaenoic acid (DHA)[1] (22:6*n*-3) esterified in membrane phospholipids is released in brain ischemia (3, 4) and is thought to yield lipid peroxides (5). Leukocyte infiltration and pro-inflammatory gene expression are mediators of ischemic stroke damage (6–8); however, there are no known messengers that down-regulate these events. The biosynthesis of oxygenated arachidonic acid messengers (9, 10) triggered by cerebral ischemia-reperfusion is preceded by an early and rapid phospholipase A_2 activation reflected in free arachidonic and docosahexaenoic acid accumulation (3, 4, 11). These fatty acids are released from membrane phospholipids where they are esterified (3). Both fatty acids are derived from dietary essential fatty acids; however, only DHA is concentrated in the central nervous system (12). It is clear that synaptic membrane and retinal photoreceptor biogenesis is dependent on liver processing of the dietary DHA or of its precursor, linolenic acid, followed by blood lipoprotein transport (13). DHA is involved in memory formation (14), excitable membrane function (15), photoreceptor cell biogenesis and function (16), and neuronal signaling (17) and has been implicated in neuroprotection (18–20). Whether DHA itself or a DHA-derived messenger is involved in these events is not known. Moreover, to date, potent bioactive autacoids from DHA acting in nanomolar concentrations have not been identified in the central nervous system. Although certain docosanoids have been identified in the retina (21) and have been proposed to be neuroprotective (12), their physiologic properties have not been explored.

To test the ability of the mouse brain to synthesize bioactive docosanoids, we used tandem LC-PDA-ESI-MS-MS-based lipidomic analysis in combination with ischemia-reperfusion. The rationale for use of this design was based upon the fact that brain ischemia releases unesterified DHA (3, 4, 11) and that during early stages of ischemia-reperfusion, endogenous signals of repair/neuroprotection may be generated.

EXPERIMENTAL PROCEDURES

Reagents—Human recombinant IL-1β (14019) was from Sigma, and 10,17-diHDHA was prepared as described previously (22, 23). Normal human neural (HN) progenitor cells (CC-2599), neural progenitor maintenance medium, human epidermal and human fibroblast growth factors, gentamicin/amphotericin B (G/A1000), and neural survival factor-1 were obtained from Clonetics (Walkersville, MD). AP1, HIF-1α, NFκBp50/p65, and STAT-1α gel-shift consensus and mutant oligonucleotides were synthesized at the Louisiana State University Health Sciences Center core facility or were purchased from Promega Life Science (Madison, WI).

Middle Cerebral Artery Occlusion (MCA-O) and Reperfusion—Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Louisiana State University Health Sciences Center, New Orleans, and followed the National Institutes of Health guidelines for experimental animal use. Mice (20–25 g body weight) were induced with 2% isoflurane in a mixture of 70% nitrous oxide and 30% oxygen. Anesthesia was maintained with 1% isoflurane.

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[1] The abbreviations used are: DHA, docosahexaenoic acid; HDHA, hydroperoxy-DHA; AP1, activator protein 1; HIF-1α, hypoxia-inducible factor 1-α; HN, human neural progenitor cells; MCA-O, middle cerebral artery occlusion; PMN, polymorphonuclear; IL, interleukin; STAT, signal transducers and activators of transcription; TTC, 2,3,5,-triphenyltetrazolium chloride; PBS, phosphate-buffered saline; LC, liquid chromatography; MS, mass spectrometry; PDA, photodiode array; ESI, electrospray ionization; SIM, selected ion monitoring.

Temperature was maintained at 36.5–37.5 °C (Harvard homeothermic blanket). P-10 polyethylene catheters were placed in the femoral artery and vein, and blood pressure was monitored. Arterial blood was analyzed for P_{O_2}, P_{CO_2}, and pH after 1 h of ischemia and 30 min after the onset of reperfusion. The common carotid and external carotid arteries were temporarily ligated with a retracting suture, and the external carotid artery was dissected just proximal to its bifurcation. The occluding filament was introduced from the external carotid artery and advanced to the internal carotid artery. The arterial venous microclip was removed, and the filament was advanced so that the blunted tip lay in the anterior cerebral artery and the side of the filament occluded the origin of the MCA. The stump of the external carotid artery was ligated, and tension on the retracting suture to the common carotid artery was gently released, restoring blood flow to the carotid system. The animals were allowed to recover from anesthesia, and 15 min before the start of reperfusion, a neurologic performance test was conducted (24). This neurologic assessment allowed definition of the degree of damage. A rating of Class III or Class IV indicated very severe damage; Class I indicated very mild damage. Thus, to enhance consistency in the group of selected animals, only mice rated as having Class II neurologic performance were included in the study. The variables minimized by this screening are anatomic variants of brain arteries and surgical procedure. About 85% of the mice had Class II neurologic performance. Occlusion of the middle cerebral artery was maintained for 1 h; reperfusion was then established by delicate retrieval of the occluding suture from the arterial lumen, restoring blood flow to the region of the MCA. The animals were killed by decapitation, and the hippocampi and brain cortices were rapidly dissected and frozen. Samples were kept at −80 °C until analysis. In some experiments, aspirin (7.5 mg/kg) was administered by gavage 15 min before MCA-O.

Assessment of Stroke Volume—Forty-eight hours after MCA-O, the mice were killed, and their brains were dissected and immersed in ice-cold saline. The brains were embedded in agar blocks and sectioned into coronal slices 1 mm thick by a vibratome (Vibratome Co., St. Louis, MO). The sections were incubated at room temperature in a 3% buffered solution of 2,3,5,-triphenyltetrazolium chloride (TTC). Once the color had developed (10–15 min), sections were fixed in 10% buffered formalin and kept at 4 °C until images were recorded by a camera (Cool-snap, Nikon) mounted to a dissecting microscope. Digital images were analyzed, and total and stroke areas were calculated and analyzed by Adobe Photoshop software. Serial sections were made for all animals.

Human Neural (HN) Progenitor Cells in Primary Culture—HN cells were grown to ~70% confluence (~50,000 cells per 3.5-cm diameter well) in 6-well culture plates (Costar) at 37 °C, 5% CO_2, 20% O_2, 75% N_2 in humidified air at 1 atmosphere (normoxic conditions) in neural progenitor maintenance medium (Clonetics CC-4241) supplemented with human fibroblast growth factor, neural survival factor-1, human epidermal growth factor, and GA-1000 as described by the manufacturer (Clonetics, Walkersville, MD). HN cells tested negative for HIV-1, hepatitis B and C, mycoplasma, bacteria, yeast, and fungi and positive for the glial and neuronal markers glial fibrillary acidic protein, mitogen-activated protein 2, and β-tubulin III (Clonetics). After 2 weeks of development, HN cells were exposed to human recombinant IL-1β (10 ng/ml) for 3 h in the presence or absence of 0.03, 0.3, 3.0, 30, and 300 nM 10,17*S*-docosatriene, DHA, or PBS, pH 7.4 (control). RNA and protein were rapidly isolated using Trizol Reagent (Invitrogen, Carlsbad, CA) and stored at −81 °C within minutes of isolation.

COX-2 RNA Abundance in Hippocampus and HN Cells—Abundance of human-specific COX-1 and COX-2 RNA message was assayed using reverse transcriptase PCR (25).

Electrophoretic Mobility Shift Assay of Human AP1, HIF-1α, NFκBp50/p65, and STAT-1α—Nuclear protein extracts were prepared from one to three 3.5-cm diameter wells of HN cells and quantitated. Nuclear protein extracts (5 μg) derived from HN cells were incubated with [γ-^{32}P]ATP (~3000 Ci/mmol)-end-labeled AP1, HIF-1α, NFκBp50/p65, or STAT-1α consensus and mutant oligonucleotides in 5-μl volumes, reacted for 30 min on ice, analyzed on 5 or 10% acrylamide, 90 mM Tris borate, pH 8.4, 1 mM EDTA gels, dried onto 2-mm Whatman filter paper at 80 °C for 2 h, and phosphorimaged using a Typhoon Variable Mode Imager (Amersham Biosciences).

