Evidence That *in Vivo* Constriction of Cerebral Arterioles by Local Application of tert-Butyl Hydroperoxide Is Mediated by Release of Endogenous Thromboxane

William I. Rosenblum and Doris Bryan

Pial arterioles of mice were suffused *in situ* with tert-butyl hydroperoxide (TBHP). This agent has been reported to stimulate synthesis or release of the constrictor, thromboxane. In the present study we observed pial arterioles by *in vivo* microscopy. Locally applied TBHP produced dose-dependent constriction, significantly inhibited by each of three drugs: acetysalicylic acid, OKY-046, and SQ-29548. These drugs are respectively a cyclooxygenase inhibitor, a thromboxane synthetase inhibitor, and a thromboxane receptor blocker. Since each acts by a different mechanism to interfere with thromboxane-mediated responses and each inhibited the contractile response to TBHP, thromboxane appears to be a mediator of this response. Platelet aggregation was not seen after local application of TBHP, and the dwell time of platelets at the site of TBHP application is less than 1 second. Thus, platelets are an unlikely source of the thromboxane mediating the local constriction. It is much more likely that the source of thromboxane is either the wall of the pial vessels or the underlying brain and/or its vessels. These data do not distinguish between the latter two possibilities, but if this suggestion is correct, then the data show for the first time that thromboxane can be released from normal brain and/or brain vessels in amounts sufficient to cause arteriolar constriction. (*Stroke* 1987; 18:195–199)

**Thromboxane** has long been known to be a potent constrictor of cerebral arteries and other vessels. However, there has been no published evidence to suggest that cerebral blood vessels can be constricted by thromboxane released from the vessels themselves and/or adjacent brain. Rather, demonstrations of constriction have involved *in vitro* application of thromboxane or its analogs. A similar situation is encountered in the literature for other vascular beds, probably because of the difficulty of selectively releasing endogenous thromboxane from the vessels or organ in question. However, Gurtner et al. found that tert-butyl hydroperoxide (TBHP) caused marked constriction of the perfused pulmonary vasculature, which was associated with the appearance of increased levels of thromboxane in the effluent. The elevated thromboxane level and the associated vasoconstriction were prevented by blocking cyclooxygenase, which was interpreted as indicating that TBHP stimulates cyclooxygenase-dependent arachidonate metabolism with a resultant increase in the production of eicosanoids like thromboxane. However, thromboxane production was increased at least eightfold while prostacyclin production was increased only threefold by the TBHP. Thus, TBHP might in addition selectively stimulate thromboxane synthetase by acting on cyclooxygenase-dependent precursors of thromboxane. In either case the thromboxane produced by TBHP caused significant constriction. The source of the thromboxane was thought to be lung "parenchyma"; however, the possibility of thromboxane released from vessel walls cannot be ruled out. Thromboxane can be made by blood vessel walls, but this fact has not been emphasized in the literature because, under the published conditions of measurement, relatively little thromboxane is made compared with other eicosanoids, particularly the potent dilator prostacyclin. We applied TBHP briefly in a superfusate over the brain surface to produce constriction of surface (pial) arterioles and continued to study the phenomenon with pharmacological probes that interfere with thromboxane production or the thromboxane receptor. These studies indicate that TBHP does produce thromboxane-mediated constriction of pial arterioles. The source of the thromboxane must be either the arterioles or the underlying brain. The data do not permit a definitive decision on this point, but the brief period (30 seconds) of exposure to TBHP and the immediate proximity of the pial arterioles to the superfusate suggest that vessel wall thromboxane might indeed be a potentially powerful vasoconstrictor of cerebral arterioles. The alternative explanation, that TBHP stimulates brain parenchyma to produce thromboxane, is equally important. In either case our data demonstrate for the first time that local release of endogenous thromboxane, whether from parenchyma or vessel, is sufficient to cause constriction.

\*From the Department of Pathology (Neuropathology), Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia.\n\*Supported in part by HL 35935.\n\*Address for reprints: Dr. Rosenblum, Medical College of Virginia, Box 17, MCV Station, Richmond, VA 23298.\n\*Received June 15, 1986; accepted August 28, 1986.
Materials and Methods

Male mice (Institute for Cancer Research strain, Flow Laboratories) weighing 22–35 g were anesthetized with urethan and subjected to tracheotomy and craniotomy as previously described.14–18 The dura was stripped as previously described,14–18 and the cerebral surface (pial) vessels lying in the subarachnoid space between the transparent arachnoid and the brain were observed through a Leitz Ultrapan microscope.15 A TV camera and monitor were employed together with an image splitter and strip-chart recorder for measurements of arteriolar diameter and diameter changes as described by Baez.19 This technique can detect diameter changes of 0.5 μm or less.19,20 In each mouse a single arteriole was arbitrarily selected for monitoring. The only criteria for monitoring were size (30–50 μm) and that the vessel could not be an anastomosis between other arterioles. The mice were maintained at 37°C, and the surface of the brain was irrigated with an artificial cerebrospinal fluid (CSF) flowing at 2 ml/min,15,18 37°C, pH 7.35 ± 0.03 (mean ± SD) as measured in the fluid passing across the craniotomy site.

