Combined therapy with COX-2 inhibitor and 20-HETE inhibitor reduces colon tumor growth and the adverse effects of ischemic stroke associated with COX-2 inhibition

Yi Zhang,1,4 Md Nasrul Hoda,2 Xuan Zheng,3,5 Weiguo Li,1 Pengcheng Luo,6 Krishna Rao Maddipati,7 Tsugio Seki,1 Adviye Ergul,1 and Mong-Heng Wang8

1Department of Physiology, Georgia Regents University, Augusta, Georgia; 2Department of Medical Laboratory, Imaging & Radiologic Sciences, College of Allied Health Sciences, Georgia Regents University, Augusta, Georgia; 3Institute of Molecular Medicine and Genetics, Georgia Regents University, Augusta, Georgia; 4Department of Orthopedics, Puren Hospital, Wahan University of Science and Technology, Wahan, China; 5Center for Gene Diagnosis, Zhongnan Hospital of Wuhan University, Wuhan, Hubei, China; 6Department of Pathology and Laboratory of Kidney Disease, Pathogenesis, and Intervention, Huangshi, Hubei, China; and 7Department of Pathology and Laboratory of Kidney Disease, Pathogenesis, and Intervention, Wuhan University, Wuhan, Hubei, China

Submitted 5 September 2013; accepted in final form 26 June 2014

Zhang Y, Hoda MN, Zheng X, Li W, Luo P, Maddipati KR, Seki T, Ergul A, Wang M. Combined therapy with COX-2 inhibitor and 20-HETE inhibitor reduces colon tumor growth and the adverse effects of ischemic stroke associated with COX-2 inhibition. Am J Physiol Regul Integr Comp Physiol 307: R693–R703, 2014. First published July 2, 2014; doi:10.1152/ajpregu.00422.2013.—20-Hydroxyeicosatetraenoic acid (20-HETE), Cyp4a-derived eicosanoid, is a lipid mediator that promotes tumor growth, as well as causing detrimental effects in cerebral circulation. We determined whether concurrent inhibition of cyclooxygenase-2 (COX-2) and 20-HETE affects colon tumor growth and ischemic stroke outcomes. The expression of Cyp4a and COXs and production of 20-HETE and PGE2 were determined in murine colon carcinoma (MC38) cells. We then examined the effects of combined treatment with rofecoxib, a potent COX-2 inhibitor, and HET0016, a potent Cyp4a inhibitor, on the growth and proliferation of MC38 cells. Subsequently, we tested the effects of HET0016 plus rofecoxib in MC38 tumor and ischemic stroke models. Cyp4a and COXs are highly expressed in MC38 cells. Respectively, HET0016 and rofecoxib inhibited 20-HETE and PGE2 formation in MC38 cells. Moreover, rofecoxib combined with HET0016 had greater inhibitory effects on the growth and proliferation of MC38 cells than did rofecoxib alone. Importantly, rofecoxib combined with HET0016 provided greater inhibition on tumor growth than did rofecoxib alone in MC38 tumor-bearing mice. Prolonged treatment with rofecoxib selectively induced circulating 20-HETE levels and caused cerebrovascular damage after ischemic stroke, whereas therapy with rofecoxib and HET0016 attenuated 20-HETE levels and reduced rofecoxib-induced cerebrovascular damage and stroke outcomes during anti-tumor therapy. Thus these results demonstrate that combination therapy with rofecoxib and HET0016 provides a new treatment of colon tumor, which can not only enhance the anti-tumor efficacy of rofecoxib, but also reduce rofecoxib-induced cerebrovascular damage and stroke outcomes.

rofecoxib; HET0016; tumor growth; stroke

COLORECTAL CANCER (CRC) is a major public health problem because it is estimated that 142,820 new cases and 50,830 deaths from CRC in the United States in 2013 (National Cancer Institute website). Early detection and chemoprevention of CRC are two major strategies against it. Moreover, early management of premalignant lesions can significantly reduce CRC-related mortality (20, 38). Coxibs are the potential chemopreventive agents in the clinical trials, because inflammation is associated with the development of CRC (41, 42).

Based on strong data that cyclooxygenase (COX)-2 is the major enzyme for the production of inflammatory PGE2 and PGJ2, and that COX-1 is a key enzyme in the production of cytoprotective PGs in the stomach (9), the Food and Drug Administration has approved the use of three coxibs: rofecoxib, celecoxib, and valdecoxib. Clinically, nonsteroidal anti-inflammatory drugs (NSAIDs) are the primary choice for the treatment of inflammation (19). However, use of conventional NSAIDs is often associated with significant gastrointestinal complications, such as ulcers and bleeding. Coxibs were developed to reduce the side effects of NSAIDs (9). While substantial evidence from clinical trials [Adenoma Prevention with Celecoxib (APC), Adenomatous PolyP Prevention on Vioxx (APPROVe), and Prevention of Colorectal Sporadic Adenomatous Polyps (PRESAP) trial] demonstrates that coxibs reduce and prevent the incidence of CRC (3–5), long-term use of rofecoxib is also associated with an increased risk of side effects, including stroke and cardiovascular events (5). Consequently, rofecoxib (Vioxx) and valdecoxib were withdrawn from the market; clinical trials of the use of rofecoxib were stopped in 2004. Currently, celecoxib (Celebrex), which is less potent than rofecoxib, is the only coxib on the market. Thus the current unmet need in this field is to develop a new strategy that can not only enhance the efficacy of coxibs, but also reduce coxib-induced cerebrovascular damage.