Polymorphonuclear (PMN) Leukocyte Infiltration Measurement by the Myeloperoxidase Assay—Inhibition of leukocyte infiltration by 10,17*S*-docosatriene or by DHA was measured in mouse hippocampi and neocortices after 1 h of MCA-O and 48 h of reperfusion. Lipids were delivered in the indicated quantities for each experiment by perfusion (250 nl/h) through Alzet mini-pumps implanted into the third ventricle. Hippocampi and neocortices from the ipsilateral (MCA-O-reperfusion) and contralateral sides were rapidly dissected. The brain samples were assayed for myeloperoxidase activity (26). Briefly, the brain tissues were homogenized in 10 mM phosphate buffer (pH 7.4) and then frozen and thawed with liquid N_2 followed by sonication. Samples were precipitated at 10,000 × *g* for 10 min, and then aliquots of supernatants were added to a 10-mM phosphate buffer (pH 6.0) and a substrate solution containing *O*-dianisidine (Sigma) and 0.025% hydrogen peroxide and finally incubated at 37 °C for 45 min. Spectrophotometric detection was obtained at 460 nm.

Immunohistochemistry of Myeloperoxidase to Assess Leukocyte Infiltration—After MCA-O, mice were infused by Alzet mini-pumps with vehicle or 10,17*S*-docosatriene. After 48 h, the mice were anesthetized and killed by intracardial perfusion of ice-cold saline followed by 10% neutral buffered formalin. The brain tissues were allowed to equilibrate overnight in 4% buffered formalin followed by 30% sucrose in 0.1 M PBS. Frozen sections were made at a thickness of 10 μm and mounted on glass slides. The sections were permeabilized with 0.6% Triton X-100 for 10 min, washed in PBS, and blocked in 2% goat serum in PBS for 30 min. Incubation with myeloperoxidase/fluorescein isothiocyanate-conjugated antibody (Dako A/S) was performed at 1:200 dilution for 2 h. The sections were washed in Tween 20 in PBS and then mounted in Vectashield (Vector, CA). Images were recorded by deconvolution microscope (Intelligent Imaging Innovations, Denver, CO).

LC-MS-MS Analysis of Docosanoids—Quantitative analysis of docosanoids by LC-MS-MS was performed in hippocampi from mice (C57/BL-6, 20–25 g body weight) killed by head-focused microwave radiation at different time points after the onset of reperfusion. The hippocampi were rapidly dissected (20–70 mg wet tissue weight), homogenized in cold methanol, and kept under nitrogen at −80 °C until purification. Purification was performed by solid-phase extraction technique (27). In short, samples pre-equilibrated at pH 3.0 were loaded onto C18 columns (Varian) and eluted with 10 ml of 1% methanol in ethyl acetate (EM Science). Samples were concentrated by a nitrogen stream evaporator before LC-MS analysis. Samples were loaded into a Surveyor MS pump (Thermo-Finnegan) equipped with a C18 discovery column (Supelco), 10 cm × 2.1 mm inner diameter, 5 μm internal phase. Samples were eluted in a linear gradient (100% solution A (60:40:0.01 methanol/water/acetic acid) to 100% solution B (99.99:0.01 methanol/acetic acid)) and run at a flow rate of 300 μl/min for 45 min. LC effluents were diverted to an electrospray ionization probe (ESI) on a TSQ Quantum (Thermo-Finnegan) triple quadrupole mass spectrometer running on negative ion detection mode. Docosanoid standards were used for calibration and optimization. The instrument was run on full-scan mode to detect parent ions and selected reaction monitoring for quantitative analysis to detect daughter ions simultaneously. The selected parent ions were 327 for DHA and 359 for 10,17*S*-docosatriene. Moreover, daughter ions were 325.1 and 297, respectively.

RESULTS

Brain Ischemia-Reperfusion Triggers the Synthesis of Docosahexaenoic Acid-Oxygenation Pathways—We used 1 h of right middle cerebral artery occlusion in mice followed by reperfusion to assess the formation of docosahexaenoic acid-oxygenation derivatives. Under these conditions there is active release of free docosahexaenoic acid from brain membrane phospholipids (2–4). This model of transient focal ischemia greatly affects the hippocampus, a brain region rich in neurons vulnerable in ischemic stroke and in other neurologic diseases (1, 2).

Fig. 1*A* shows the time course of formation of novel docosanoids in the ipsilateral hippocampus during ischemia-reperfusion. There was generation of 17-hydroperoxy-DHA, the 15-lipoxygenase-like action on DHA (22), and a novel 10,17*S*-docosatriene accumulated up to 8 h of reperfusion. The MS-MS spectrum of 10,17*S*-docosatriene (Fig. 1, *B* and *C*) corresponded to a dihydroxy-containing DHA with prominent fragment ions at *m/z* 359 (Fig. 1*C*, [M-H]). There were also fragment ions at *m/z* 323 ([M-H]-$2H_2O$), 315 ([M-H]-CO_2), 297 ([M-H]-H_2O-CO_2), and 277 ([M-H]-$2H_2O$-CO_2-2H). Other diagnostic ions were essentially identical to those recently documented (22) in murine brain, human blood, and glial cells. In addition, there was a time-dependent formation of the carbon 22-ω hydroxylation product, 4,17di-HDHA (Fig. 1*A*) as determined by analysis (at *m/z* 375) of the MS-MS spectrum (Fig. 1*D*).

