ATTENUATION OF POSTISCHEMIC BRAIN HYPOPERFUSION AND REPERFUSION INJURY BY THE CYCLOOXYGENASE–LIPOOXYGENASE INHIBITOR BW755C

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Arachidonic acid metabolites are believed to be important mediators of tissue injury during reperfusion after cerebral ischemia. To determine whether inhibiting the oxygen-dependent metabolism of arachidonic acid would reduce reperfusion injury, we administered the mixed cyclooxygenase–lipoxygenase inhibitor BW755C (3-amino-1-[m(trifluoromethyl)phenyl]-2-pyrazoline) near the time of reperfusion in a rat model of temporary focal ischemia. The duration of ischemia + reperfusion was 2 hours + 22 hours, 3 hours + 3 hours, or 3 hours + 21 hours. The effects of drug or saline treatment on infarct volume, blood-brain barrier permeability, and blood flow were determined. Cortical blood flow was monitored with laser Doppler flowmetry and blood-brain barrier permeability was evaluated by the Evans blue dye method. Infarct volume was determined in all groups by computerized image analysis of Nissl-stained sections. We found that BW755C treatment significantly attenuated postischemic hypoperfusion in the 3 + 3 group (p < 0.05) and reduced the volume of Evans blue dye staining in the cortex (p < 0.01) and basal ganglia (p < 0.05). Hemispheric swelling was reduced in all treatment groups (p < 0.01), as was total infarct volume in the ischemic hemisphere (p < 0.05). These results support the hypothesis that arachidonic acid metabolites contribute to acute postischemic reperfusion injury and suggest that using a mixed cyclooxygenase–lipoxygenase inhibitor as an adjunct to thrombolytic or revascularization therapy could lengthen the ischemia time after which reperfusion is beneficial.

KEY WORDS • cerebral ischemia • reperfusion • permeability • infarction • eicosanoids • cerebral blood flow
monooxygenase inhibition; the agent has nonspecific antioxidant effects and may induce histamine release.23,35,48 To determine if combined blockage of lipoxygenases and cyclooxygenase could reduce cerebral reperfusion injury, the effects of administering BW755C near the time of onset of reperfusion were studied in a rat model of temporary focal ischemia.48 The effects of drug or saline treatment on infarction volume, BBB permeability, and blood flow were determined.

Materials and Methods

Animal Preparations

Sixty-six male Sprague-Dawley rats weighing between 280 and 300 g were anesthetized with 3.5% isoflurane, intubated, and then ventilated with 1.5% isoflurane in an air/oxygen mixture (90%: 10%). One femoral artery and one femoral vein were cannulated for administering drugs, determining arterial blood gases, and monitoring blood pressure. The contralateral temporalis muscle temperature was continuously measured until it remained stable for 30 minutes. The rats were decapitated. The brains were fixed by intracardiac perfusion through the ascending aorta with heparinized 0.9% saline followed by 4% formaldehyde in a pH 7.4, 0.1 M phosphate-buffered saline solution. The brains were then removed and sliced coronally at 1, 3, 5, 7, 9, and 11 mm from the frontal pole. The slices were photographed with ASA 100 negative film, which was developed using the C41 process. The dried areas of the cortex and the basal ganglia in each coronal section were measured with a computerized image analysis system (MCID, St. Catharines, Ontario, Canada). The abnormal permeability volume of the dye in each brain was estimated by adding the dried areas of the six sections and multiplying the sum by the thickness of the sections (2 mm).

Histological Studies

Hemisphere Infarct Volume. The volume of infarction in the ischemic hemisphere was determined in the rats that survived until sacrifice in each of the three groups (13 in the 3 + 3 group, 14 in the 3 + 21 group, and 19 in the 2 + 22 group). The brains were rapidly removed and frozen in −30°C 2-methylbutane, then placed in powdered dry ice. Twenty-micrometer-thick serial coronal sections were obtained every 0.4 mm from the anterior limit of the caudate to the posterior hippocampus (20 to 22 sections per brain). The sections were stained with cresyl violet for computerized image analysis of infarct volume. To minimize the influence that swelling in the infarcted brain has on quantification, the area of infarction in each section was determined by subtracting the area of normally staining brain in the ischemic hemisphere from that in the contralateral hemisphere.42 The infarct volume was then calculated by adding the infarct areas measured in each section and multiplying the sum by the distance between sections (0.4 mm).

