Effect of Thromboxane Synthase Inhibition on Eicosanoid Levels and Blood Flow in Ischemic Rat Brain

L.C. Pettigrew, MD, J.C. Grotta, MD, H.M. Rhoades, PhD, and K.K. Wu, MD

Reperfusion of ischemic brain is associated with production of thromboxane A\textsubscript{2} (TXA\textsubscript{2}), a proaggregatory vasoconstrictor. We used an animal model of transient forebrain ischemia to study the effects of 1-benzylimidazole (1-BI), a selective inhibitor of thromboxane synthase, upon cerebral eicosanoid levels and cerebral blood flow. Male Wistar rats were subjected to 30 minutes of four-vessel occlusion. The mean±SEM brain level of TXB\textsubscript{2}, the stable metabolite of TXA\textsubscript{2}, determined after 60 minutes of reperfusion was 101±20 pg/mg brain protein in five ischemic control rats. Infusion of 10 \textmu g/g 1-BI reduced mean±SEM cerebral TXB\textsubscript{2} concentration to 11±3 pg/mg brain protein in five rats (p<0.002). Mean±SEM hemispheric cerebral blood flow measured in four ischemic control rats after 60 minutes of reperfusion was 42±9 ml/100 g brain/min compared with 104±13 ml/100 g brain/min in three 1-BI-treated rats (p<0.001). Mean±SEM hippocampal blood flow in four ischemic control rats after 60 minutes of reperfusion was 51±14 ml/100 g brain/min compared with 125±25 ml/100 g brain/min in three 1-BI-treated rats (p<0.04). We conclude that selective inhibition of thromboxane synthase may alleviate ischemic brain damage by reducing cerebral TXA\textsubscript{2} concentrations and elevating cerebral blood flow. (Stroke 1989;20:627-632)

Thromboxane A\textsubscript{2} (TXA\textsubscript{2}), an enzymic product of arachidonic acid (AA), has been implicated in causing platelet aggregation and vasoconstriction leading to the reduction of microcirculatory blood flow in various organ systems. The synthesis and release of TXA\textsubscript{2} from activated platelets may promote secondary aggregation by interacting with TXA\textsubscript{2}/prostaglandin H\textsubscript{2} (PGH\textsubscript{2}) receptors on platelet membranes.\textsuperscript{1,2} The vasoconstrictive properties of TXA\textsubscript{2} have been demonstrated in bioassay experiments showing contraction of vascular smooth muscle and reduction of renal and coronary blood flow.\textsuperscript{3,4}

Cerebral ischemia causes a massive release of AA, stearic acid, and other free fatty acids cleaved by phospholipases from phospholipid membranes in damaged cells.\textsuperscript{5-8} The release of AA is considered to be the rate-limiting step in eicosanoid metabolism within ischemic brain. The cyclooxygenase and peroxidase activities of prostaglandin endoperoxide synthase convert AA to the proaggregatory endoperoxide PGH\textsubscript{2}. Thromboxane synthase (TX synthase) in activated platelets converts PGH\textsubscript{2} to TXA\textsubscript{2}, thereby promoting secondary platelet aggregation, arterial thrombosis, and vasoconstriction in brain subjected to ischemia or subarachnoid hemorrhage.\textsuperscript{9-11}

Selective inhibition of TX synthase during early reperfusion of ischemic brain may augment recovery of microcirculatory blood flow. We tested this hypothesis by studying the effects of 1-benzylimidazole (1-BI), a selective inhibitor of TX synthase,\textsuperscript{12} in an animal model of transient forebrain ischemia.

**Materials and Methods**

We divided 33 male Wistar rats into two groups for the determination of eicosanoid levels in brain (n=20, Group A) and measurement of cerebral blood flow (CBF) (n=13, Group B). We used the four-vessel occlusion model of transient forebrain ischemia described by Pulsinelli and Brierley\textsuperscript{13} to subject the rats to 30 minutes of brain hypoperfusion before measurement of eicosanoid concentrations or CBF.