Because aspirin is often used prophylactically as well as

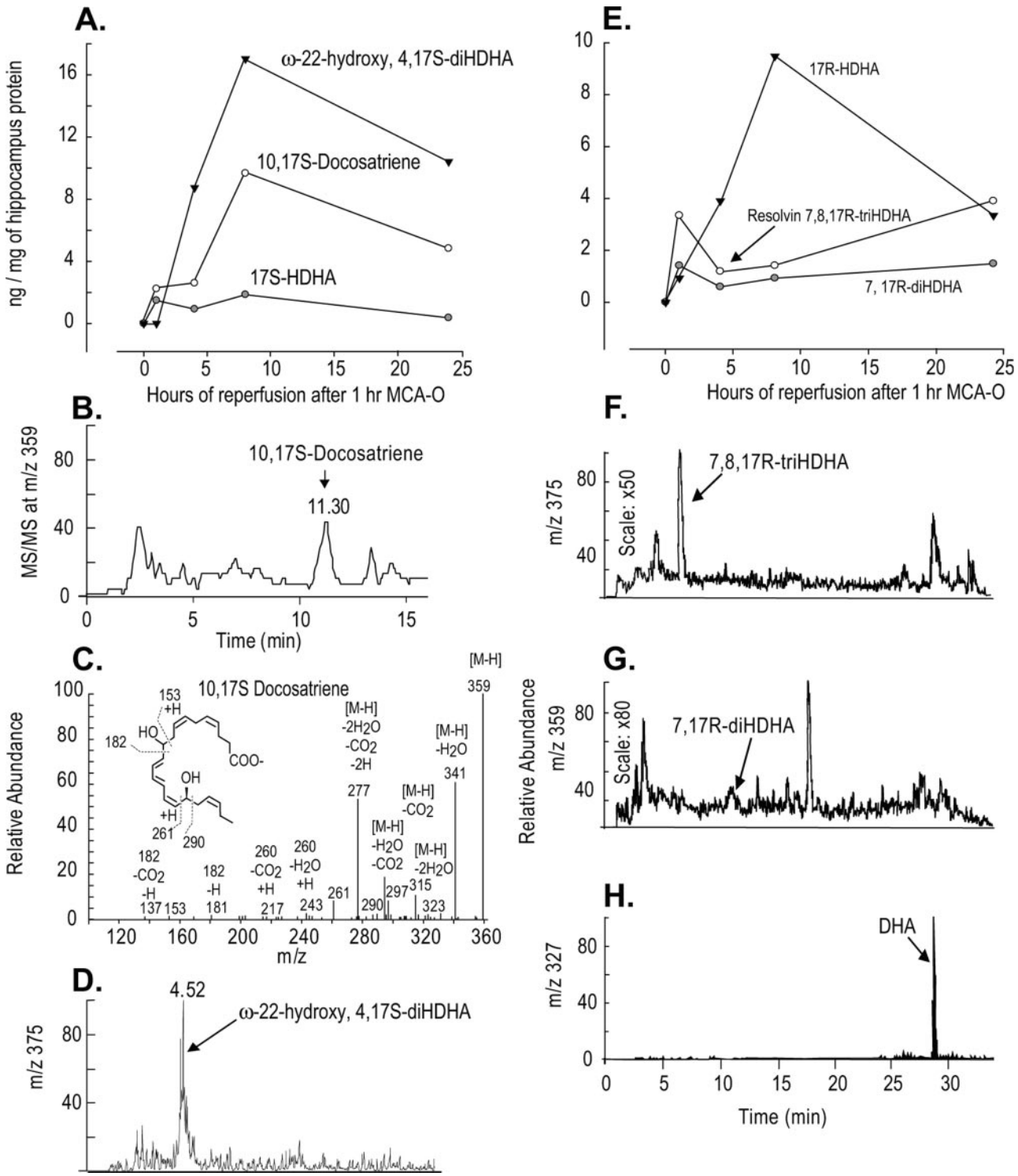

FIG. 1. **Synthesis and metabolism of docosanoids in the ipsilateral mouse hippocampus during reperfusion following transient ischemia.** Structural elucidation of DHA and docosanoids was performed by lipidomic analysis by LC-PDA-MS-MS as described previously (22, 23). *A*, time course of accumulation of 17*S*-HDHA, 10,17*S*-docosatriene, and ω-22-hydroxy-4,17*S*-diHDHA in the ipsilateral hippocampus during reperfusion after 1 h of MCA-O. There was an enhanced accumulation of ω-22-hydroxy-4,17*S*-diHDHA, a product of an oxidative pathway, and 10,17*S*-docosatriene. *B*, selected ion monitoring (SIM) chromatogram (*m/z 359*) showing 10,17*S*-docosatriene. *C*, MS-MS spectrum for 10,17*S*-docosatriene. *D*, SIM (*m/z 75*) chromatogram showing ω-22-hydroxy-4,17*S*-diHDHA. *E*, time course for the accumulation of 17*R*-series resolvins in hippocampi of mice pretreated with aspirin before MCA-O. Aspirin triggered a metabolic shift toward accumulation of 17*R*-docosatrienes, 7,8,17*R*-triHDHA, and 7,17*R*-diHDHA, products of acetylated COX-2 enzyme. *F*, representative SIM (*m/z 375*) chromatogram with peak for 7,8,17*R*-triHDHA. *G*, representative SIM (*m/z 359*) chromatogram with peak for 7,17*R*-diHDHA. *H*, typical SIM (*m/z 327*) chromatogram with peak for DHA.

therapeutically to manage cerebrovascular diseases, we next asked whether brain biosynthesis of DHA messengers is modified in the presence of aspirin *in vivo*. Moreover, in non-neural tissues, aspirin triggers the biosynthesis of anti-inflammatory lipid mediators (23, 28). We examined the formation of DHA-derived docosanoids in the presence of aspirin during reperfusion after an ischemic stroke (Fig. 1). Fig. 1*E* shows the time course of docosanoid formation with aspirin treatment. There

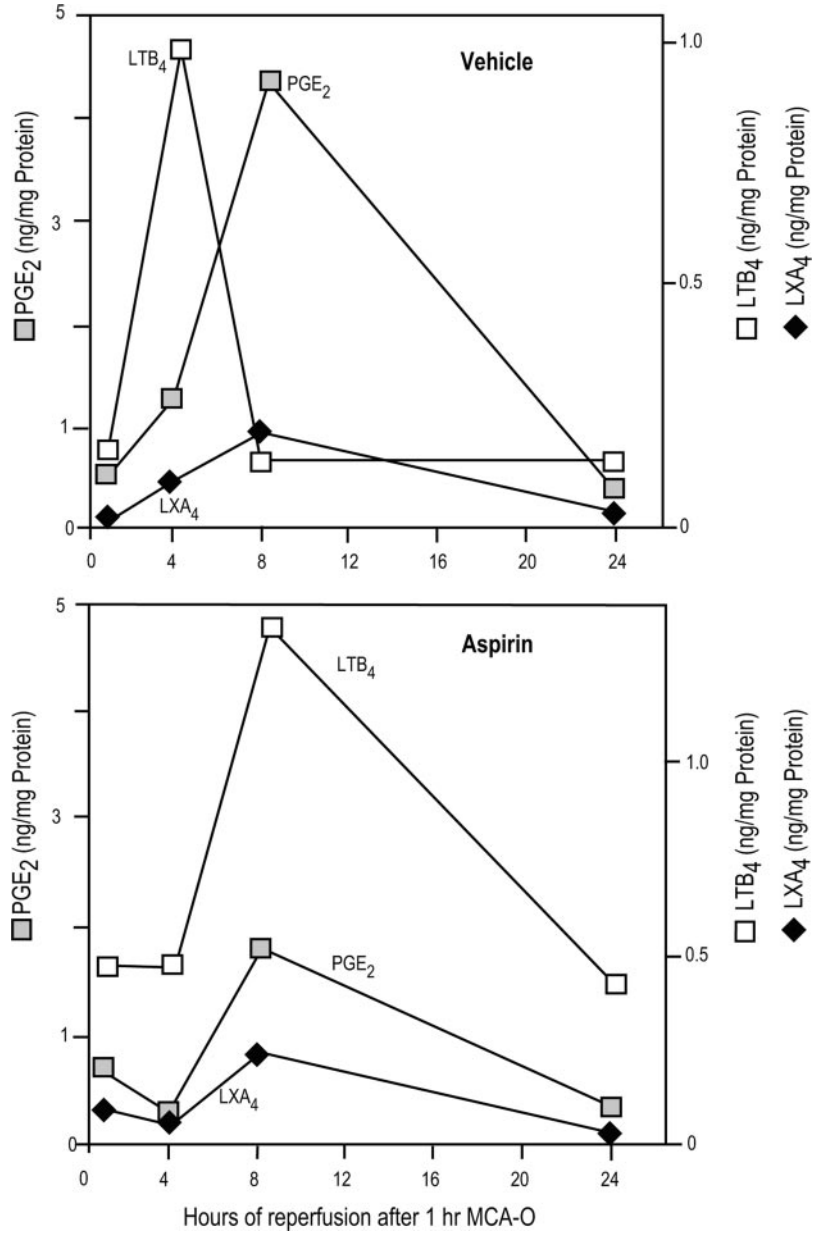

FIG. 2. **Time course of prostaglandin E_2, leukotriene B_4, and lipoxin A_4 formation in mouse hippocampus after 1 h of MCA-O followed by reperfusion with or without aspirin treatment.** Detection was performed by sensitive and specific enzyme-linked immunosorbent assay, in tandem, for prostaglandin E_2 (*PGE*$_2$), leukotriene B_4 (*LTB*$_4$), and lipoxins (*LXA*$_4$) (Neogen, Lexington, KY). This study showed inhibitory changes in the eicosanoids when animals were pretreated with aspirin (15 min) before MCA-O. C57BL/6 mice were treated by gavages with vehicle (sterile saline) or aspirin (7.52 mg/kg).

was a shift away from the products generated from endogenous sources of DHA in the absence of aspirin (Fig. 1*A*) toward products that include the novel 17*R*-series resolvins, in particular 7,17*R*-diHDHA and 7,8,17*R*-triHDHA, which were formed and were present at the earliest time intervals. There was a marked accumulation in 17*R*-HDHA, shown recently to be a product of aspirin-acetylated cyclooxygenase-2 (28).

Fig. 1, *F* and *G,* shows a representative LC-MS-MS of the ipsilateral hippocampus following treatment with aspirin and depicts the chromatographic profile of products identified via MS-MS and lipidomic analyses. Here we also confirmed the release of DHA under these conditions (3, 4, 11) (Fig. 1*H*). To assess that the aspirin dose administered by gavage did reach the brain, the time courses for prostaglandin E_2, leukotriene B_4, and lipoxin A_4 production were determined in the hippocampi in parallel assays (Fig. 2, *PGE*$_2$, *LTB*$_4$, and *LXA*$_4$). The rapid accumulation of prostaglandin E_2 within 8 h of reperfusion was inhibited by aspirin treatment. Maximal leukotriene B_4 was generated in aspirin-treated hippocampi by 8 h, as was the case with lipoxin A_4, which also peaked within 8 h. Together these results clearly indicate that the dose of aspirin used *in vivo* did access the central nervous system.

