Cerebral ischemia–reperfusion injury is characterized by a profound transient reduction in cerebral blood flow (CBF) that is followed by reperfusion. During the reperfusion period, ischemic edema and physiological abnormalities of neuroelectrical function may develop progressively. Clinically, ischemic edema formation is a major factor contributing to poor outcome after a transient cerebral ischemic insult. One approach to reducing the clinical effects of cerebral ischemia–reperfusion injury is to develop therapies that protect the neuronal cells from its secondary metabolic effects.

Several studies have demonstrated that the onset of cerebral ischemia is associated with the breakdown of cellular phospholipids and the subsequent accumulation of free fatty acids including arachidonic acid. In contrast to the generation of arachidonate, the conversion of arachidonic acid to the prostaglandins by cyclooxygenase and to the leukotrienes by lipoxygenase is greatly enhanced in the brain during reperfusion following ischemia. Higher levels of leukotrienes, with their strong vasoconstrictor activity and ability to increase vascular permeability, may contribute to the development of ischemic edema. The efficacy of lipoxygenase inhibitors in modifying edema formation has been tested in several models of brain injury. The studies of Unterberg, et al., indicated that BW755C, a nonspecific inhibitor of lipoxygenases, did not attenuate edema formation after a freezing cortical injury in rats. However, the BW755C that was used in those studies inhibits 5-, 12-, and 15-lipoxygenases as well as cyclooxygenase. On the other hand, studies in our laboratory have demonstrated that nordihydroguaiaretic acid, another nonspecific inhibitor of lipoxygenases, reduced edema formation after transient ischemia in gerbils. Unlike nonspecific lipoxygenase inhibitors, specific 5-lipoxygenase inhibitors appear to be more consistent in reducing edema formation in several models of cerebral ischemia. Thus, the studies of Minamisawa and coworkers indicate that ONO-LP-016 and AA-861, specific 5-lipoxygenase inhibitors, decreased edema formation after transient ischemia in spontaneously hypertensive rats. Recently Mabe and colleagues also showed that AA-861 decreased edema formation after ischemia in the rat forebrain. In the present study, we examined the effect of AA-861 on brain levels of leukotriene C4 (LTC4) and correlated those changes with changes in edema formation and regional cerebral blood flow (rCBF) after transient ischemia in gerbils.
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Materials and Methods

In these studies, we carefully adhered to the animal welfare guidelines set forth in the Guide for the Care and Use of Laboratory Animals, U.S. Department of Health and Human Services, Publication No. 85-23, 1985.

Preparation of Animals

Forebrain ischemia was induced in Mongolian gerbils weighing from 47 g to 80 g. The animals were anesthetized by means of an intraperitoneal dose of combined anesthetic medications (87 mg/kg ketamine hydrochloride and 13 mg/kg xylazine). In each gerbil, both common carotid arteries were isolated and dental floss was looped around each artery. The end of a double-lumen vinyl tube (2.5 cm long, 1.1 mm inner diameter, 2.5 mm outer diameter) was placed next to each carotid artery with one end exiting the gerbil just posterior and inferior to the ear. The tube had a double lumen to avoid twisting of, or adhesion between, the lengths of floss. Two double-lumen tubes were used per animal. The tubing was held in place with cyanocrylate adhesive applied to the skin of the exit wound. Each end of the dental floss was passed through a separate tube lumen to form a snare by which ischemia could be produced by applying traction to the floss. Two aneurysm clips were used to hold the floss. Bilateral carotid occlusion was initiated in this fashion in the experimental groups of gerbils. The surgical procedure to occlude the carotid artery was performed as previously described.13

At the end of 20 minutes, the aneurysm clips were removed and the carotid blood flow was reestablished. The control animals underwent identical surgical procedures, but their carotid arteries were not occluded.