The 20 rats in Group A were subdivided further into an ischemic-treated subgroup given intravenous infusions of 10 \textmu g/g 1-BI during the 30 minutes of ischemia and for the first 30 minutes of reperfu-
sion (Subgroup A1), an ischemic-control subgroup that received vehicle intravenously by the same paradigm (Subgroup A2), a normal subgroup that did not undergo surgery (Subgroup A3), and a sham-ischemic subgroup that underwent surgical preparation and nonocclusive manipulation of the carotid arteries after 24 hours of recovery (Subgroup A4); each subgroup contained five rats. The brain of each rat was freeze-fixed in situ and concentrations of TXB$_2$ and 6-ketoprostaglandin F$_{1\alpha}$ (6kPGF$_{1\alpha}$), the stable metabolite of prostacyclin, were determined by radioimmunoassay (RIA). The brains of rats in subgroups A1 and A2 were freeze-fixed and analyzed after 60 minutes of reperfusion.

The indicator-fractionation technique of Van Uitert and Levy$^{14}$ was used to measure CBF in the 13 rats in Group B. Ischemic-treated (Subgroup B1, n=3) and ischemic-control (Subgroup B2, n=4) rats were given intravenous infusions of 1-BI or vehicle according to the paradigm for Subgroups A1 and A2. Rats in Subgroups B1 and B2 underwent measurement of CBF after 60 minutes of reperfusion following 30 minutes of ischemia. The remaining six rats (Subgroup B3) did not undergo surgery.

Male Wistar rats (Hilltop Farms, Scottsdale, Pennsylvania) weighing 250–300 g were prepared for transient forebrain ischemia by our modification of the four-vessel occlusion model.$^{13,15,16}$ The rats were anesthetized with 0.5 mg/g i.p. chloral hydrate. The common carotid arteries were tagged with loose ligatures, and the vertebral arteries were cauterized. A 10-cm length of 0-gauge surgical silk was passed through each side of the neck posterior and lateral to the common carotid artery, external jugular vein, esophagus, and trachea but medial to the cervical muscles. The ends of the sutures were secured to the dorsum of the neck. The rats were fasted during 24 hours of recovery.

Forebrain ischemia was initiated by locating the common carotid arteries again and occluding them with surgical clips. The neck sutures were tightened. Ischemia continued for 30 minutes, during which time a Grass model 79D electroencephalography (EEG) machine (Grass Instrument Company, Quincy, Massachusetts) recorded EEG data from subdermal electrodes by the method of Willmore et al.$^{17}$ Rats not demonstrating isoelectric EEG and loss of righting reflex were excluded from further study. Ischemia was terminated by removing the clips from the carotid arteries and cutting the sutures in the neck.

All rats in Subgroups A1 and A2 were anesthetized again with 0.5 mg/g i.p. chloral hydrate and paralyzed with 2 mg/kg i.p. d-tubocurarine after removal of the carotid clips. Tracheostomy tubes were inserted, and the rats were ventilated with room air administered by an animal respirator (Model 681, Harvard Bioscience, South Natick, Massachusetts). The scalp was incised along the sagittal suture and resected to expose the calvaria. After 60 minutes of reperfusion, liquid nitrogen was poured into a funnel mounted on the exposed calvaria of each rat for 3 minutes before the rat was decapitated and the head was immersed in liquid nitrogen according to the method of Pontén et al.$^{18}$ Rats in Subgroups A3 and A4 were fasted for 24 hours and treated similarly, although Subgroup A4 rats underwent nonocclusive manipulation of the carotid arteries before being anesthetized.

The details of eicosanoid measurement have been reported.$^{16,19}$ The frozen brains were chiseled out of the skulls on dry ice, weighed, and stored at −80°C. Each sample consisted of forebrain dissected free of dura, venous sinuses, olfactory bulbs, cerebellum, and brainstem. The sample was homogenized in 10 ml of 0.5% glacial acetic acid in cold methanol (MeOH). The homogenate was diluted with 35 ml of 0.5% acetic acid in distilled water to a concentration of 30% MeOH, and a 500-µl aliquot was removed for protein quantification. The remaining homogenate was centrifuged at 400g for 30 minutes at 4°C. The supernatant was passed through glass filter paper to remove proteinaceous debris. The filtrate was eluted over a reverse-phase Sep-Pak C-18 column (Waters Associates, Milford, Massachusetts). The column was then washed with 5 ml of 0.5% acetic acid in 25% MeOH and eluted three times with 1 ml of 0.5% acetic acid in 90% MeOH. All eluate was flushed from the column with air and taken to dryness under nitrogen at 37°C. The residue was dissolved in 1 ml of RIA buffer (0.1% gelatin in 0.05 M Tris). Our previous studies have shown that extraction efficiency is 68.6% for TXB$_2$ and 73.5% for 6kPGF$_{1\alpha}$. Protein in the aliquots of the homogenates was quantified by the method of Lowry et al.$^{20}$