20-Hydroxyeicosatetraenoic acid (20-HETE), the ω-hydroxylation product of arachidonic acid (AA), is a lipid mediator in cerebral microvasculature and has detrimental effects on cerebral circulation (8, 23, 29, 33, 43). In addition, several studies have demonstrated that 20-HETE acts as a mitogenic and angiogenic factor that promotes the growth of different types of tumors (1, 12, 45). In the present study, we determined whether concurrent inhibition of COX-2 and 20-HETE affects colon tumor growth and ischemic stroke outcomes.

Address for reprint requests and other correspondence: M.-H. Wang, Dept. of Physiology, Georgia Regents Univ., 1120 15th St., Augusta, GA 30912 (e-mail: mwang@gru.edu).
METHODS

Animals. Eight-week-old male C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). All mice were maintained on a 12:12-h light-dark cycle and were housed five mice to a cage. All animal protocols were approved by the Institutional Animal Care and Use Committee and were in accord with the requirements of the National Research Council Guide for the Care and Use of Laboratory Animals.

Cell culture of MC38 cells, HMCHEL, RBMEC, and NCM460. Murine colon carcinoma (MC38) cells were obtained from Dr. Michael R. Shurin (University of Pittsburgh, Pittsburgh, PA). MC38 cells were maintained in complete medium consisting of DMEM supplemented with 10% heat-inactivated FBS, 100 mg/ml streptomycin, and 100 U/ml penicillin in a humidified incubator at 37°C with 5% CO2. Subconfluent cultured cells were harvested by washing T-75 flasks two times with Ca2+ and Mg2+-free Earl’s balanced salts, followed by 2-min incubation with 0.25% trypsin-EDTA (Life Technologies, Grand Island, NY) at 37°C. A human cerebral microvascular endothelial cell line (HMCHEL) was obtained from Dr. Jason Zastre (University of Georgia, Athens, GA). Primary rat brain microvascular endothelial cells (RBMEC) were prepared as described previously (30). HCMEL and RBMEC were maintained in MCDB-131 complete medium (VEC Technologies, Rensselaer, NY). NCM460, a normal human colon mucosal epithelial cell line, cells were obtained from Dr. Kebin Liu (Georgia Regents University) and were maintained in RPMI-1640 medium (Corning Cellgro, Manassas, VA).

Measurement of 20-HETE and PGE2 levels in the media and cell lysates of MC38 cells. MC38 cells were collected using 0.25% trypsin-EDTA. After centrifugation, a cell pallet was resuspended in complete medium (VEC Technologies, Rensselaer, NY). NCM460, a human cerebral microvascular endothelial cell line (HCMECL) was obtained from Dr. Kebin Liu (Georgia Regents University) and were main-
HETE-d8 was used as the internal standard for recovery and quantitation. The spectrometer (QTRAP5500, ABSCIEX) in the negative ion mode using multiple reaction monitoring under optimized conditions. 15(S)-HETE-d8 was used as the internal standard for recovery and quantitation. The concentrations of eicosanoids were determined by comparing the ratio of ion intensity of each eicosanoid vs. that of 15(S)-HETE-d8, as described previously (7, 22).

Assessment of neurovascular injury and functional outcome after ischemic stroke. MC38 tumor mice were divided into three treatment groups and treated for 3 wk with the following: rofecoxib (50 mg/l), rofecoxib (50 mg/l) plus HET0016 (5 mg·kg⁻¹·day⁻¹ ip), or vehicle. We also used age-matched non-tumor mice as control. At the end of the treatment period, mice were subjected to thromboembolic stroke. Under isoflurane anesthesia, the right common carotid artery, the right external carotid artery, and the right internal carotid artery were exposed through a middle incision on the ventral side of the neck. A PE10 catheter containing a fibrin-rich clot was gently inserted from the external carotid artery stump into the distal internal carotid artery just proximal to the origin of the middle cerebral artery. Each clot (~1 cm), prepared from a donor mouse, was injected with 75 µl of sterile PBS; after 2 min, the catheter was withdrawn as described previously (14). Occlusion was confirmed by a ≥70% drop in cerebral blood flow compared with the preischemic value. The success rate of thromboembolic middle cerebral artery occlusion was 95% based on changes in cerebral blood flow.

At day 3 after surgery, mice were first evaluated for functional outcome and then killed for evaluation of neurovascular injury, including infarct size and bleeding. Extracted brains were analyzed for infarct size in coronal slices of 2 mm thickness, labeled A-G, from front to back. Hemorrhagic transformation (HT), secondary bleeding into the brain after ischemic stroke, was evaluated by the presence of visible macroscopic bleeding and measured in a binary fashion: yes or no. Number of animals that had HT was reported per group. 2,3,5-Triphenyltetrazolium chloride, a mitochondria stain, was used to outline the infarct area. The captured images were numerically labeled and analyzed using specialized KS300 software. Infarct size was expressed as a percentage of the contralateral hemisphere. Neurological deficits in mice given different treatments were assessed at 24 and 72 h after stroke, using a 5-point scale for scoring: 0, no deficit; 1, forelimb flexion deficit on contralateral side; 2, decreased resistance to lateral push and torus turning to the ipsilateral side when held by tail; 3, significant circling to the affected side and reduced capability to bear weight on the affected side; and 4, rarely moved spontaneously and preferred to stay at rest.

Statistical analysis. All values are expressed as means ± SE. All data were analyzed by GraphPad Instat Software (LaJolla, CA). We used one-way ANOVA and Tukey-Kramer tests for multiple comparisons or independent Student’s t-test for unpaired groups. Bleeding was analyzed by Fisher’s exact test. Statistical significance was set at P < 0.05 or 0.01.