Chemicals were applied to the cerebral surface as a bolus of 1.0 ml at 37°C delivered in 30 seconds. The chemicals used in this way were norepinephrine bitartrate (levarterenol), tert-butyl hydroperoxide (Aldrich), SQ-29548 ([15-[1α,2β(5Z),3β,4α]-7-[3-[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid); a gift from Squibb, Princeton, N.J.), and U-46619 (9,11-didecenoxy,9α,11α-methaneepoxy-PGF2α; Cayman, Denver, Colo.). The diluent for the norepinephrine and tert-butyl hydroperoxide was the artificial CSF, with pH maintained at 7.35. Stock solutions of 10^-2 M SQ-29548 and 10^-4 M U-46619 were made in ethanol and tert-butyl hydroperoxide was the artificial CSF, with pH maintained at 7.35. Stock solutions of 10^-2 M SQ-29548 and 10^-4 M U-46619 were made in ethanol and further dilutions made in the artificial CSF. Vehicle controls for SQ-29548 omitted the drug but were identical in volume (1 ml) and contained ethanol equivalent to the highest concentration used in the drug-diluent mixture.

In other experiments intraperitoneal (i.p.) injections of acetylsalicylic acid (ASA; Sigma, St. Louis), OKY-046 ([sodium (E)-3-4-Cl-imidazolylimethyl]phenyl-2-propanoate; Ono Pharmaceutical Co., Osaka, Japan), and SQ-29548 were used. The first two were soluble in normal saline, the former with pH adjusted to 7.4–7.5 by adding NaHCO3. These diluents served as controls. SQ-29548 was initially dissolved in ethanol and subsequently diluted in saline for i.p. injection. Controls received the equivalent ethanol–saline mixture.

In all the experiments in which i.p. injections were employed, the injection was given prior to craniotomy. Following craniotomy, the pial surface was suffused with artificial CSF for a 20-minute period of equilibration prior to beginning the experiment itself. A similar equilibration period was employed in the experiments in which only locally applied drugs were employed.

At the end of each experiment, 100 μl of blood was obtained from carotid artery for CO2, O2, and pH analysis with a Radiometer Ultramicro Blood Gas Analyzer (Cleveland, Ohio).

Results

Constriction by TBHP and Inhibition by an Inhibitor of Cyclooxygenase

In this experiment 5 mice were treated with vehicle and 5 mice with 100 mg/kg ASA i.p. 1 hour before the test. ASA is a well-established inhibitor of cyclooxygenase2,3 and should therefore reduce the capacity of TBHP to elicit thromboxane production. On the test day TBHP was applied to the brain surface in molar concentrations of 2 × 10^-3, 2 × 10^-4, and 2 × 10^-5. Three applications of 30 seconds each were separated by a 15-minute washout period during which baseline diameter was reestablished. Prior to these experiments, preliminary studies showed no tachyphylaxis to doses of TBHP given 15 minutes apart. In the experiments comparing ASA-treated with vehicle-treated mice the arteriolar diameter was 40 ± 0.5 μm in each group of mice (mean ± SEM). As shown in Table 1, TBHP constricted pial arterioles in a dose-dependent manner (p < 0.01, analysis of variance), and constriction was significantly reduced by ASA. Arterial CO2, pH, and O2 levels (mean ± SEM) were the same in both groups (31 ± 1 mm Hg, 7.37 ± 0.04, 113 ± 6 mm Hg vs. 33 ± 1, 7.36 ± 0.02, 106 ± 7). In a control study to establish the specificity of the ASA effect, the contractile response to norepinephrine was not affected by the ASA treatment. This negative result with norepinephrine duplicates data we have previously reported.16

Table 1. ASA, a Cyclooxygenase Inhibitor, Inhibits Constriction of Pial Arterioles by TBHP

<table>
<thead>
<tr>
<th>Dose TBHP (molar)</th>
<th>Diameter reduction as percent of baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle-treated</td>
</tr>
<tr>
<td>2 × 10^-3</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>2 × 10^-4</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>2 × 10^-5</td>
<td>8 ± 2</td>
</tr>
</tbody>
</table>

Values as mean ± SEM. *Significantly (p < 0.05) smaller responses than those in vehicle-treated mice (analysis of variance).
responses to TBHP was produced by OKY-046 (Table 2). In a control study to establish the specificity of the OKY effect, the constriction produced by norepinephrine was not affected by the OKY treatment.