Measurement of Leukotriene C₄

In this study, the gerbils were treated intragastrically with AA-861 in the amount of 200 mg/kg (four animals), 500 mg/kg (four animals), or 1000 mg/kg (28 animals) or with vehicle (1 ml of 7.5% gum arabic solution, 28 animals). The doses of AA-861 were dissolved in 1 ml of vehicle. One hour later, the experimental gerbils were subjected to a 20-minute occlusion followed by a reperfusion period lasting 10 minutes, 1 hour, 2 hours, or 6 hours. Twenty-four gerbils were used as a control group. With the exception of the 10-minute reperfusion period, all gerbils were reanesthetized with the anesthetic mixture at the end of each reperfusion period. The gerbils were then decapitated and their heads placed in liquid nitrogen. The brains were removed and the frontal cortex region was dissected and frozen in liquid nitrogen to measure brain levels of LTC₄. The frozen brains from each group were homogenized in 10 ml of a mixture of 0.1% acetic acid/100% methanol. After centrifugation of the homogenates at 35,000 rpm at 4°C for 1 hour, the supernatants were brought to a 25% methanol concentration by addition of 0.1% acetic acid. The resulting extracts were loaded onto 2-ml Sep-Pak columns that previously had been washed with 5 ml of 0.1% acetic acid in methanol and 5 ml of 0.1% acetic acid. After washing the Sep-Pak columns with 5 ml of 0.1% acetic acid and 5 ml 0.1% acetic acid containing 25% methanol, LTC₄ was eluted with 1 ml of 0.1% acetic acid containing 90% methanol. The eluates were dried under nitrogen and resuspended in enzyme-linked immunosorbent assay (ELISA) buffer. Brain levels of LTC₄ were measured according to the ELISA technique. Our preliminary studies revealed an approximately 85% rate of recovery when the leukotriene standards also underwent the same extraction procedures.

Measurement of Specific Gravity

Cerebral edema formation was assessed by measuring specific gravity by the method described earlier.10 The animals were treated with vehicle (12 animals) or 1000 mg of AA-861 (12 animals) 1 hour before surgery. Then the animals were subjected to a 20-minute occlusion of the bilateral carotid artery and decapitated after 1 minute. The cerebral edema tissues were quickly removed and 2-mm³ samples of the cortical and subcortical brain regions were dissected away. The striatum and caudoputamen were included in the subcortical brain sample. These regions were placed in a continuous density gradient column of kerosene and bromobenzene, and their specific gravity was recorded after 1 minute.

Measurement of Regional Cerebral Blood Flow

In this study, all animals were treated with vehicle or drug 1 hour before surgery. After the carotid arteries were isolated, the gerbil was placed on a stereotactic frame and a 2.3-mm diameter hole was made in the skull to expose the left frontal cortex. The concentric Doppler probe was placed on the exposed cortex. The temperature of the gerbil was maintained at 37°C. The CBF was monitored with a blood perfusion monitor. After obtaining steady readings, ischemia was induced by occluding the carotid arteries in six animals treated with vehicle and six animals treated with 1000 mg/kg of AA-861. The gerbils were kept anesthetized throughout the 6-hour reperfusion period during which rCBF was measured. The anesthetic mixture (14 mg/kg ketamine and 2 mg/kg xylazine administered intraperitoneally) was supplemented hourly throughout the experiment. The flow measurements in the control animals were performed similarly but with no occlusion (six animals treated with vehicle and six animals treated with 1000 mg/kg of AA-861).

Statistical Analysis

All measurements were expressed as the mean ± standard error of the mean and data were analyzed across treatment groups using one-factor analysis of variance (ANOVA). If probability was less than 0.05 for the ANOVA, group comparisons were made using the Fisher’s protected least-significant difference with significance assigned to probability less than 0.05. All analyses were performed using a commercially available statistical software program.

Sources of Supplies and Equipment

In the LTC₄ measurement experiments, we used 2-ml Sep-Pak columns available from Waters & Associates, Milford, MA. The ELISA technique was accomplished using a kit obtained from Cayman Co., Ann Arbor, MI. Solvents used in the isolation of LTC₄ from the brain were obtained from Fisher Scientific Products, Pittsburgh, PA.

Cerebral blood flow was monitored with a Laserflo blood perfusion monitor, model BPM 403A, Vasamedics Inc., St. Paul, MN. All statistical analyses were performed using the StatView 512+ statistical package from Brain Power, Inc., Agoura Hills, CA, for use with the Macintosh SE computer, available from Apple Computer, Inc., Cupertino, CA.

Results

Effect of AA-861 Pretreatment on Brain Levels of Leukotriene C₄

Global cerebral ischemia–reperfusion injury in gerbils produced a significant increase in brain levels of LTC₄; this increase was most prominent at 2 hours of reperfusion (22 ± 7 ng/g wt, p < 0.05) (Fig. 1). Pretreatment with doses of both 500 and 1000 mg/kg AA-861 significantly inhibited brain levels of LTC₄ at 2 hours of reperfusion; however, inhibition was almost complete with the 1000-mg/kg dose (1.05 ± 0.2 ng/g wt, p < 0.05).