RESULTS

Cyp4a expression and 20-HETE production in MC38 cells. It is well established that Cyp4a isoforms are important for the production of 20-HETE (34). To determine whether MC38, the murine colon carcinoma, cells have the capacity to synthesize 20-HETE, cell lysates were prepared from MC38 cells for Western blot analysis. We used normal mouse colon tissue homogenates as a control. Intriguingly, the expression of Cyp4a is absent in normal colon tissue, whereas its expression is upregulated in MC38 cells (Fig. 1A).

To test whether 20-HETE production occurs in MC38 cells, we determined 20-HETE levels in the lysates and media of cultured MC38 cells. We detected 20-HETE predominantly in the cell media and found much lower amounts in cell lysates (Fig. 1B). To determine the effect of HET0016, the selective inhibitor of Cyp4a enzymes, on 20-HETE production, we incubated MC38 cells with HET0016 (1 µM) or vehicle. We found that HET0016 was effectively to block 20-HETE production in the media of cultured MC38 cells (Fig. 1C).

COX-2 expression and PGE₂ production in MC38 cells. To determine whether MC38 cells have the capacity to generate PGs, we did Western blot analysis of COX enzymes in the lysates of MC38 cells. MC38 cell lysates contained a 72-kDa protein that cross-reacted with COX-2 antibody (Fig. 2A), which is in agreement with a previous report that COX-2 is expressed in MC38 cells (17). Similarly, COX-1 is also expressed in MC38 cells. Experiments were also done to compare PGE₂ production in MC38 cells. PGE₂ was detected mainly in
the media of cultured MC38 cells (72 ng/10^6 cells in the media vs. 0.8 ng/10^6 cells in cell lysates) (Fig. 2B). Moreover, we found that rofecoxib dose dependently inhibited PGE2 production in MC38 cells (Fig. 2C), indicating that rofecoxib effectively blocks PGs synthesis in these cells.

Combination of rofecoxib with HET0016 causes an additive inhibitory effect on the growth and proliferation of MC38 cells. It is well known that HET0016 avidly binds to serum proteins (13). To determine whether HET0016 alters the inhibitory effects of rofecoxib on the proliferation of colon cancer cells, MC38 cells were cultured in serum-free medium. The growth of these cells was stimulated with EGF, a mitogen of many cells, at a concentration of 200 ng/ml, as described previously (13). Using the same culture conditions as those described earlier, both HET0016 alone (Fig. 3A) and rofecoxib alone (Fig. 3B) inhibited cell growth. Interestingly, combining rofecoxib (1 μM) with HET0016 (1 μM) increased the inhibitory
HET0016 AND ROFECOXIB ON TUMOR GROWTH AND STROKE

Effect on the growth of MC38 cells compared with rofecoxib alone (Fig. 3C). These results demonstrate that inhibition of 20-HETE enhances the inhibitory effect of rofecoxib on the proliferation of MC38 cells.

In complementary experiments, we determined whether HET0016, rofecoxib, or HET0016 + rofecoxib affect the proliferation of a normal human colon mucosal epithelial cell line, NCM460 cells. We found that neither HET0016 nor rofecoxib alone nor HET0016 + rofecoxib affected cell growth (Fig. 5A) and BrdU incorporation (Fig. 5B) in NCM460 cells. These results indicate that blockade of COX-2 and 20-HETE does not affect the proliferation of normal colon epithelial cells.

Inhibition of 20-HETE enhances anti-tumor effects of rofecoxib in MC38 colon tumor-bearing mice. To study the effects of combination therapy with rofecoxib and HET0016 on colon tumor, we subcutaneously injected 8-wk-old male C57BL/6J mice with MC38 cells (1.4 × 10^6 cells/mouse) to induce colon tumor. The effects of HET0016 alone, rofecoxib alone, and HET0016 + rofecoxib on tumor growth were determined by assessing tumor volumes at 9 days and 18 days after the implantation of MC38 cells. Treatment with HET0016 did not

Fig. 4. Effects of HET0016, rofecoxib, and rofecoxib + HET0016 on bromodeoxyuridine (BrdU) incorporation into MC38 cells. MC38 cells growing in 96-well plates were treated with HET0016 (100 nM to 10 μM; A), rofecoxib (100 nM to 10 μM; B), or HET0016 (1 μM) + rofecoxib (1 μM) (C) for 48 h. In the last 3 h, BrdU was added (10 μM final concentration). Incorporation of BrdU was determined using an ELISA kit. Results are expressed as %control and are means ± SE; n = 5. *P < 0.01 vs. vehicle. **P < 0.01 vs. other groups.

Fig. 5. Effects of HET0016, rofecoxib, and rofecoxib + HET0016 on cell numbers (A) and BrdU incorporation in NCM460 cells (B). NCM460 cells were treated with HET0016 (1 μM), rofecoxib (1 μM), HET0016 (1 μM) + rofecoxib (1 μM), or vehicle for 48 h. A: cell proliferation by counting cell numbers was determined as described in Fig. 3. B: BrdU incorporation was determined using an ELISA kit as described in Fig. 4. Values are means ± SE; n = 4 (A), n = 5 (B).

To confirm the effects of rofecoxib and HET0016 on the proliferation of colon cancer cells, MC38 cells were cultured in serum-free medium with added EGF (200 ng/ml). The effects of HET0016 alone, rofecoxib alone, and HET0016 + rofecoxib on the proliferation of MC38 cells were determined by assessing the incorporation of a thymidine analog, BrdU. HET0016 alone (Fig. 4A) concentration-dependently inhibited the incorporation of BrdU. Rofecoxib at 1 μM significantly inhibited BrdU incorporation (Fig. 4B). Notably, HET0016 (1 μM) + rofecoxib (1 μM), compared with rofecoxib alone, displayed an enhanced inhibitory effect on BrdU incorporation.