**Constriction by TBHP and Inhibition by an Antagonist of the Thromboxane Receptor**

In one experiment 5 mice were treated with vehicle and 5 with SQ-29548, a selective antagonist of the endoperoxide–thromboxane receptor. SQ-29548 was given i.p. at 10 mg/kg 30 minutes before the test with a single dose of TBHP (2 x 10^{-3} M) applied for 30 seconds in the suffusate over the brain surface. In the vehicle-treated mice, TBHP reduced diameter 21 ± 3% (mean ± SEM) while diameter was reduced only 6 ± 1% in the group treated with the thromboxane-blocking drug (p = 0.01, Students t). The arteriolar diameter was 37 ± 2 μm in the controls and 39 ± 2 μm in the drug-treated group, and both groups had identical arterial gas and pH levels (32 ± 1 mm Hg, 7.39 ± 0.01, 118 ± 3 mm Hg vs. 32 ± 2, 7.37 ± 0.02 and 117 ± 3).

A second study of the SQ-29548 effect was performed on 5 mice. The brain surface of each mouse was suffused for 30 seconds with each of 3 different treatments given 15 minutes apart. The standard suffusate of artificial CSF was used during the 15-minute washout period, which restored normal diameter (38 ± 2 μm, mean ± SEM). In the presence of only 2 x 10^{-6} M TBHP, arteriolar diameter was reduced by 11 ± 1% (mean ± SEM). The addition of 10^{-6} M SQ-29548 to the TBHP had no effect: Arteriolar diameter was still reduced by 10 ± 3%. However, a higher concentration of SQ-29548 (10^{-5} M) added to the TBHP in the suffusate diminished the constriction obtained by half so that diameter was reduced by only 5 ± 0.5% (mean ± SEM). This effect was highly significant (p<0.001, paired t).

Even higher concentrations of the thromboxane receptor blocker (10^{-6} M) failed to alter contractile responses to 1 μg/ml norepinephrine, confirming the selectivity of the blocking activity. However, 10^{-6} M SQ-29548 did significantly reduce the contractile response to U-46619, a stable agonist of the endoperoxide thromboxane receptor (Table 3). The latter result establishes the fact that SQ-29548 is indeed an antagonist of the endoperoxide–thromboxane receptor in the pial microcirculation.

We also tested whether SQ-29548 would relax pial arterioles in the absence of a stimulus for thromboxane release. Three doses (10^{-6}, 10^{-7}, 10^{-8} M) were locally suffused in each of 5 mice. No change in diameter was detected in 11 of the 15 trials. The average (mean ± SD) change detected for each dose was not significantly different from zero (-2 ± 4%, -2 ± 5%, 0 ± 4%).

**Discussion**

Our data clearly show a dose-dependent constriction of mouse pial arterioles by TBHP, an agent known to release large amounts of thromboxane from perfused lung. In the lung, thromboxane was thought to mediate the constriction that accompanied perfusion by TBHP. Other hydroperoxy compounds are reported to constrict a variety of vessels, including brain vessels, but no consideration has been given by others to a possible role of thromboxane in mediating these effects. We have carried the pharmacologic analysis much further. Our data show not only that a cyclooxygenase inhibitor, ASA, diminishes the effect of TBHP but also that constriction was diminished by OKY-046, a selective antagonist of the thromboxane receptor. All these data taken together strongly suggest that thromboxane is a mediator of the constriction produced by application of TBHP to the cerebral surface in our study.

The source of the thromboxane cannot be definitely established. The most commonly considered source of thromboxane is aggregating platelets. However, no platelet aggregates ("white bodies") were observed during in vivo microscopy. Moreover, no stimulus to aggregation was present unless it was thromboxane itself released by the TBHP. It is unlikely that the source of this thromboxane was the platelets, because not only was platelet aggregation absent in vivo but also the exposure time of platelets to TBHP was too short. The blood moves at several millimeters per second as derived, for example, from shear rate data. Therefore, contact of the platelets with TBHP was limited to less than a second, and such platelets would by then have left the exposed field. Similarly, the

**Table 2. OKY-046, a Thromboxane Synthesis Inhibitor, Inhibits Constriction of Pial Arterioles by TBHP**

<table>
<thead>
<tr>
<th>Dose TBHP (molar)</th>
<th>Diameter reduction as percent of baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 x 10^{-3}</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>2 x 10^{-4}</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>2 x 10^{-5}</td>
<td>7 ± 1</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. *Significantly (p < 0.01) smaller responses than in vehicle-treated mice (analysis of variance).