Fig. 3 depicts the proposed pathway to generate the novel 10,17*S*-diHDHA ω-oxidation product measured in the absence of aspirin. In our experiments, 4,17*S*-diHDHA did show bioactivity in PMN infiltration, but these were less than those evoked by equimolar concentrations of 10,17*S*-docosatriene in a non-neural experimental model (Fig. 4). Fig. 4, therefore, confirms that 4,17*S*-diHDHA inhibits PMN leukocyte exudate formation in murine peritonitis models (28). This ω-oxidation product is the likely inactivation product for 10,17*S*-diHDHA. Fig. 3 also depicts the resolvin or 17*R* series pathways. These are the products formed in the presence of aspirin.

Polymorphonuclear Leukocyte Infiltration Mediated by Focal Ischemic Stroke in Mice Is Inhibited by 10,17S-Docosatriene—We monitored PMN infiltration, a major factor in mediating brain ischemia-reperfusion damage (7, 8, 29, 30). PMN infiltration is a complex multistep process that is modulated by the coordinated expression of adhesion and signaling molecules (26). DHA-derived messengers were very recently reported to inhibit PMN invasion outside of the central nervous system in the air-pouch model (23, 26, 28).

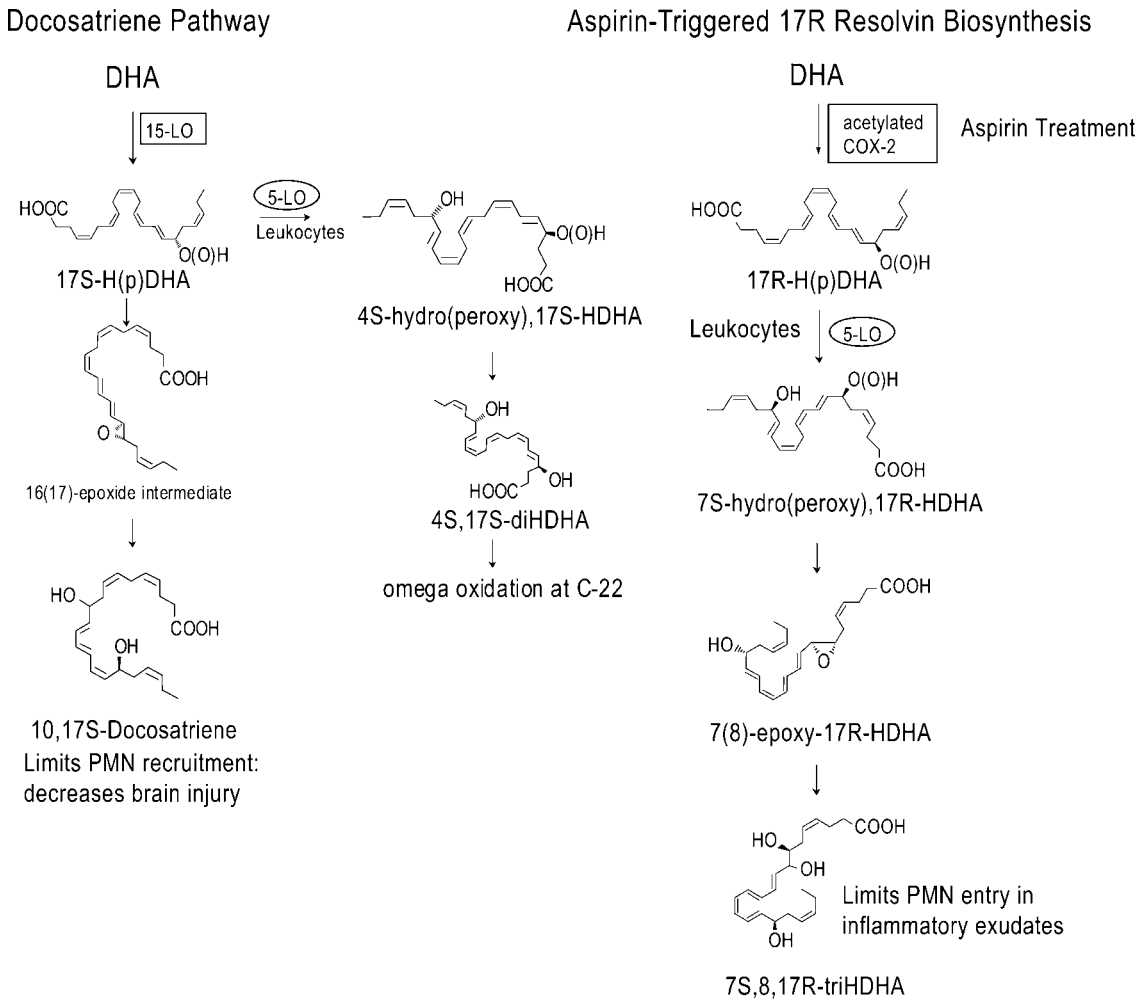

FIG. 3. **Proposed biosynthetic pathways for 10,17*S*-docosatriene and the aspirin-triggered 17*R*-series resolvins.** The stereochemistry for compounds in both pathways is based on the biogenic total synthesis, lipidomic analyses, and alcohol-trapping profiles (22, 23).

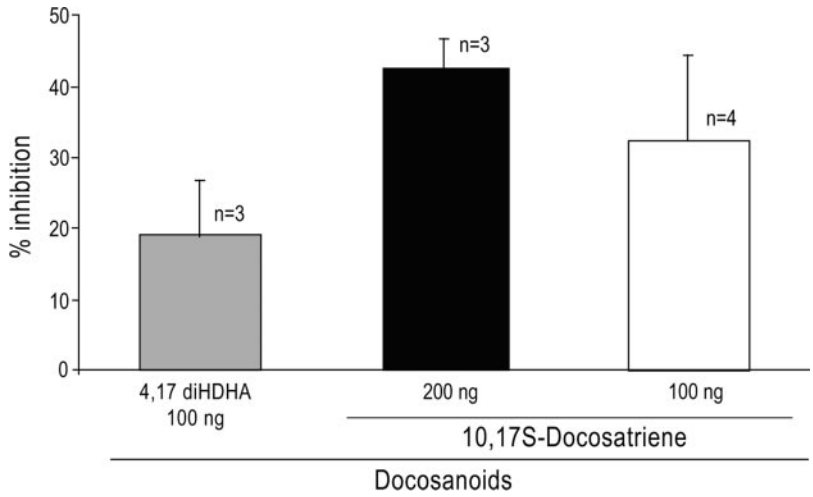

FIG. 4. **Inhibition of leukocyte infiltration in zymosan-induced peritonitis by 4,17*S*-diHDHA and 10,17*S*-docosatriene.** The 4,17*S*-diHDHA caused dose-dependent inhibition of polymorphonuclear leukocyte infiltration. Here 100-ng 10,17*S*-docosatriene caused potent inhibition. Peritonitis was induced in 6–8-week-old male FVB mice (Charles River Laboratories) by peritoneal injection of 1 mg of zymosan A. Compounds 4,17*S*- and 10,17*S*-diHDHA were injected by intravenous bolus injection, 1.5 min before zymosan A treatment. Two h after induction of peritonitis, rapid peritoneal lavages were collected, and cell type enumeration was performed.

To determine whether 10,17*S*-docosatriene displays counteractive bioactivity during brain ischemia-reperfusion-induced PMN infiltration, this docosanoid was constantly infused into the third ventricle during 48 h of superfusion in the amounts shown in Fig. 5. By immunostaining of myeloperoxidase as a marker of PMN leukocytes, we demonstrated a marked inhibition by 10,17*S*-docosatriene on the ipsilateral side of the brain (Fig. 5*A*). Myeloperoxidase is a marker of PMN infiltration in the brain (7, 8, 29, 30) and is depicted with a characteristic cytoplasmic granular pattern found only in the focal ischemic side (Fig. 5*B*). In addition, we dissected both the hippocampus and the neocortex from the focal stroke side (ipsilateral) as well as from the contralateral side and quantified myeloperoxidase enzyme activity. Both the free acid and the carboxymethyl ester of 10,17*S*-docosatriene inhibited the appearance of myeloperoxidase (Fig. 5, *C* and *D*). This was the case both in the ipsilateral hippocampus and in the ipsilateral neocortex. However, the contralateral brain regions did not display increased enzyme activity, nor did they show immunostaining for leukocytes. Infusion of DHA also elicited inhibition of PMN infiltra-