AJP-Regul Integr Comp Physiol • doi:10.1152/ajpregu.00422.2013 • www.ajpregu.org
Prolonged treatment with rofecoxib selectively increases circulating 20-HETE levels in MC38 colon tumor-bearing mice. To determine whether treatment with rofecoxib and rofecoxib + HET0016 affects circulating 20-HETE levels during anti-tumor therapy, we measured the levels of plasma 20-HETE and other eicosanoids in MC38 tumor mice given different treatments. As shown in Fig. 7, rofecoxib selectively increased 20-HETE levels in MC38 tumor mice. It is possible that the increased 20-HETE levels were caused by elevation of

affect tumor size on day 9 postimplantation, but dose dependently reduced tumor size on day 18 postimplantation (Fig. 6A). Treatment with rofecoxib significantly reduced tumor size on both day 9 and day 18 postimplantation (Fig. 6B). Interestingly, on days 9 and 18 postimplantation, treatment with HET0016 + rofecoxib increased the inhibition of tumor growth achieved with rofecoxib alone (Fig. 6B), clearly demonstrating that inhibition of 20-HETE enhances the anti-tumor efficacy of rofecoxib in MC38 tumor-bearing mice.

In a complementary experiment, we determined the water intake and body weight of mice treated with water, vehicle, and rofecoxib (50 mg/kg). We found that neither vehicle (3.02 ± 0.17 vs. 3.16 ± 0.16 ml·24 h−1·mouse−1, n = 5) nor rofecoxib (3.07 ± 0.1 vs. 3.16 ± 0.16 ml·24 h−1·mouse−1, n = 5) affected water intake. Likewise, there is no significant change of body weight among water (24 ± 1.63 g/mouse, n = 5), vehicle (23.8 ± 1.1 g/mouse, n = 5), and rofecoxib (24.5 ± 1.0 g/mouse, n = 5) groups. These results indicate that neither vehicle nor rofecoxib treatment has significant impact on eating/drinking habits.

Fig. 6. A: dose effects of HET0016 treatment on subcutaneous MC38 tumors in C57B6 mice. MC38 colon tumor-bearing mice (1.4 × 10⁶ cells/mouse, injected subcutaneously) were divided into vehicle, HET0016 (5 mg·kg−1·day−1 ip), or HET0016 (10 mg·kg−1·day−1 ip) treatment groups. After 9 or 18 days postimplantation, tumor volumes were determined using a sliding caliper. B: effects of rofecoxib + HET0016 on tumor growth in MC38 tumor mice. MC38 tumor mice were divided into vehicle, rofecoxib (50 mg/l), HET0016 (5 mg·kg−1·day−1 ip), or rofecoxib + HET0016 treatment groups. After 9 and 18 days postimplantation, tumor volumes were determined. Values are means ± SE; n = 6. *P < 0.05, **P < 0.01 vs. vehicle. #P < 0.05 vs. other groups.

Fig. 7. A: MC38 colon tumor-bearing mice were divided into 3 treatment groups: vehicle, rofecoxib (50 mg/l), or rofecoxib + HET0016 (5 mg·kg−1·day−1 ip). After 3 wk of treatment, plasma levels of 20-HETE, epoxyeicosatrienoic acids (EETs)/dihydroxyeicosatrienoic acids (DHETs), and other HETEs in tumor mice given different treatments were determined by LC/MS/MS. The levels of different eicosanoids were quantified by comparing the ratio of eicosanoids vs. 15(S)-HETE-d₈. The plasma 20-HETE, 5-HETE, 8-HETE, 11-HETE, and 15-HETE levels were 2.84 ± 0.5, 10 ± 1.4, 4 ± 0.9, 5.23 ± 0.8, and 1.34 ± 0.5 ng/ml, respectively, in the vehicle-treated group. The EETs-to-DHETs ratio in the vehicle-treated group was 0.9 ± 0.2. Values are means ± SE; n = 4. *P < 0.05 vs. vehicle. #P < 0.05 vs. rofecoxib group. B: MC38 colon tumor-bearing mice were treated with HET0016 (5 mg·kg−1·day−1 ip) or vehicle for 3 wk. At the end of treatment, plasma PGs levels were determined by LC/MS/MS. Values are means ± SE; n = 4. *P < 0.05 vs. vehicle. #P < 0.05 vs. rofecoxib group. C: effects of 3-wk treatment of rofecoxib on Cyp4a expression in the liver and kidney of MC38 tumor mice. C, control; Rof, rofecoxib treatment.
the adverse effects of rofecoxib on ischemic stroke. However, we found no significant changes in levels of EETs/DHETs, 5-HETE, 8-HETE, 11-HETE, or 15-HETE after rofecoxib treatment (Fig. 7A). Notably, HET0016 selectively reduced plasma 20-HETE without affecting EETs/DHETs, 5-HETE, 8-HETE, 11-HETE, or 15-HETE in MC38 tumor mice (Fig. 7A). To exclude that HET0016 affects COX pathway, we determined plasma PGs levels after HET0016 administration. HET0016 did not affect plasma PGE2, TxB2, and 15-keto-PGE2 levels (Fig. 7B), suggesting that HET0016 does not alter COX pathway in vivo. These results demonstrate that prolonged treatment with rofecoxib selectively induces circulating 20-HETE levels. To exclude the possibility that rofecoxib affects the expression of 20-HETE synthesizing enzyme, we determined the expression of Cyp4a in the liver and kidney. Rofecoxib had no effect on Cyp4a expression (Fig. 7C), suggesting that rofecoxib does not affect 20-HETE levels through altering the expression of Cyp4a levels.