All RIAs were performed in duplicate with serial dilutions and generation of standard curves. Antibody sera were generous gifts from Elizabeth R. Hall, PhD. The antibody to TXB$_2$ had a 0.02% rate of cross-reactivity with other eicosanoids and a sensitivity of 15 pg/200 µl (B/B$_0$=80%).$^{21}$ The cross-reactivity rate for anti-6kPGF$_{1\alpha}$ was <7.7%, with a sensitivity of 15 pg/200 µl (B/B$_0$=80%).$^{21}$

Data were calculated and are presented as mean±standard errors of the mean (SEM) picograms of metabolite per milligram of brain protein and underwent two-way analysis of variance (ANOVA), treating metabolite concentration as a repeated measure to identify significant interactions between subgroups and metabolite levels. A separate two-way ANOVA comparing metabolite concentrations in Subgroups A3 and A4 showed no significant differences, permitting values from the two subgroups to be combined for all other statistical analyses. Student’s $t$ test for independent samples was then used with Bonferroni’s correction for multiple comparisons to determine significant differences in metabolite levels as a function of subgroup. The ANOVAs and Bonferroni’s corrections applied to the $t$ tests required $p≤0.05$ for statistical significance.
Hemispheric and hippocampal CBF were measured by using the indicator-fractionation technique with \([^{14}C]\)butanol as the diffusible tracer. Each rat in Subgroups B1 and B2 was anesthetized, paralyzed, and ventilated after reversal of ischemia as in Subgroups A1 and A2. After 60 minutes of reperfusion, a bolus of 2.5 μCi \([^{14}C]\)butanol (specific activity 0.88 mCi/mmol; New England Nuclear, Boston, Massachusetts) was injected intravenously as arterial blood was withdrawn through PE-50 tubing into a 250-μl Hamilton syringe using a syringe pump (Model 351, Sage Instruments, Boston, Massachusetts) set at 250 μl/min. The rat was decapitated as the syringe pump was turned off after 10 seconds of blood collection. One cerebral hemisphere and the contralateral hippocampus were dissected, weighed separately, and dissolved in Protosol (New England Nuclear). The blood in the Hamilton syringe and arterial catheter was dissolved in 2% sodium dodecylsulfate. The carbon-14-labeled blood and brain samples were counted in a liquid scintillation spectrometer (Beckman LS6800, Beckman Instruments, Fullerton, California) with appropriate corrections for quenching. The counts and the tissue weights were used to compute hemispheric and hippocampal CBF as milliliters per 100 grams brain per minute. Arterial blood gases and rectal temperature were obtained for all 13 Group B rats. Subgroup B3 rats were subjected to the same procedure after being fasted for 24 hours.

Values for hemispheric and hippocampal CBF were analyzed in a fashion similar to that for TXB2 and 6-ketoprostaglandin F1a, with two-way ANOVA treating blood flow in brain regions as a repeated measure. ANOVA was used to analyze arterial blood gas and temperature data.

Results

Preliminary ANOVA showed significant interactions between metabolite levels and subgroups \((p<0.001)\). Post hoc analysis by t test specified the nature of the differences between subgroups. Figure 1 gives the concentrations of TXB2 and 6kPGF1a in brain and identifies significant differences in TXB2 concentrations. Subgroup A1 rats had brain TXB2 levels of \(11\pm3\) pg/ml protein compared with \(101\pm20\) pg/ml protein in Subgroup A2 \((p<0.002)\) and \(4\pm1\) pg/ml protein in the combined Subgroups A3 and A4 \((p<0.01)\). The brain TXB2 level in Subgroup A2 was significantly greater than that in the combined Subgroups A3 and A4 \((p<0.001)\), showing the robust synthesis of thromboxane metabolites in ischemic parenchyma. Treatment with 1-B1 inhibited TX synthase in ischemic brain by 90% and only minimally reduced 6kPGF1a concentration in Subgroup A1 \((10\pm3\) pg/ml protein) compared with Subgroup A2 \((19\pm2\) pg/ml protein, difference not significant). The 6kPGF1a level in Subgroup A2 exceeded that in the combined Subgroups A3 and A4 \((4\pm1\) pg/ml protein, \(p<0.001)\).