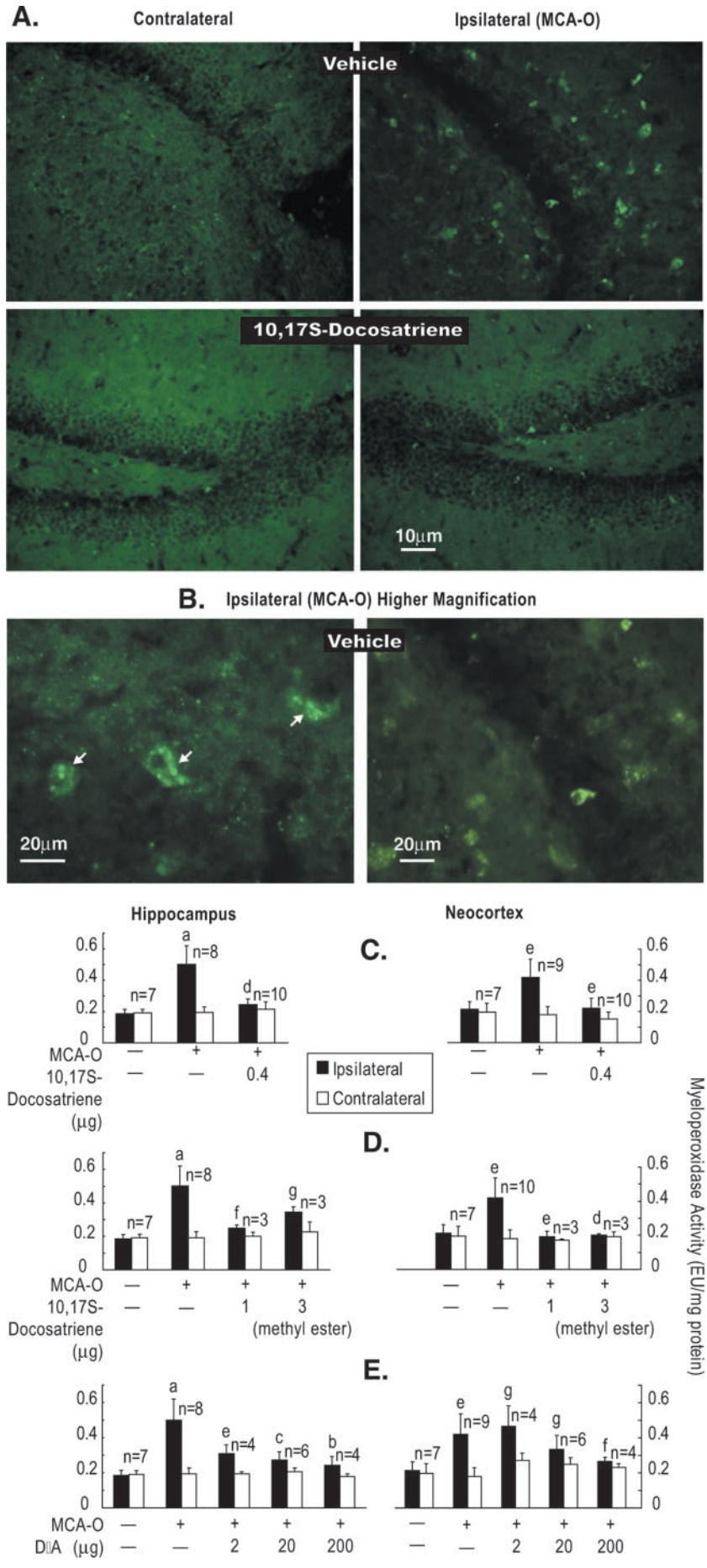

FIG. 5. **Inhibition of leukocyte infiltration by 10,17*S*-docosatriene in mouse hippocampus and neocortex after 1 h of MCA-O and 48 h of reperfusion.** Data shown represent averages ± S.D. of the indicated number (*n*) of individual mice. *A* and *B*, immunocytochemical visualization of polymorphonuclear leukocytes (*green* fluorescence) that exhibit myeloperoxidase immunoreactivity. Ipsilateral brain areas (ischemic area) show positive green fluorescence (*Vehicle*) as compared with contralateral tissue. The areas shown correspond to the dentate gyrus of the hippocampus. In *A*, 10,17*S*-docosatriene-infused animals (0.4 μg over 48 h) exhibited a large reduction in green fluorescence. *B* shows higher magnifications of the ipsilateral stroke area from vehicle-treated animals, with the cytoplasmic granular appearance of immunoreactivity (*arrows*) that is characteristic of PMN leukocytes. *C*, cerebroventricular perfusion of 0.4 μg of 10,17*S*-docosatriene over 48 h resulted in 80% inhibition of ipsilateral myeloperoxidase activity as compared with vehicle-treated animals. *D*, perfusion as in *A*, but with 1 μg or 3 μg of 10,17*S*-docosatriene methyl ester over 48 h. The free acid exerted more potent inhibition (80 and 99% in hippocampus and cortex, respectively) as compared with the methyl ester derivative, which resulted in 49% inhibition in hippocampus and 97% inhibition in neocortex. *E*, same as in *A*, but perfusion with 2, 20, or 200 μg of DHA (free acid) over 48 h. In the hippocampus, dose-dependent inhibition of myeloperoxidase activity was observed (60, 72, and 82%, respectively). In the neocortex, no inhibition was found with 2 μg, but there were 40 and 73% inhibition with 20 or 200 μg, respectively. Statistically significant differences determined by the factorial analysis of variance with comparison of treatment means done by protected Student's *t* test (37) are represented as follows: *a*, $p < 0.0001$; *b*, $p < 0.001$; *c*, $p < 0.003$; *d*, $p < 0.006$; *e*, $p < 0.01$; *f*, $p < 0.03$; *g*, no significant difference.