Expression of COX-1, COX-2, and Cyp4a in HCMECL, primary RBMEC, and MBM. It is possible that increased circulating 20-HETE levels by rofecoxib are a result of a decrease in metabolism by COX-2 because 20-HETE can be metabolized by COX enzymes (6, 28, 37). To investigate whether the cerebrovasculature has the capacity to synthesize and metabolize 20-HETE, we determined the expression of COX-1, COX-2, and Cyp4a in HCMECL, primary RBMEC, and MBM. We found that COX-1 (~70 kDa), COX-2 (~72 kDa), and Cyp4a (~52 kDa) were expressed in HCMECL (Fig. 8A), primary RBMEC (Fig. 8B), and MBM (Fig. 8C). These results provide evidence that the cerebrovasculature is involved in the synthesis and metabolism of 20-HETE across species.

Combined treatment with rofecoxib and HET0016 reduces the adverse effects of rofecoxib on ischemic stroke. Since chronic treatment with rofecoxib significantly increased plasma 20-HETE levels by rofecoxib are a result of a decrease in metabolism by COX-2 because 20-HETE can be metabolized by COX enzymes (6, 28, 37). To investigate whether the cerebrovasculature has the capacity to synthesize and metabolize 20-HETE, we determined the expression of COX-1, COX-2, and Cyp4a in HCMECL, primary RBMEC, and MBM. We found that COX-1 (~70 kDa), COX-2 (~72 kDa), and Cyp4a (~52 kDa) were expressed in HCMECL (Fig. 8A), primary RBMEC (Fig. 8B), and MBM (Fig. 8C). These results provide evidence that the cerebrovasculature is involved in the synthesis and metabolism of 20-HETE across species.

Although substantial evidence from APC, APPROVe, and PreSAP clinical trials demonstrates that coxibs reduce the incidence of CRC or even prevent it (3–5), the increased risk of side effects, including stroke and cardiovascular events, stopped the use of coxibs as cancer preventive agents. The prevailing theory to explain the adverse cardiovascular effects of coxibs is that they reduce the production of PGI2, a potent inhibitor of platelet aggregation, but do not affect the production of TxA2, a potent platelet-aggregating agent (9). Thus stroke and the cardiovascular complications caused by coxibs might be a consequence of a shifting balance between the levels of PGI2 and TxA2 (10). Although this theory is attractive, it cannot fully explain why other nonselective COX inhibitors, including diclofenac, ibuprofen, naproxen, and indomethacin, also significantly increase the risk of side effects (21). Hence, more complex mechanisms may be responsible for the coxib-induced side effects.

Interestingly, Liu et al. (21) recently reported that chronic administration of rofecoxib greatly increases 20-HETE levels without affecting other eicosanoid pathways. 20-HETE has well-characterized detrimental effects on cerebral circulation (33), and it promotes the growth of tumors in the brain (13), kidney (1), and lung (45). Since 20-HETE inhibitor effectively blocks tumor growth and reduces detrimental effects in cerebral circulation, we hypothesized that cotreatment with COX-2 and 20-HETE inhibitor could be a novel strategy, not only enhancing the anti-tumor efficacy of coxibs, but also attenuating the adverse effects of rofecoxib on ischemic stroke.

DISCUSSION

Although substantial evidence from APC, APPROVe, and PreSAP clinical trials demonstrates that coxibs reduce the incidence of CRC or even prevent it (3–5), the increased risk of side effects, including stroke and cardiovascular events, stopped the use of coxibs as cancer preventive agents. The prevailing theory to explain the adverse cardiovascular effects of coxibs is that they reduce the production of PGI2, a potent inhibitor of platelet aggregation, but do not affect the production of TxA2, a potent platelet-aggregating agent (9). Thus stroke and the cardiovascular complications caused by coxibs might be a consequence of a shifting balance between the levels of PGI2 and TxA2 (10). Although this theory is attractive, it cannot fully explain why other nonselective COX inhibitors, including diclofenac, ibuprofen, naproxen, and indomethacin, also significantly increase the risk of side effects (21). Hence, more complex mechanisms may be responsible for the coxib-induced side effects.

Interestingly, Liu et al. (21) recently reported that chronic administration of rofecoxib greatly increases 20-HETE levels without affecting other eicosanoid pathways. 20-HETE has well-characterized detrimental effects on cerebral circulation (33), and it promotes the growth of tumors in the brain (13), kidney (1), and lung (45). Since 20-HETE inhibitor effectively blocks tumor growth and reduces detrimental effects in cerebral circulation, we hypothesized that cotreatment with COX-2 and 20-HETE inhibitor could be a novel strategy, not only enhancing the anti-tumor efficacy of coxibs, but also attenuating the adverse effects of rofecoxib on ischemic stroke.

Although the substrate, AA, after blocking COX-2. However, we found no significant changes in levels of EETs/DHETs, 5-HETE, 8-HETE, 11-HETE, or 15-HETE after rofecoxib treatment (Fig. 7A). Notably, HET0016 selectively reduced plasma 20-HETE levels without affecting EETs/DHETs, 5-HETE, 8-HETE, 11-HETE, or 15-HETE in MC38 tumor mice (Fig. 7A). To exclude that HET0016 affects COX pathway, we determined plasma PGs levels after HET0016 administration. HET0016 did not affect plasma PGE2, TxB2, and 15-keto-PGE2 levels (Fig. 7B), suggesting that HET0016 does not alter COX pathway in vivo. These results demonstrate that prolonged treatment with rofecoxib selectively induces circulating 20-HETE levels. To exclude the possibility that rofecoxib affects the expression of 20-HETE synthesizing enzyme, we determined the expression of Cyp4a in the liver and kidney. Rofecoxib had no effect on Cyp4a expression (Fig. 7C), suggesting that rofecoxib does not affect 20-HETE levels through altering the expression of Cyp4a levels.