ANOVA showed significant interactions between hemispheric CBF and subgroup \((p<0.001)\) and hippocampal CBF and subgroup \((p<0.02)\). Figure 2 shows hemispheric and hippocampal CBF and the results of post hoc t tests on hemispheric CBF. Hemispheric CBF in subgroup B2 \((42\pm9\) ml/100 g brain/min) was depressed below that of Subgroup B3 \((118\pm9\) ml/100 g brain/min, \(p<0.001)\). Subgroup B1 rats demonstrated significantly elevated hemispheric CBF \((104\pm13\) ml/100 g brain/min) compared with Subgroup B2 \((p<0.009)\). There were no significant differences in hemispheric CBF between Subgroups B1 and B3. Hippocampal CBF reflected the trends seen in the cerebral hemispheres. Hippocampal CBF in Subgroup B2 was \(51\pm14\) ml/100 g brain/min compared with \(123\pm14\) ml/100 g brain/min in Subgroup B3 \((p<0.008)\). The elevation in hemispheric CBF observed in Subgroup B1 was associated with a similar increase in hippocampal CBF \((125\pm25\) ml/100 g brain/min) to greater than that in Subgroup B2 \((51\pm14\) ml/100 g brain/min, \(p<0.04)\). There was no significant difference between hippocampal CBF of Subgroups B1 and B3.

Physiologic data from arterial blood samples taken from the 13 Group B rats revealed a pH of \(7.31\pm0.02\), a PO2 of \(92\pm3\) mm Hg, a PCO2 of \(37\pm2\) mm Hg, and
ferent from Group B2. Significance not shown for hippo-
cortex23 as well as in membrane fractions of plate-
benzylimidazole-treated rats (Group Bl), four ischemic
procedure (after 60 minutes of reperfusion in Groups Bl and
any variable by ANOVA.
ject.24-26 TX synthase isolated from human
The low levels of 6kPGFia in our rats
Our paradigm for administering 1-BI was develop-
linked to the 1 position of the imidazole molecule.
These results show that 1-BI was effective in pro-
ment has a molecular weight of 58,800 and has
Our CBF measurements suggest that inhibition of
five normal rats (Group B3) measured by indicator-fractionation tech-

discussed by Softer et al31 has shown that imidazole

Discussion
TX synthase has been found in rat cerebral
cortex23 as well as in membrane fractions of plate-

a rectal temperature of 37.0±0.4° C. There were no

Our choice of 10 μg/g 1-BI was based on exper-
emission followed by 30 minutes of reperfusion.19 We

We found no difference between the two dosages in that
earlier study and chose to use the 10 μg/g regimen
to maintain consistency with the protocol estab-
ished by Hall et al.12

Our CBF measurements suggest that inhibition of
TX synthase during reperfusion of ischemic brain
enhances microcirculatory blood flow. We were
unable to determine the mechanism by which 1-BI
increases posts ischemic recirculation. It may retard
the vasoconstriction that would contribute to depres-
sion of global CBF observed during reperfusion.29
Alternatively, 1-BI may prevent microvascular occlu-
sion by inhibiting secondary aggregation of platelets
stimulated by toxic products released during
ischemia.30 The low levels of 6kPGFia in our rats
with 1-BI suggest that the elevated CBF was
not secondary to global vasodilation produced by
enhanced synthesis of prostacyclin.

Previous attempts to alter blood flow or metabo-
ism in ischemic brain by inhibition of TX synthase
have produced conflicting results. An abstract pre-
sented by Sofer et al31 has shown that imidazole
had no effect in a rabbit model of global cerebral
ischemia. Moufarrij et al32 gave intravenous injec-
tions of UK-38,485 to cats before middle cerebral
artery occlusion; found no difference between treated
and control cats in CBF measured by xenon-133
clearance, in infarct size, in EEG amplitude, or in
extravasation of vital dye in the brain; and con-
cluded that UK-38,485 had no effect on the evolu-
tion of cerebral infarction (although they provided

in platelets leads to "shunting" of endoperoxides
into endothelial cells, lymphocytes, or damaged
vessel walls for formation of prostacyclin.28 We did
not observe a significant increase in cerebral 6kPGFia
levels in ischemic rats treated with 1-BI, indicating
that there was no measurable shunting of PGH2
away from TX synthase.