No changes were observed in brain levels of LTC₄ in the control animals throughout the experiment (Fig. 2). In the experimental animals an increase in brain levels of LTC₄ began at 1 hour, peaked at 2 hours, and declined between 2 and 6 hours of reperfusion. A dose of 1000 mg/kg AA-861 inhibited these increases of LTC₄ at 1, 2, and 6 hours of reperfusion (p < 0.05).
Effect of AA-861 Pretreatment on Regional Cerebral Edema

Increased cerebral edema corresponds to decreased specific gravity. A comparison of regional cerebral edema in the cortex and subcortex was made among control animals, experimental animals pretreated with vehicle, and experimental animals pretreated with 1000 mg/kg AA-861. At 6 hours of reperfusion, significant edema formation was found in the cortices of experimental animals pretreated with vehicle (p < 0.05) but not in the cortices of animals pretreated with AA-861 at a dose of 1000 mg/kg (p < 0.05) (Fig. 3). At 6 hours of reperfusion, significant edema formation was also found in the subcortex; this edema formation was found to be decreased with AA-861 pretreatment (data not shown). At 2 hours of reperfusion, no significant edema formation was observed in the cortices or subcortices of any animals.

Effect of AA-861 Pretreatment on the Regional Cerebral Blood Flow

Regional CBF was compared among groups of control animals pretreated with vehicle, control animals pretreated with AA-861, experimental animals pretreated with vehicle, and experimental animals pretreated with AA-861. No significant differences in rCBF were found in control animals pretreated with vehicle or with AA-861. The rCBF was reduced to 10% and 7.6% of baseline by bilateral carotid occlusion in experimental animals treated with vehicle and AA-861, respectively. The rCBF was increased to 73% and 68.5% of baseline, respectively, at 1 hour after reperfusion was established. Pretreatment with AA-861 did not improve rCBF throughout the experiment (Fig. 4).

Discussion

Several studies, including those from our laboratory, have demonstrated that brain levels of leukotrienes B₄ (LTB₄), C₄, and D₄ (LTD₄) are increased during reperfusion after transient cerebral ischemia in gerbils and rats.²⁹,³₀,³¹,³² Although the increase in LTB₄ is modest in gerbil brains during reperfusion, previous studies from our laboratory have indicated that pretreatment with nordihydroguaiaretic acid lowered LTB₄ levels and reduced cerebral edema after transient ischemia. Although increases in LTB₄ in gerbil brains during reperfusion are modest, increases in LTC₄ and LTD₄ are marked.⁹,¹⁰,²² This study was designed to examine the role of LTC₄ in transient ischemia-induced changes in CBF and the development of ischemic cerebral edema. Other studies have shown that a nonspecific lipoxygenase inhibitor, such as BW755C, did not decrease the development of edema after a freezing cortical injury in rats;³₀ therefore, in the present study we used AA-861, a specific 5-lipoxygenase inhibitor.

The present results indicate that more than 200 mg/kg of AA-861 is required to inhibit the marked reperfusion-induced increases in LTC₄ in gerbils. The reduction was approximately 58% at 500 mg/kg and 95% at 1000 mg/kg. These observations are somewhat different from those of Minamisawa, et al.,²¹ who found that 200 mg/kg AA-861 reduced the increase in LTC₄ by 65% in rats. This discrepancy may arise either from differences in species or from differences in the regimen of the AA-861 administration. Minamisawa, et al.,²¹ administered a total of 200 mg/kg AA-861 in two doses through the same route used in this study. The present results also show that the maximum accumulation of LTC₄ occurred at approximately 2 hours of reperfusion after 20 minutes of ischemia. These observations are somewhat different from those of Moskowitz and colleagues,²² who observed maximum increases in leukotriene at 15 minutes of reperfusion after 15 minutes of ischemia; however, their study measured total leukotrienes. These observations indicate that the maximum generation of different leukotrienes may occur at...
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Gerbils were pretreated with either vehicle or AA-861 (1000 mg/kg) and then subjected to 20 minutes of occlusion. Gerbils in the control group were not pretreated or subjected to occlusion. Specific gravity was significantly different between the control animals and the ischemic animals pretreated with vehicle ($p < 0.05$); pretreatment with AA-861 increased the specific gravity of the cortex ($p < 0.05$), $* p < 0.05$ compared to control.