Expression of COX-1, COX-2, and Cyp4a in HCMECL, primary RBMEC, and MBM. It is possible that increased circulating 20-HETE levels by rofecoxib are a result of a decrease in metabolism by COX-2 because 20-HETE can be metabolized by COX enzymes (6, 28, 37). To investigate whether the cerebrovasculature has the capacity to synthesize and metabolize 20-HETE, we determined the expression of COX-1, COX-2, and Cyp4a in HCMECL, primary RBMEC, and MBM. We found that COX-1 (~70 kDa), COX-2 (~72 kDa), and Cyp4a (~52 kDa) were expressed in HCMECL (Fig. 8A), primary RBMEC (Fig. 8B), and MBM (Fig. 8C). These results provide evidence that the cerebrovasculature is involved in the synthesis and metabolism of 20-HETE across species.

Combined treatment with rofecoxib and HET0016 reduces the adverse effects of rofecoxib on ischemic stroke. Since chronic treatment with rofecoxib significantly increased plasma 20-HETE levels by rofecoxib are a result of a decrease in metabolism by COX-2 because 20-HETE can be metabolized by COX enzymes (6, 28, 37). To investigate whether the cerebrovasculature has the capacity to synthesize and metabolize 20-HETE, we determined the expression of COX-1, COX-2, and Cyp4a in HCMECL, primary RBMEC, and MBM. We found that COX-1 (~70 kDa), COX-2 (~72 kDa), and Cyp4a (~52 kDa) were expressed in HCMECL (Fig. 8A), primary RBMEC (Fig. 8B), and MBM (Fig. 8C). These results provide evidence that the cerebrovasculature is involved in the synthesis and metabolism of 20-HETE across species.

Although substantial evidence from APC, APPROVe, and PreSAP clinical trials demonstrates that coxibs reduce the incidence of CRC or even prevent it (3–5), the increased risk of side effects, including stroke and cardiovascular events, stopped the use of coxibs as cancer preventive agents. The prevailing theory to explain the adverse cardiovascular effects of coxibs is that they reduce the production of PGI2, a potent inhibitor of platelet aggregation, but do not affect the production of TxA2, a potent platelet-aggregating agent (9). Thus stroke and the cardiovascular complications caused by coxibs might be a consequence of a shifting balance between the levels of PGI2 and TxA2 (10). Although this theory is attractive, it cannot fully explain why other nonselective COX inhibitors, including diclofenac, ibuprofen, naproxen, and indomethacin, also significantly increase the risk of side effects (21). Hence, more complex mechanisms may be responsible for the coxib-induced side effects.

Interestingly, Liu et al. (21) recently reported that chronic administration of rofecoxib greatly increases 20-HETE levels without affecting other eicosanoid pathways. 20-HETE has well-characterized detrimental effects on cerebral circulation (33), and it promotes the growth of tumors in the brain (13), kidney (1), and lung (45). Since 20-HETE inhibitor effectively blocks tumor growth and reduces detrimental effects in cerebral circulation, we hypothesized that cotreatment with COX-2 and 20-HETE inhibitor could be a novel strategy, not only enhancing the anti-tumor efficacy of coxibs, but also attenuating the adverse effects of rofecoxib on ischemic stroke.

Fig. 8. Expression of COX-1, COX-2, and Cyp4a in human cerebral microvascular endothelial cell line (HCMECL; A), rat brain microvascular endothelial cells (RBMEC; B), and mouse brain microvessels (MBM; C). Microscopic images shown on the right of A and B are views of cultured HCMECL and RBMEC, respectively. We used 30–50 μg of protein from different samples for Western blot analysis. Based on protein standards (Bio-Rad), the respective sizes of COX-1, COX-2, and Cyp4a are ~70, ~72, and ~52 kDa, respectively.
HET0016 and rofecoxib on tumor growth and stroke

Fig. 9. MC38 colon tumor-bearing mice treated with vehicle, rofecoxib (50 mg/l), or rofecoxib + HET0016 (5 mg·kg⁻¹·day⁻¹ ip), as well as control mice (nontumor), were subjected to ischemic stroke using a modified thromboembolic model. A: presence of bleeding was evaluated as a measure of vascular injury. Four out of five animals in the rofecoxib group developed macroscopic bleeding into the brain, while there was no visible bleeding in any other groups. #P = 0.0008 (Fisher’s exact test). B: infarct size was significantly smaller in rofecoxib-treated groups. *P < 0.05 vs. vehicle. A representative image from each group is shown on top. C: functional outcome was greatly improved in the rofecoxib + HET0016 group, as indicated by a lower neurological deficit score. **P < 0.01 vs. other groups. Values are means ± SE; n = 4 in control and n = 5–7 in other groups.

To determine the role of 20-HETE in colon cancer, we addressed the following questions. Is 20-HETE produced in colon cancer cells? If so, what is the action of 20-HETE in these cells? In addressing these questions, we found that the expression of Cyp4a is absent in normal colon tissue, whereas its expression is upregulated in colon carcinoma cells (Fig. 1A). These results suggest that increasing 20-HETE may play an important role in CRC development because previous reports have shown that 20-HETE not only promotes the proliferation of renal adenocarcinoma cells (1) and human glioma cells (12), but also stimulates angiogenesis in non-small lung cancer cells (45). Further investigation is needed to determine whether Cyp4a is also upregulated in human colon cell lines and whether 20-HETE inhibitor is effective to block human colon tumor growth. Interestingly, a previous report (15) has demonstrated that the expression of Cyp epoxygenase 2J2 is undetectable in normal tissues, whereas its expression is upregulated in human carcinoma tissues. The exact mechanisms whereby the upregulation of Cyp4a and Cyp2J2 occurs in cancer tissues are still not clear, and it is required for further investigation.