Our paradigm for administering 1-BI was devel-
oped to maximize penetration of the intracranial
compartment and to provide high tissue concentra-
tions of inhibitor during the hyperemic phase of
posts ischemic reperfusion.29 We chose to measure
CBF after 60 minutes of reperfusion because previ-
ous investigators using the four-vessel occlusion
model have shown that CBF reaches its nadir at this
time.29 We coupled our determinations of brain
eicosanoid concentrations with CBF measurements
to highlight the effects of TX synthase inhibition
upon cerebral thromboxane levels, which have been
shown to rise early during reperfusion of ischemic
brain.9,11,16,19

Our choice of 10 μg/g 1-BI was based on exper-
iments performed by Hall and colleagues12 to study
the effects of TX synthase inhibition on in vitro
platelet aggregation and adhesion of platelets to
deeothelialized aorta. We have confirmed the
effectiveness of this dose of 1-BI in our animal
model by conducting a feasibility study comparing 7
and 10 μg/g 1-BI given during 20 minutes of ischemia
followed by 30 minutes of reperfusion.19 We
found no difference between the two dosages in that
earlier study and chose to use the 10 μg/g regimen
to maintain consistency with the protocol estab-
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tion of cerebral infarction (although they provided

![FIGURE 2. Histogram showing mean±SEM hemi-
spheric (shaded bars) and hippocampal (open bars) cere-
bral blood flow in ml/100 g brain/min from three 1-
benzylimidazole-treated rats (Group B1), four ischemic
control rats given vehicle (Group B2), and six normal rats
(Group B3) measured by indicator-fractionation tech-
nique (after 60 minutes of reperfusion in Groups Bl and
B2). *p£0.001 different from Group B3; **p£0.009
different from Group B2. Significance not shown for hippo-
campal blood flow.](http://stroke.ahajournals.org/DownloadedFrom)
no evidence of successful penetration of the compound into the intracranial compartment. A recent study by Prough et al. using UK-38,485 in a dog model of global cerebral ischemia demonstrated reduction of TXB₂ concentrations in jugular venous blood, implying pharmacologic penetration into the brain; these investigators measured CBF by xenon-133 clearance and found no significant difference between treated and control dogs.

Three studies have described beneficial effects of selective TX synthase inhibition in animal models of brain ischemia. Black et al. infused eicosapentaenoic acid, the precursor of triene prostaglandins, into gerbils given intraperitoneal injections of sodium 5-(3'-pyridinylmethyl)benzofuran-2-carboxylate (U-63557A) before being subjected to 15 minutes of bilateral carotid artery occlusion followed by 2 hours of reperfusion. Gerbils given a combination of the two agents showed significantly elevated CBF measured by hydrogen clearance after 90 and 120 minutes of reperfusion, although brain TXB₂ levels were not affected. Katayama et al. performed bilateral common carotid artery occlusion on spontaneously hypertensive rats given intraperitoneal injections of Trapidil, a pyrimidine TX synthase inhibitor, and found significant increases in brain adenosine 5'-triphosphate and pyruvate concentrations after 3 hours of reperfusion. Roy et al. gave imidazole to cats subjected to permanent occlusion of the middle cerebral artery and found that reduced TXB₂ levels correlated with increased regional CBF in the ischemic penumbra. Their findings suggest that TX synthase inhibition may be important for the preservation of neurons threatened, but not destroyed, by hypoperfusion.

The discrepancies between these results may be influenced by the choice of animal model, the timing and dosage of inhibitor, the effectiveness of the inhibitor in penetrating the blood–brain barrier, and the unaltered proaggregatory effects of prostaglandin endoperoxide intermediates accumulated as a result of TX synthase inhibition. It is of interest to note that the beneficial effects of 1-BI, U-63557A, and Trapidil appear most obvious during reperfusion of ischemic brain, when oxygen-dependent prostaglandin endoperoxide synthase would be actively producing substrate for TX synthase. These findings indicate that rapid infusion of an inhibitor with long-lasting binding affinity for TX synthase could enhance perfusion of tissue within the ischemic penumbra, as shown by Roy et al. Alternatively, the combination of a TX synthase inhibitor with a TXA₂/PGH₂ receptor antagonist might constitute ideal therapy for limiting all known effects of TXA₂ and its proaggregatory endoperoxide precursors. Experiments now underway in our laboratory will determine the effect of TX synthase inhibition, alone or in combination with TXA₂/PGH₂ receptor antagonism, on cerebral metabolism and histologic change studied after reperfusion of ischemic brain.

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References


KEY WORDS • cerebral blood flow • cerebral ischemia • thromboxane synthase • rats
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