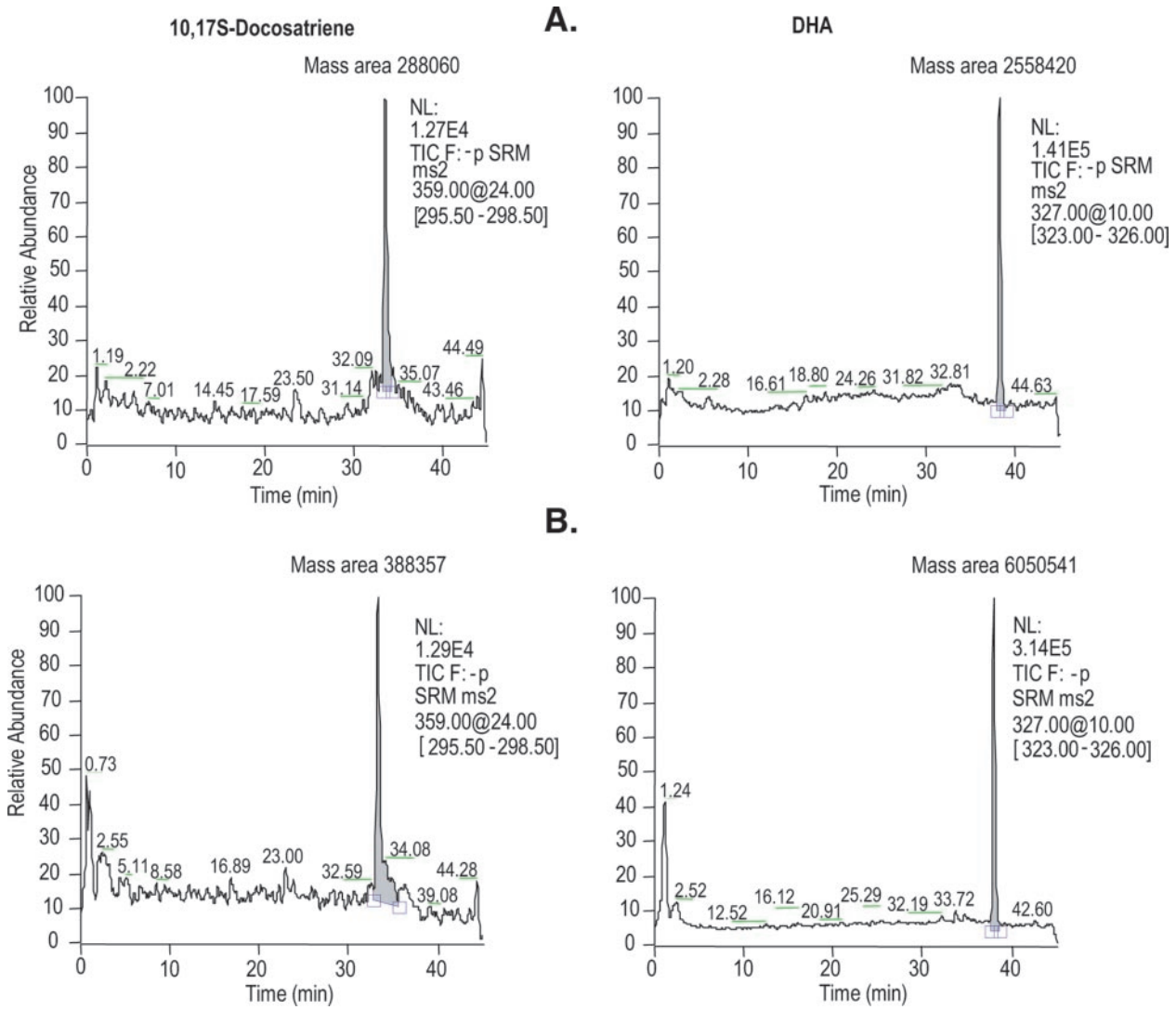

FIG. 6. **LC-MS-MS quantitative analysis of 10,17*S*-docosatriene and DHA levels in mouse hippocampus after 1 h of MCA-O and 6 h of reperfusion.** Shown are representative chromatograms of 10,17*S*-docosatriene selected reaction monitoring (*SRM*) for the parent ion 359 *m/z* on quadrupole 1 (Q1) and the daughter ion 297 *m/z* on Q3. Also shown are the DHA SRM for parent ion 327 *m/z* and daughter ion 325 *m/z* on Q1 and Q3, respectively. *A*, animals treated by cerebroventricular perfusion of cerebrospinal fluid vehicle for 6 h produced consistent endogenous amounts of 10,17*S*-docosatriene and DHA. *B*, animals that were perfused with DHA (20 μg) exhibited greater formation of 10,17*S*-docosatriene and at least a 3-fold increase in DHA levels.

tion mainly in the hippocampus (Fig. 5*E*). One interpretation of this effect is that exogenously infused DHA is being utilized for the synthesis of the bioactive docosanoid, 10,17*S*-docosatriene. This hypothesis is supported by our finding that after 1 h of MCA-O followed by 6 h of reperfusion with DHA, 10,17*S*-docosatriene was unequivocally generated in the hippocampus at higher levels than in vehicle-perfused mice (Fig. 6).

10,17S-Docosatriene Elicits Neuroprotection in Mouse Focal Ischemic Stroke—To test for *in vivo* bioactivity of 10,17*S*-docosatriene in brain tissue during focal ischemic stroke, we devised a combined approach. We implanted Alzet mini-pumps into the third ventricle to deliver the lipid messenger over the 48-h reperfusion following 1 h of MCA-O. Then serial coronal sections of the brain were obtained and incubated with TTC (Figs. 7 and 8). TTC is actively taken up by cells into mitochondria resulting in a reddish color. The focal infarcted right side of the brain, the result of ischemic injury, displays colorless areas indicating severe mitochondrial and cell damage (Fig. 8*A*). The focal infarcted volume in mice infused with vehicle was about 30% of the total volume, whereas, when 10,17*S*-docosatriene was infused, there was a reduction to less than 15% of the stroke volume (Fig. 8*B*).

The Ischemia-induced Up-regulation of COX-2 Expression and NFκB Activation in Brain Tissue Is Inhibited by 10,17S-Docosatriene—Pro-inflammatory gene expression is an important promoter of ischemic brain injury. NFκB is activated in MCA-O (31, 32) as is COX-2 expression, which in turn generates prostaglandin H_2, the substrate for prostaglandin synthetases and a contributor to oxidative stress (33). To investigate whether pro-inflammatory gene expression is also a target of the novel docosanoid messenger 10,17*S*-docosatriene, we measured DNA binding activity of NFκB and other transcription factors as well as COX-1 and -2 expression in the hippocampus after MCA-O. NFκB was increased over 2-fold in the ipsilateral hippocampus after MCA-O, and the infusion of DHA or 10,17*S*-docosatriene inhibited ischemia-reperfusion-induced NFκB activation by 28 and 48%, respectively (Fig. 9*A*). This effect was selective, because DNA bindings for AP1, HIF1α, and STAT-1, which were slightly enhanced by MCA-O, were unaffected by lipid messengers, except for a small effect of 10,17*S*-docosatriene on STAT-1-DNA binding (Fig. 9*B*). To determine COX-1 and COX-2 expression we measured mRNA abundance in the ipsilateral hippocampus and found a 3-fold increase of COX-2 (Fig. 9*C*). The infusion of the precursor DHA

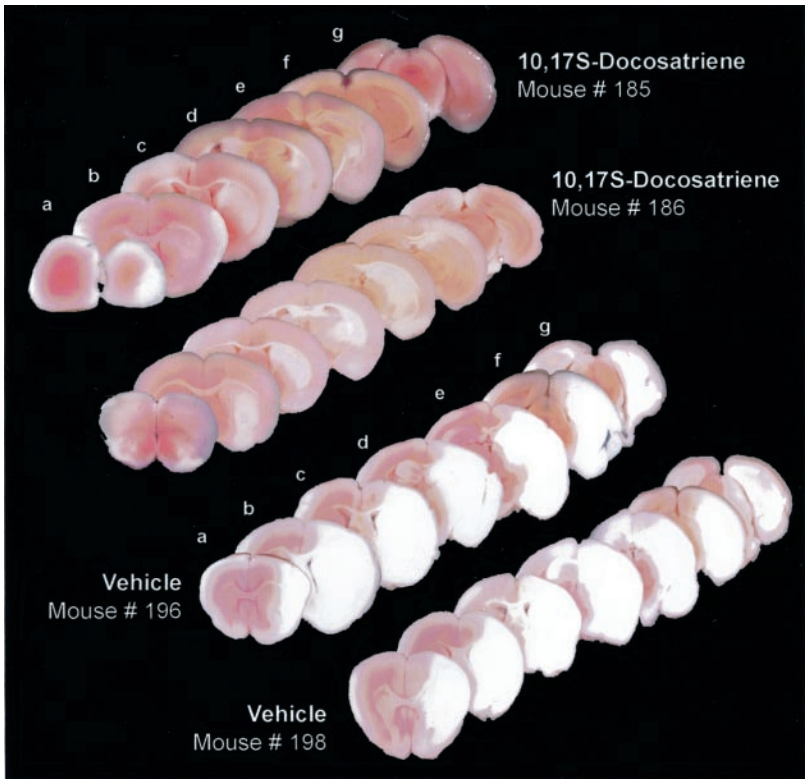

FIG. 7. **Serial sections of the mouse brain exposed to TTC.** Sections for TTC staining were made with a vibratome and handled as described under "Experimental Procedures." The identification *numbers* of individual mice as well as *letters* that designate each serial section are shown. Two of the mice depicted were injected with vehicle and two with 10,17*S*-docosatriene as described under "Experimental Procedures." *Colorless* areas indicate mitochondrial damage reflecting the inability to metabolize TTC.