**Table 3. SQ-29548, a Blocker of Thromboxane Receptors, Inhibits Constriction of Pial Arterioles by U-46619, a Thromboxane Receptor Agonist**

<table>
<thead>
<tr>
<th>Dose U-46619 (molar)</th>
<th>Diameter reduction as percent of baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.4 x 10^{-8}</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>1.4 x 10^{-9}</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>1.4 x 10^{-10}</td>
<td>8 ± 1</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. *Significantly (p < 0.01) smaller responses than in the mice given U-46619 plus vehicle (analysis of variance).
released by TBHP of thromboxane from vessel wall or brain parenchyma would not be expected to aggregate passing platelets or to stimulate them to release additional thromboxane, again because contact of the moving platelets with locally produced thromboxane would last less than a second. For these reasons it seems most probable that the local constriction produced by TBHP is a result of thromboxane produced from the stationary vessel wall and/or brain, but not from rapidly passing platelets.

Thromboxane has been produced in both brain slices and homogenates, and from large cerebral vessels as well as from larger vessels of other organs. While brain parenchyma may produce thromboxane, it is more likely that the effects of a 30-second bolus of TBHP in the suffusate of the pial vessels can best be explained by release of thromboxane from the pial vessels immediately exposed to the suffusate. However, the suffusate does reach the brain parenchymal surface, so release of thromboxane from brain cells or from vessels within the underlying brain cannot be ruled out. Whatever the site of thromboxane production and release, the present data appear to be the first evidence that thromboxane may be mobilized from brain vessels and/or normal brain in amounts sufficient to cause arteriolar vasocostriction.

Additional findings of interest in our study include the demonstration of dose-dependent constriction of pial arterioles by U-46619, a thromboxane receptor agonist. Others have shown constriction of cerebral arteries in vitro by thromboxane or its stable analogs, but we are unaware of studies showing constriction of pial arterioles by thromboxane receptor agonists in situ. We used U-46619 to establish that SQ-29548, a selective blocker of the thromboxane receptor at other sites, did block the thromboxane receptor in pial arterioles. In fact, it has not been possible to separate the endoperoxide receptor from the thromboxane receptor, and therefore SQ-29548 might interfere with endoperoxide action as well as with thromboxane’s action. However, in our study the constriction elicited by TBHP must be related to production of thromboxane because the constriction was inhibited by OKY-046, a thromboxane synthetase inhibitor.

SQ-29548, the endoperoxide–thromboxane antagonist, worked not only when applied locally with TBHP, but also when given intraperitoneally. Therefore, it must cross either the blood–brain or the blood–CSF barriers in amounts sufficient to reach effective concentrations.

The present data, suggesting the constricting potential of thromboxane mobilized from brain or brain vessels, provides evidence that endogenous thromboxane could play a role in controlling cerebrovascular tone. Whether such control is actually manifest in either health or disease remains an open question. Others have suggested a role for thromboxane in producing cerebral vasocostriction seen in pathologic states. However, the brain is frequently injured in these experimental models of pathologic states, and many different putative vasoconstrictors are simultaneously mobilized. It becomes difficult to then ascribe constriction to the thromboxane. The present study is unique in that it provides a demonstration of cerebral vasocostriction elicited by an agent thought to selectively release thromboxane, and this agent, TBHP, has been applied in the absence of brain injury.

Acknowledgments

Martin L. Ogletree, PhD, of Squibb Laboratories, provided invaluable advice during the course of these studies. SQ-29548 was a gift of Squibb Laboratories. OKY-046 was a gift of Ono Pharmaceutical Corp., Osaka, Japan.

References

17. Rosenblum WI, El-Sabban F: Dimethyl sulfoxide (DMSO) and glycerol, hydroxyl radical scavengers, impair platelet aggregation within and eliminate the accompanying vasodilation of, injured mouse pial arterioles. *Stroke* 1982;13:35-39

**KEY WORDS** • cerebral microcirculation • pial arterioles • vasoconstriction • thromboxane synthetase inhibitor • thromboxane receptor blocker
Evidence that in vivo constriction of cerebral arterioles by local application of tert-butyl hydroperoxide is mediated by release of endogenous thromboxane.

W I Rosenblum and D Bryan

Stroke. 1987;18:195-199
doi: 10.1161/01.STR.18.1.195

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/18/1/195