To the best of our knowledge, the present study is the first to show that 20-HETE is produced in MC38 cells (Fig. 1). It is well known that 20-HETE synthesis is primarily catalyzed by the Cyp4a gene family (34). In the mouse, four isoforms have been identified: Cyp4a10, Cyp4a12a, Cyp4a12b, and Cyp4a14. Using baculovirus and Sf9 insect cells, Muller et al. (26), in a study of the hydroxylation of AA by mouse Cyp4a isoforms, demonstrated that AA ω-hydroxylation is catalyzed by Cyp4a10, Cyp4a12a, and Cyp4a12b. Cyp4a12a and Cyp4a12b have similar catalytic activity for 20-HETE production, with a Vmax value ~10 min⁻¹ and a Km value ~20–40 μM. Furthermore, the ω-hydroxylase activity of AA for Cyp4a10 is ~25- to 75-fold lower than that of Cyp4a12 isoforms. Therefore, it is possible that one of the mouse Cyp4a isoforms is responsible for 20-HETE synthesis in MC38 cells.

To determine the action of 20-HETE in colon cancer cells, we demonstrated that HET0016 dose-dependently inhibited the growth and proliferation of MC38 cells (Figs. 3A and 4A). Interestingly, a previous study (12) showed that overexpression of CYP4A1, a potent 20-HETE synthase, while having negligible ability to form EETs (12, 27), induces hyperproliferative phenotype in human glioma cells (U251). They also demonstrated that elevated 20-HETE levels not only increases oxidative stress and promote angiogenesis, but also induce the phosphorylation of extracellular signal-regulated kinase-1/2, cyclin D-1/2, and vascular endothelial growth factor in U251 cells. Therefore, it is possible that 20-HETE stimulates proliferation of MC38 cells through similar molecular mechanisms. Importantly, we found that 20-HETE blockade did not affect the proliferation of NCM460 cells, a normal human colon mucosal epithelial cell line (Fig. 5). These data demonstrate that the action of 20-HETE is mainly mediated in the colon cancer cells rather than in normal colon cells.

To determine whether 20-HETE inhibition enhances the anti-tumor effects of COX-2 inhibitor in colon cancer cells, we addressed the question of whether the combined therapy with rofecoxib and HET0016 would have an increased inhibitory effect on the proliferation of colon cancer cells in vitro and...
tumor growth in vivo. Our data showed that, although mono-
therapy with either HET0016 or rofecoxib reduced the prolif-
eration of MC38 cells and tumor growth, greater anti-prolifera-
tive and anti-tumor effects were obtained when rofecoxib was
administered in combination with HET0016 (Figs. 4 and 6).

Although the present study provided new information about
the additional inhibitory effect of rofecoxib + HET0016 on the
growth of colon tumor, the exact mechanisms whereby this
combination blocked tumor growth were still not known. It is
possible that PGE2, the major COX-2-derived PG, was mainly
present in the medium of MC38 cells (Fig. 2B), which is
consistent with the notions that PGE2 is secreted by cancer
cells, and that multidrug resistance protein-4 is involved in this
process (32). Thus the action of PGE2 to promote MC38 tumor
growth may have been mediated through its paracrine and
autocrine action. It is well known that PGE2 promotes the
growth of CRC by stimulating angiogenesis and cell prolifera-
tion, as well as inhibiting apoptosis (42). Therefore, it is
possible that the enhanced inhibitory effect of rofecoxib by
HET0016 (Fig. 6B) on tumor growth was because this combi-
nation blocked both the paracrine/autocrine action of PGE2 and
mitogenic and angiogenic action (1, 12, 45) of 20-HETE in
colon cancer cells. Moreover, because CRC is a heterogeneous
disease, involving multiple dysregulated pathways (40, 42), the
cotreatment therapy with rofecoxib and HET0016 may in-
crease therapeutic efficacy.

To determine whether 20-HETE inhibition reduces the ad-
verse effects of rofecoxib on stroke during anti-tumor therapy,
we addressed the following questions. Does prolonged use of
rofecoxib elevate 20-HETE levels? If so, does 20-HETE in-
hibitor attenuate the adverse effects on stroke during anti-
tumor therapy? We found that 3 wk of anti-tumor therapy with
rofecoxib significantly increased circulating 20-HETE levels
(Fig. 7). This increase was highly selective because rofecoxib
treatment did not affect levels of EETs/DHETs, 5-HETE,
8-HETE, 11-HETE, or 15-HETE (Fig. 7). These results are
consistent with the finding in a previous report (21) that, in
C57BL/6J mice, the administration of rofecoxib causes greatly
induction of 20-HETE levels without affecting circulating
levels of LOX- and COX-derived eicosanoids.

The exact mechanisms whereby rofecoxib induces 20-HETE
are still unknown. Since rofecoxib treatment does not affect the
expression of Cyp4a in the liver and kidney of MC38 tumor
mice (Fig. 7C), the reason for the induction of circulating
20-HETE levels by rofecoxib (Fig. 7A) may not be a conse-
quence of the increases in endogenous 20-HETE synthesis. It
is well established that 20-HETE can be metabolized by COXs
into PGs, such as 20-OH PGE2 and 20-OH PGF2-α (6, 21).
Therefore, it is possible that the increase in 20-HETE levels
induced by prolonged use of rofecoxib in vivo is caused by
inhibition of the metabolizing pathways of 20-HETE by COX
enzymes in brain microvessels. To test this possibility, we
determined whether the cerebrovasculature has the capacity to
carry out 20-HETE synthesis and metabolism. We found that
COX-1, COX-2, and Cyp4a are expressed in HCMECL, RBMECs,
and MBM (Fig. 8), providing evidence that the cerebrovascu-
lature is involved in 20-HETE synthesis and metabolism.