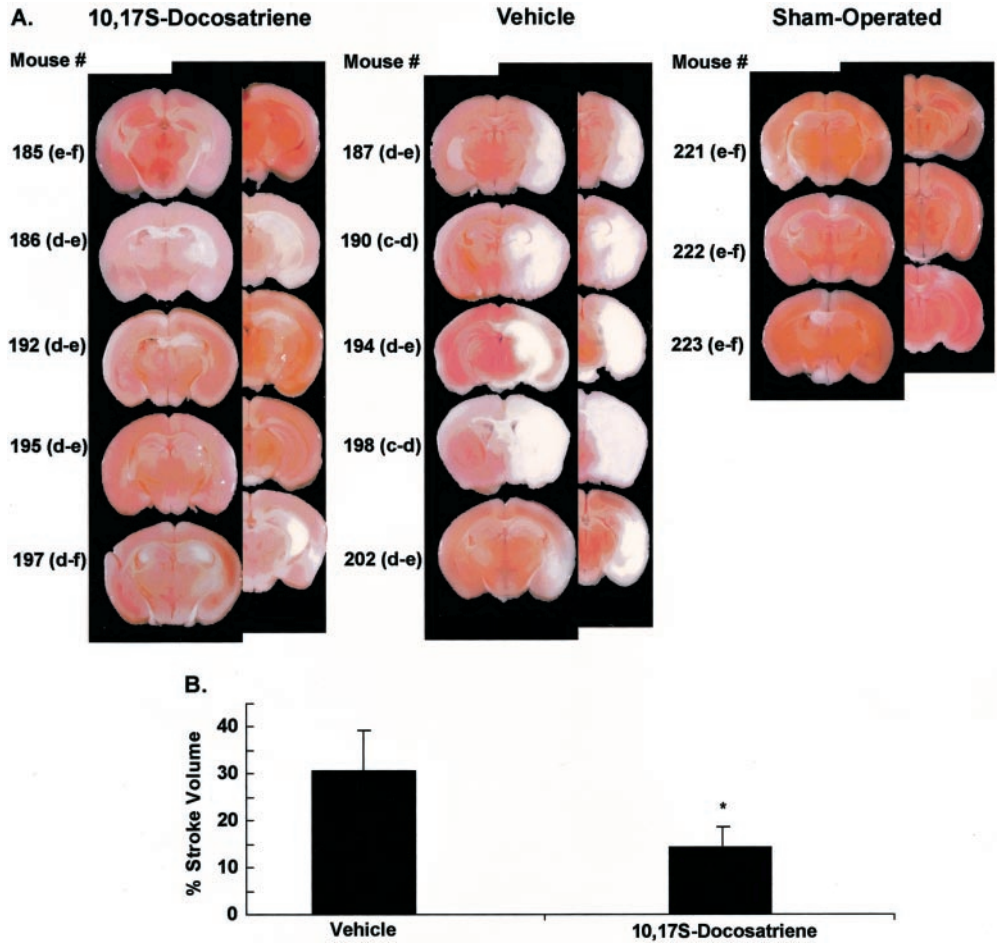

FIG. 8. **Neuroprotection by 10,17*S*-docosatriene in transient focal stroke in mice.** *A*, selected sections from different mice (each mouse is identified by a *number* and each brain section by a *letter*) in three groups: sham-operated, vehicle-infused, and 10,17*S*-docosatriene-infused. Sectioning and TTC staining were performed as described under "Experimental Procedures." Infusion was performed through an Alzet mini-pump implanted into the third ventricle (see "Experimental Procedures"). *B*, the graph depicts the percentage of the TTC-stained area with respect to total brain coronal area. *Bars* are averages ± S.D. from 6 animals each. *Asterisk* indicates $p < 0.001629$, Student's t test.

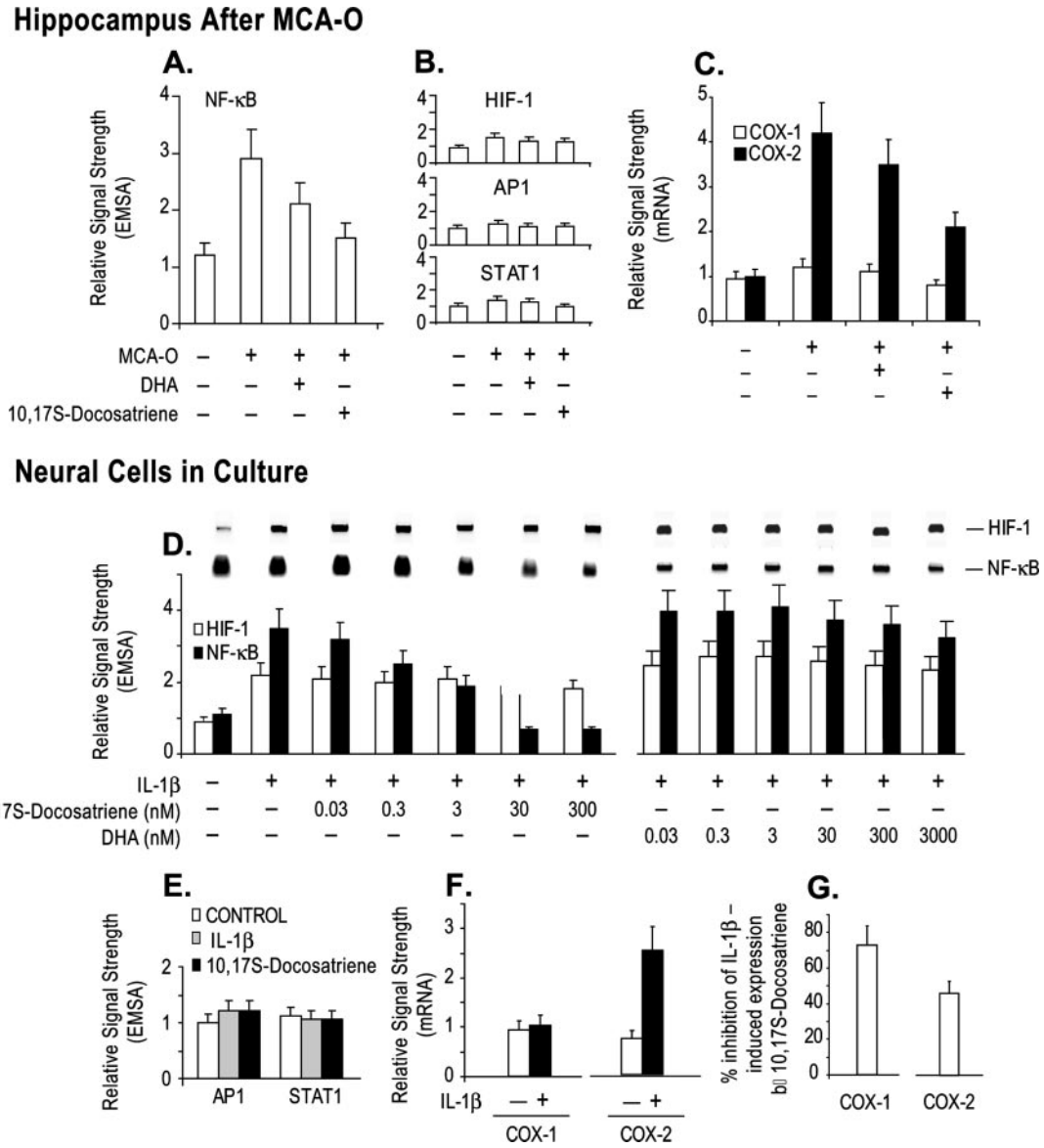

FIG. 9. **10,17*S*-docosatriene inhibited MCA-O- and IL-1β-induced NFκB activation and COX-2 expression.** *A–C*, mouse hippocampus after 1 h of MCA-O followed by 2 h of reperfusion. Vehicle or 10,17*S*-docosatriene (0.16 μg/ml) was infused into the third ventricle for 3 h at 0.25 μl/h. *A*, enhanced NFκB-DNA binding activity, determined by electrophoretic mobility shift assay after MCA-O, was inhibited by 10,17*S*-docosatriene or DHA. *B*, AP1 and STAT-1, unlike HIF-1α, were not affected by MCA-O. Only STAT-1-DNA binding was reduced by 10,17*S*-docosatriene. *C*, COX-2 expression was greatly increased by MCA-O-reperfusion in hippocampus, and 10,17*S*-docosatriene or DHA inhibited this enhanced expression. *D*, IL-1β-induced NFκB activation, but not IL-1β-induced HIF-1α activation, was inhibited by 10–17*S*-docosatriene. Free docosahexaenoic acid added in concentrations up to 30 μM was ineffective in modulating either NFκB or HIF-1α induction by IL-1β. *E*, as monitored by electrophoretic mobility-shift assay, 10,17*S*-docosatriene did not affect the small increase in IL-1β-induced AP1-DNA binding activity; STAT-1 was unaffected by IL-1β or the lipid under these conditions. *F*, IL-1β prominently activates COX-2 expression but not that of COX-1. *G*, IL-1β-induced expression of COX-2 mRNA was inhibited by 10,17*S*-docosatriene ($p < 0.05$). There was also a small effect of the lipid on Il-1β induction of COX-1 ($p > 0.05$, analysis of variance).

as well as its product, 10,17*S*-docosatriene, inhibited COX-2 expression by 14 and 52%, respectively. The infusion of 10,17*S*-docosatriene also decreased COX-1 expression by 19%.