Brain edema is an important contributing factor in the evolution of tissue damage after ischemia–reperfusion injury, causing progressive microcirculatory compression and eventual death. One approach to reducing the brain edema induced by ischemia–reperfusion injury is pharmacological therapy, which may prevent secondary metabolic events that lead to brain edema formation, such as inhibition of leukotrienes with AA-861. The present results indicate that pretreatment with AA-861 (1000 mg/kg) in gerbils subjected to 20 minutes of transient ischemia resulted in increases in specific gravity (that is, less edema formation) in both the cortical and subcortical regions. This effect was best seen after 6 hours of reperfusion. Although the specific gravity of the cortical and subcortical regions was greater in AA-861–pretreated gerbils than in vehicle-pretreated gerbils at 2 hours reperfusion, the differences did not reach statistical significance. However, at 6 hours reperfusion, the differences in the specific gravities of both the cortical and subcortical regions between the AA-861–pretreated gerbils and the vehicle-pretreated gerbils did reach statistical significance. This observation indicates that pretreatment of gerbils with AA-861 inhibited the ischemia-induced development of edema. It is possible that the use of the anesthetic agent ketamine (87 mg/kg), known as a partial N-methyl-D-aspartate receptor antagonist, might have caused reduction in brain edema; however, those reductions would probably be modest because ketamine produced only marginal attenuation of ischemia-induced neuronal cell injury and behavioral deficits at a dose of 100 mg/kg.

The present observation that pretreatment of gerbils with AA-861, a specific 5-lipoxygenase inhibitor, reduced the reperfusion-induced increase of brain LTC$_4$, inhibited the development of edema indicates that LTC$_4$ is involved in the development of ischemic edema. This suggestion is also consistent with other reports that AA-861 pretreatment inhibited the development of edema following ischemia in normotensive and hypertensive rats. However, such a suggestion of involvement of LTC$_4$ in brain injury is not consistent with the observations of Un-terberg and colleagues that BW755C, a nonspecific inhibitor of lipoxygenases, failed to attenuate edema formation after a freezing cortical injury in the rat. This discrepancy could have occurred because BW755C caused an insufficient inhibition of brain LTC$_4$; its order of inhibitory potencies from highest to lowest is cyclooxygenase, 12-lipoxygenase, 5-lipoxygenase, and 15-lipoxygenase. Therefore, in addition to LTB$_4$, LTC$_4$ is likely to be involved in the development of ischemic brain edema.

The generation of LTC$_4$ enhanced by 5-lipoxygenase after cerebral ischemia is probably a consequence of the accumulation of arachidonic acid during ischemia. Arachidonic acid is metabolized by cyclooxygenase and lipoxygenase to generate the prostaglandins and leukotrienes, respectively. It is possible that the inhibition of leukotriene synthesis may result in an increased conversion of arachidonate to prostaglandins, which may effect CBF. Therefore, in this study we also examined the effect of pretreatment with AA-861 on CBF after ischemia. The present results indicate that pretreatment with AA-861 did not change CBF during reperfusion after ischemia. Thus, the decrease in ischemic brain edema caused by AA-861 pretreatment may primarily involve LTC$_4$. The decrease in LTC$_4$ induced by pretreatment with AA-861, probably caused a decrease in ischemia-induced changes in vascular permeability and a subsequent increase in specific gravity, consistent with our previous observations that maximum inhibition of edema formation after a freezing cortical injury in the rat. This discrepancy could have occurred because BW755C caused an insufficient inhibition of brain LTC$_4$; its order of inhibitory potencies from highest to lowest is cyclooxygenase, 12-lipoxygenase, 5-lipoxygenase, and 15-lipoxygenase. Therefore, in addition to LTB$_4$, LTC$_4$ is likely to be involved in the development of ischemic brain edema.

**Fig. 3.** Bar graph displaying specific gravities of the cortex at 2 hours and at 6 hours of reperfusion after a 20-minute occlusion. Gerbils were pretreated with either vehicle or AA-861 (1000 mg/kg) and then subjected to 20 minutes of occlusion. Gerbils in the control group were not pretreated or subjected to occlusion. Specific gravity was significantly different between the control animals and the ischemic animals pretreated with vehicle ($p < 0.05$); pretreatment with AA-861 increased the specific gravity of the cortex ($p < 0.05$). $* p < 0.05$ compared to control.

**Fig. 4.** Graph showing cerebral blood flow as a function of reperfusion after 20 minutes of occlusion. Ischemic gerbils were pretreated with either vehicle or AA-861 (1000 mg/kg) and then subjected to 20 minutes of occlusion. Control gerbils were pretreated with either vehicle or AA-861 (1000 mg/kg) and were not subjected to occlusion.
reduction in the development of edema. This suggestion is consistent with the observations of Baba and associates that the intracarotid infusion of LTC_4 selectively increased blood-brain barrier permeability after focal ischemia in rats.

In conclusion, the findings reported in this study indicate that LTC_4 production during reperfusion after ischemia may be an important contributing factor to the development of brain edema because edema is attenuated by using pretreatment with AA-861 to inhibit LTC_4 production. Future studies are required to obtain the therapeutic window for AA-861.

References


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