Although the clinical trials have established that regular use
of coxibs over 10–15 yr reduces the risk of CRC by 40–50%
(42), prolonged use of coxibs was associated with higher risk
of stroke and cardiovascular events. Thus a great deal of effort
has focused on reducing the side effects of coxibs. One ap-
proach to achieve this goal is to use lower doses of coxibs in
combination with other complementary agents. Accumulating
evidence has demonstrated that combination treatment pro-
vides better efficacy in anti-cancer therapy with coxibs. For
example, combining celecoxib with sorafenib, a multikinase
inhibitor, enhanced inhibition of the growth of hepatocellular
carcinoma cells (24). Combined therapy of rofecoxib with S-1,
a oral fluoropyryazine drug, produced more potent efficacy to
restrain liver metastasis of LM-H3 cells than each agent alone
(39); and celecoxib combined with AEE788, a tyrosine kinase
inhibitor, potentiated celecoxib-mediated inhibition of prolif-
eration and angiogenesis in HCT 15 colon cancer cells (40). In
the present study, we showed that rofecoxib + HET0016
provides better blockage of MC38 tumor growth than does
rofecoxib alone (Fig. 6). Therefore, combined treatment with
rofecoxib and HET0016 could be clinically important for the
treatment of CRC because it would allow the use of a lower
dose of rofecoxib, which could reduce complications induced
by its prolonged use.

To determine whether chronic treatment with rofecoxib
causes the adverse effects associated with stroke, nontumor
mice and MC38 tumor mice treated with vehicle, rofecoxib, or
rofecoxib + HET0016 were subjected to embolic stroke using
a humanized clot model. HT, which is secondary bleeding into
the brain after an ischemic event, is an important complication
of ischemic stroke. Studies have shown that disability and
death occur more frequently in ischemic stroke patients who
develop HT (18). We found that HT was significantly in-
creased in rofecoxib-treated mice. Four out of the five animals
in that group developed HT, whereas there was no visible
bleeding in the other groups (Fig. 9). Thus chronic adminis-
tration of rofecoxib was associated with increased complica-
tions of ischemic stroke. These results are in agreement with
clinical data showing long-term use of rofecoxib is associated
with a risk of stroke (5, 36). It is well known that 20-HETE is
a potent vasoconstrictor of cerebral arteries (11); it also in-
creases platelet aggregation and shortens bleeding time (21); it
contributes to the development of cerebral vasospasm (35); it
also contributes to neuronal cell death after ischemia-reperfu-
sion injury (33); and it is involved in neuronal excitotoxicity
in vivo (43). It is possible that elevated levels of 20-HETE
induced by rofecoxib during anti-tumor therapy (Fig. 7) trigger
the detrimental action of 20-HETE in cerebral circulation,
thereby causing the adverse effects associated with ischemic
stroke. Notably, combined treatment with rofecoxib + HET0016
attenuated the adverse effects associated with ischemic stroke
induced by rofecoxib (Fig. 9). These results demonstrate that
this combination therapy can enhance anti-tumor effects, but
reduce deleterious cerebrovascular events. Since the relative
degree of selectivity for COX-2 inhibition is rofecoxib >>
celecoxib (2), further investigation is needed to determine
whether combination therapy with 20-HETE inhibition and
celecoxib is also effective to prevent celecoxib-induced side
effects.

Previous studies documented that 20-HETE analogs, includ-
ing WIT0013 and N-[2-hydroxyeicosa-5(Z),14(Z)-dienoyl-
lycine (5,14–20-HEDGE), and 20-HETE antagonist (WIT002)
are important tools to study the function of 20-HETE pathway
in vivo. For example, Regner et al. (31) found that 5,14,20-
HEDGE protects kidney from ischemia-reperfusion injury.
Another report (44) demonstrated that WIT0013 increases cerebral vascular tone, whereas WIT0012 attenuates 20-HETE-induced vasoconstriction response in vascular smooth muscle cells. Thus it will be interesting to determine the roles of 20-HETE analogs and antagonists in coxib-induced stroke event.

**Perspectives and Significance**

We have made the novel findings that 20-HETE is produced in colon cancer cells, where it promotes the proliferation of cancer cells and tumor growth. In vitro and in vivo, combined treatment with rofecoxib + HET0016 displayed greater anti-proliferative and anti-tumor effects than did rofecoxib alone. Our LC/MS/MS analysis supported the notion that prolonged use of rofecoxib increases circulating 20-HETE levels, which could trigger cerebrovascular events, during anti-tumor therapy. It appears that combination with rofecoxib and HET0016 significantly reduces rofecoxib-induced cerebrovascular damage and stroke outcomes. Therefore, this study provides a novel intervention strategy that concurrent inhibition of 20-HETE and COX-2 can not only enhance anti-tumor efficacy of coxibs, but also prevent coxib-induced adverse stroke events.

**ACKNOWLEDGMENTS**

The authors thank Jeanne D. Cole for editorial assistance.

**GRANTS**

This study was supported by the following grants: American Heart Association Grant-in-Aid grants (AHASE00054; AHASE00090) to M.-H. Wang; National Institute of Neurological Disorders and Stroke Grant NS-054688 to A. Ergul; National Natural Science Foundation of China (NSFC81100341) to P. Luo; and Division of Research Resources Grant S10-RR-027926 to K. R. Maddipati.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