Hemispheric Swelling. The amount of hemispheric swelling was used as a measure of histological edema. The two areas of the normal and ischemic hemispheres were determined using the image analysis system. The total hemispheric volume was calculated from all 20 to 22 sections as described above. The percentage of enlargement of the ischemic hemisphere was calculated by dividing the dif-
Treatment of reperfusion injury with BW755C

Data Analysis

All histological analyses were performed by an individual blinded to the animal’s experimental protocol. Changes in cortical blood flow between groups were analyzed using repeated-measures analysis of variance with pairwise contrasts. Differences in infarct volume, Evans blue dye volume, and hemispheric swelling between groups were examined with one-tailed t-tests. These differences were considered significant at \( p < 0.05 \). The data are reported as the mean \pm standard error. Differences in physiological variables were assessed using repeated-measures analysis of variance with contrasts. Fisher’s exact test was used to compare differences in mortality between drug- and saline-treated groups.

Results

Physiological Characteristics

There was no significant difference in mean arterial blood pressure or blood gases observed between BW755C-treated animals and controls (data not shown). The mortality for the 3 + 21 group was 40% for control and 20% for BW755C-treated animals. The mortality rate in the 2 + 22 group was 10% for control and 0% for BW755C-treated animals. In the 3 + 3 group, there was no mortality among drug-treated rats and 28% mortality in the control animals. None of these differences was statistically significant.

Cortical Blood Flow

The effect of BW755C on cortical blood flow during 3 hours of MCA occlusion and 3 hours of reperfusion is shown in Fig. 2. Occlusion reduced cortical blood flow to approximately 20% of baseline in the ipsilateral cortex of rats treated with BW755C and control rats. Cortical blood flow remained at this level throughout the occlusion period, with no significant difference between the groups. When the MCA was reperfused, initial temporary hyperemia resulted (cortical blood flow increased to approximately 120% of baseline) in both groups. This cortical hyperemia lasted approximately 30 minutes in both groups, giving way to delayed hypoperfusion. Delayed hypoperfusion was significantly attenuated by BW755C; by 80 minutes of reperfusion, the BW755C-treated animals had significantly higher cortical blood flow than control animals (\( p < 0.01 \)). Significant conservation of cortical blood flow by BW755C continued for the rest of the experiment.

Permeability of the Blood-Brain Barrier

The permeability of the BBB was reduced in rats treated with BW755C. The volume of Evans blue dye found in the cortex and basal ganglia was significantly less in the rats treated with BW755C than in control animals (\( p < 0.05 \); Fig. 3). Representative sections of the cortex and basal ganglia stained with Evans blue dye are shown in Fig. 4.

![Fig. 2. Graph showing the effect of BW755C on laser Doppler estimates of regional cortical blood flow over time, expressed as a percentage of baseline values in rats in the 3 + 3 group. *\( p < 0.05 \) vs. saline group. Error bars indicate standard errors.](image1)

![Fig. 3. Graph showing the effect of BW755C on Evans blue dye volume in the cortex and basal ganglia after 3 hours ischemia and 3 hours reperfusion. *\( p < 0.05 \), **\( p < 0.01 \) vs. saline group. Error bars indicate standard errors.](image2)

![Fig. 4. Photographs of sectioned rat cortex and basal ganglia showing the effect of BW755C on Evans blue dye staining in representative animals from the 3 + 3 group. Left: Evans blue dye in a saline-treated rat. Right: Evans blue dye in a BW755C-treated rat.](image3)
Hemispheric Swelling

Rats treated with BW755C had significantly reduced hemispheric swelling manifested by fractional enlargement of the ischemic hemisphere (Fig. 5 upper). These results confirm the Evans blue dye permeability in the 3 + 3 group, and reduced hemispheric swelling and infarct volume in all groups.

Histological Studies

Total infarct volume was significantly reduced by BW755C in all groups (Fig. 5 lower). These results indicate that BW755C treatment that is initiated at the onset of reperfusion remains effective in reducing infarction volume after 2 or 3 hours of ischemia.

Discussion

In this study we found that BW755C prevented delayed postischemic hypoperfusion after 3 hours of ischemia, reduced the extent of BBB disruption manifested by Evans blue dye permeability in the 3 + 3 group, and reduced hemispheric swelling and infarct volume in all groups.