Selective Inhibition by 10,17S-Docosatriene of IL-1β-mediated COX-2 Expression and NFκB Activation in Human Neural Progenitor Cells—We tested whether 10,17*S*-docosatriene affects pro-inflammatory gene signaling in human neural progenitor cells in culture exposed to IL-1β. These cells display dendrites and express neuronal markers (25). IL-1β prominently activated NFκB, and 10,17*S*-docosatriene down-regulated NFκB to a level below that of unstimulated cells in a concentration-dependent manner (Fig. 9*D*). HIF1-α was not affected even at 10-fold higher concentrations of DHA. Although IL-1β slightly increased AP1-DNA binding activity, 10,17*S*-docosatriene did not affect the level of this transcription factor or that of STAT-1 under these conditions (Fig. 9*E*). The action of 10,17*S*-docosatriene on intact cells strongly suggests the presence of a receptor-mediated mechanism, which would be consistent with the observation in other cells of evidence for a receptor-mediated event activated by this docosanoid (22). Moreover, the lack of effect of DHA implies that, unlike in whole brain, where infused DHA shows some inhibition of PMN infiltration induced by ischemia-reperfusion, these neural cells in culture exogenous DHA might not be converted to docosanoids in high enough concentrations. Alternatively, other cells may be required for this conversion to occur (*e.g.* glial cells).

Fig. 9, *F* and *G*, show that IL-1β-induced expression of COX-2 in neural progenitor cells was decreased by 10,17*S*-docosatriene. It is interesting that IL-1β did not activate COX-1 expression, but the docosanoid seemed to decrease the basal level of COX-1 mRNA. Taken together, these observa-

tions demonstrate that, after MCA-O, as well as in cytokine-stimulated neural cells in culture, 10,17*S*-docosatriene produced an overall attenuation of pro-inflammatory gene activation by inhibiting cytokine-induced (and brain ischemia-reperfusion-induced) NFκB and COX-2 expression.

DISCUSSION

Here we have demonstrated for the first time in brain tissue undergoing ischemia-reperfusion the generation of stereospecific DHA-oxygenation pathways that lead to the formation of novel messengers. We found two DHA-oxygenation pathways: the first pathway is responsible for the formation of the messenger 10,17*S*-docosatriene, and the second pathway, which is active in the presence of aspirin, leads to the formation of the resolvin-type messengers (17*R*-DHA). The pathways described have the potential for exerting counter-regulatory actions on cellular and molecular signaling that promotes brain injury. In the presence of aspirin and during ischemia-reperfusion, we found the formation of characteristic DHA messengers that in non-neural tissues are potent mediators of inflammation resolution (28). These resolvin-type DHA-derived messengers may elicit additional neuroprotective actions in brain ischemia-reperfusion. Our present results demonstrate that the novel 10,17*S*-docosatriene is a potent inhibitor of ischemia-reperfusion-induced PMN infiltration and pro-inflammatory gene induction. This novel DHA messenger also inhibits cytokine-mediated pro-inflammatory gene activation in neural cells in culture. Overall, 10,17*S*-docosatriene potently elicited neuroprotection *in vivo* by reducing the stroke infarct volume 48 h after MCA-O.

It is important to note that, in the presence of aspirin, there was enhanced formation in brain tissue of 17*R*-series resolvins that have recently been found to be cytoprotective and are counter-regulators of inflammation outside the nervous system (23). It could be argued that these compounds enhance the actions of endogenously generated docosatrienes, which are the counter-regulatory substances generated from DHA to decrease leukocyte recruitment to brain and to limit leukocyte-mediated inflammation and brain damage. The implied switch, from endogenous to aspirin-triggered DHA-derived lipid mediators that enhance this protective action, is of great interest for future studies, given the wide use of aspirin.

The synthesis of 10,17*S*-docosatriene after 1 h of MCA-O coincides with free DHA availability that results from phospholipase A_2 activation (2–4) as well as with reperfusion reoxygenation. The endogenous brain synthesis of 10,17*S*-docosatriene that peaks at 8 h of reperfusion may be a response of such insufficient magnitude as to counteract leukocyte infiltration and pro-inflammatory gene induction under the present experimental conditions. Thus the relatively large ischemic insult produced by 1 h of MCA-O followed by several h of reperfusion (Fig. 8) may overcome the ability of the endogenously generated docosanoids to elicit neuroprotection. Thus exogenous administration of 10,17*S*-docosatriene directly into the cerebroventricular system through continuous infusion during the initial 2 days of reperfusion did indeed exert neuroprotection. During 24–72 h of reperfusion is when most brain leukocyte infiltration occurs (7, 8, 29, 30). Leukocytes accumulate in an area surrounding the brain infarct (Fig. 5, *A* and *B*) and are thought to possess a multifactorial ability to promote injury in brain ischemia-reperfusion (34). Moreover, PMN infiltration as well as amoeboid microglia along the edges of the stroke infarct may be responsible for expansion of the penumbra region, which occurs from 16 h onward after MCA-O followed by reperfusion (35). Here we demonstrate that 10,17*S*-docosatriene administered continuously by intracerebroventricular perfusion inhibited such infiltration and subsequently reduced the stroke volume by 50% after 48 h. The amount of docosanoid infused was 1 μg over a 48-h period at a rate of 250 nl/h. This observation implies that 10,17*S*-docosatriene is neuroprotective. Once these leukocytes infiltrate the brain, they release IL-1β, tumor necrosis factor-α, and other cytokines, as well as myeloperoxidase, which in turn catalyzes the formation of additional reactive oxygen species. Myeloperoxidase in intercellular spaces of the brain is potentially a highly effective enzymatic catalyst for the initiation of lipid peroxidation (36), and brain cells are richly endowed with a major target of that process, polyunsaturated fatty acyl phospholipids (2, 12).

The bioactivity of the novel docosanoid 10,17*S*-docosatriene was further studied at the cellular level. We chose IL-1β as the trigger, because this cytokine increases during brain ischemia-reperfusion as a result of PMN infiltration as well as activation of microglia and macrophages (35). Whether 10,17*S*-docosatriene bioactive inhibition of PMN infiltration and its blocking of pro-inflammatory gene expression are independent events or part of the same signaling remains to be defined. What is clear is that the outcome of infusing 10,17*S*-docosatriene is neuroprotection from ischemia-reperfusion damage.

These findings have several implications related to both the understanding of how the brain modulates inflammatory injury responses and designing new experimental therapeutics for neurologic diseases. Therefore we have demonstrated novel DHA-signaling pathways that may lead to answers to clinically important questions regarding undiscovered mechanisms in stroke, traumatic head injury, spinal cord injury, and other diseases that involve a neuroinflammatory component. The potent bioactivity of 10,17*S*-docosatriene suggests the existence of a potentially important target for therapeutic neuroprotective interventions in these diseases.

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Novel Docosanoids Inhibit Brain Ischemia-Reperfusion-mediated Leukocyte Infiltration and Pro-inflammatory Gene Expression

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Page 43811, Fig. 4: Two concentrations of 4,17*S*-diHDHA are shown. The data were labeled incorrectly. The correct figure is shown below.

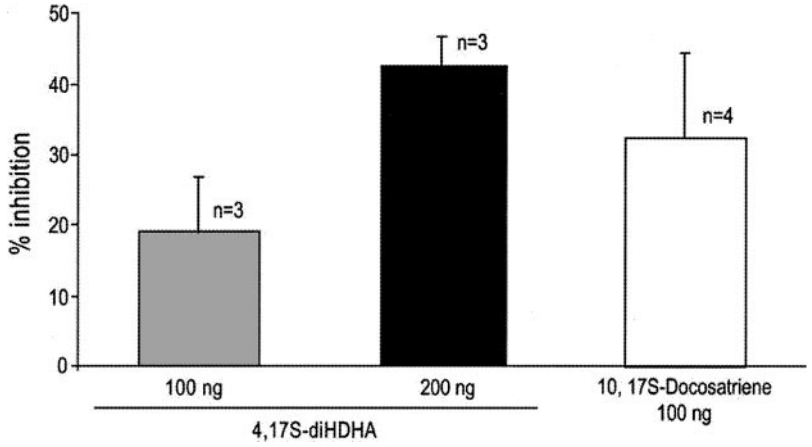

FIG. 4

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