Postischemic Hypoperfusion

Postischemic hypoperfusion may occur as a result of direct effects of vasoconstrictive compounds on vessels or as a secondary effect of reduced metabolic demand by the dying brain. The present study cannot differentiate between these mechanisms, but other studies have supported a direct role of arachidonic acid metabolites in postischemic hypoperfusion. The cyclooxygenase products, prostaglandins and thromboxane A₂, and the lipoxygenase products, leukotrienes C₄, D₄, and E₄, as well as 5-, 12-, and 15-HETE, are potential constrictors of the cerebral vessels. The concentrations of these metabolites are significantly elevated in the brain after ischemia and reperfusion. The time course is the same for the development of delayed postischemic hypoperfusion and for increases in metabolite concentrations, whereas delayed neuronal necrosis after ischemia occurs many hours or days after reperfusion. These results support those of other studies that have found that the vasoconstrictive action of arachidonic acid metabolites may be important in the development of postischemic hypoperfusion. One possible mechanism by which BW755C may reduce infarction is by improving blood flow during reperfusion by preventing vasoconstriction that is mediated by lipoxygenase and cyclooxygenase metabolites.

Disruption of the Blood-Brain Barrier

Disruption of the BBB after reperfusion may be mediated at least in part by arachidonic acid metabolites. Arachidonic acid and the lipoxygenase metabolites, leukotriene B₄, C₄, and E₄, produce BBB damage in brain parenchyma. Leukotriene B₄ is a potent chemoattractant and may induce the infiltration of leukocytes following cerebral ischemia and reperfusion. Leukocyte-mediated inflammation may be an important component of BBB degradation. These results support the hypothesis that arachidonic acid metabolites may mediate increases in BBB permeability during reperfusion.

Infarct Volume

Our study shows that BW755C reduces infarct volume. Although this may be due to the drug’s effect on postischemic hypoperfusion and on BBB disruption, some arachidonic acid metabolites may be important mediators of neurotoxic injury from ischemia and reperfusion. Leukotrienes and hepoxillins can induce a prolonged excitation of the Purkinje neurons. Moreover, hydroxide, a lipoxigenase inhibitor, can block calcium-induced long-term potentiation in the dentate gyrus and in area CA1 of the hippocampus; it also blocks glutamate-induced neurotoxicity in vitro. The 12-lipoxygenase metabolites may play a role in the activation of N-methyl-D-aspartate (NMDA) receptors, probably by acting as second messengers or bioregulators of ion channels. Furthermore, 12-HETE is the primary eicosanoid produced in neurons exposed to NMDA. However, because

Fig. 5. Graphs showing the effects of BW755C. Upper: Hemispheric swelling as determined by the percentage enlargement of the ischemic hemisphere. Lower: Total infarct volume. 3 + 3 = 3 hours of ischemia followed by 3 hours of reperfusion; 2 + 22 = 2 hours of ischemia followed by 22 hours of reperfusion; 3 + 21 = 3 hours of ischemia followed by 21 hours of reperfusion. *p < 0.05, **p < 0.01 vs. saline group. Error bars indicate standard errors.
BW755C does not block 12-lipoxygenase, these latter results suggest that the drug’s mechanism of action may be unrelated to direct neuronal protection. This view is supported by the ineffectiveness of BW755C in a gerbil model of global ischemia. Limitations of the Study

Many complex, interdependent pathophysiological and biochemical changes occur after ischemia. Our observation that BW755C prevents some of these pathophysiological changes does not rule out the possibility that other mechanisms could be at work. Indeed, BW755C may have nonspecific antioxidant effects not limited to the mono-oxygenase enzymes. There are also technical limitations with our study design. First, although LDF has the advantage of providing continuous measures of blood flow, it does not provide absolute quantification. In addition, our measurements were restricted to the superficial cortex and therefore may not reflect flow in deep structures. Second, because of a high mortality rate, we did not measure infarction volume at times later than 24 hours after ischemia. Consequently, we cannot definitively exclude the possibility that the drug delays rather than prevents infarction. However, although delayed neuronal death occurs for many days after global ischemia, there are no differences between infarct volumes at 24 hours, 72 hours, and 7 days after temporary focal ischemia when a correction for edema is applied. Furthermore, in this model the loss of glucose utilization is complete throughout the region of the MCA by 24 hours, suggesting that infarction is maximum at that time.

Conclusions

Despite its limitations, our study demonstrates that BW755C administered to rats immediately prior to the onset of reperfusion significantly attenuates posts ischemic cerebral hypoperfusion, limits BBB permeability, and reduces infarction volume at 24 hours, thus protecting the brain from reperfusion injury after temporary MCA occlusion. Our previous finding that infarct volume after 2 hours of ischemia is temporary/no less than that produced by permanent MCA occlusion indicates that BW755C extends, to at least 3 hours, the time during which infarction may be reduced by reperfusion. Thus, treatments such as BW755C may be useful adjuncts to thrombolytic agents. In addition, such treatments may be useful to protect the brain after temporary intraoperative cerebrovascular occlusion. These results are consistent with the hypothesis that reperfusion injury may be mediated, at least in part, by arachidonic acid metabolites.

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