Pharmacological Modification of Bradykinin Induced Breakdown of the Blood-brain Barrier
Jennifer J. Raymond, David M. Robertson and Henry B. Dinsdale

ABSTRACT: Internal carotid artery infusion of bradykinin caused extensive breakdown of the blood-brain barrier to protein as demonstrated by the extravasation of the marker, horseradish peroxidase, into vessel walls and the adjacent parenchyma. Pretreatment of the animals with indomethacin, trifluoperazine, or imidazole significantly reduced the quantity of abnormally permeable vessels as determined by light microscopy. By electron microscopy, it was determined that bradykinin caused an intense increase in the number of pinocytotic vesicles in the permeable segments, but no change in the interendothelial junctions. After imidazole pretreatment, although the extent of the permeability change was markedly reduced, the intensity of pinocytotic activity in the involved areas was not altered.

METHODS
Experiments were performed on 33 female Wistar rats weighing 180-200 g.

1. Drug Pretreatment
Animals were divided into five groups. All groups received 3 pretreatment injections (0.2 ml/100 g i.p.). These were given intraperitoneally at 24 hours, 16 hours, and 1 hour before internal carotid infusion. The number of animals in each group is given in brackets.

Group 1: (6) 0.9% NaCl
Group 2: (9) 0.9% NaCl
Group 3: (9) 0.25% trifluoperazine (TFP)
Group 4: (6) 1.0% imidazole
Group 5: (6) 0.9% NaCl and 0.25% TFP

Imidazole was dissolved in 0.9% NaCl before injection. TFP was dissolved in dimethyl sulfoxide (DMSO) and diluted to 0.25% with 0.9% NaCl immediately before injection. The final DMSO concentration in the mixture was 0.1%.

Disclosures
No disclosures.

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From the Departments of Pathology and Medicine, Queen’s University and Kingston General Hospital, Kingston
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Reprint requests to: Dr. David M. Robertson, Professor and Head, Department of Pathology, Queen’s University, Kingston, Ontario, Canada K7L 3N6
Group 3: (6) Imidazole in 0.9% NaCl (150 mg/kg in 1st and 3rd and 100 mg/kg in 2nd injection)
Group 4: (6) 1 mg/kg trifluoperazine (TFP) in 0.9% NaCl
Group 5: (6) 2.5 mg/kg indomethacin (Sigma) in 0.83 M Na2CO3 buffered to pH 7.6. Additional control animals were tested with 0.83 M Na2CO3 alone and were found to be the same as Group 1.

2. Carotid Infusion

Anesthesia was induced by an intraperitoneal injection of sodium amytal (10 mg/100 g) approximately 15 minutes before the operation. Body temperature was maintained at 37°C using a heating pad. Blood pressure was monitored by placing a polyethylene catheter (PE90) attached to a Grass polygraph into the abdominal aorta. The left internal carotid artery was infused by retrograde insertion of a PE10 catheter into the external carotid artery and advancing it to the origin of the internal carotid artery. Other arterial branches including the pterygopalatine, ascending pharyngeal and superior thyroid were ligated to ensure the infusate reached the left hemisphere of the brain. In initial experiments, trypan blue was infused into the internal carotid artery and its distribution throughout the left hemisphere was observed two minutes later.

All animals were infused at the rate of 0.39 ml min⁻¹ for 3 minutes. Control animals (Group 1) were infused with 0.9% NaCl containing 12.5 mg HRP (horseradish peroxidase type II, Sigma) per ml. Animals in Groups 2-5 received 0.9% NaCl, 1 x 10⁻⁵ M bradykinin, and 12.5 mg HRP per ml. Thirty seconds later blood gases were measured and the rats perfused via the ascending aorta with 50 ml 0.9% NaCl followed by Karnovsky’s fixative.

3. Tissue Processing

The cortex from left and right hemispheres was embedded in 6% agar and approximately fifty 50 μm sections were cut using a Sorval TC-2 tissue sectioner. The sections were washed in 0.05 M Tris HCl (pH 7.6) and incubated for 20 minutes in 20 ml buffer containing 20 mg 3,3-diaminobenzidine tetrahydrochloride (Sigma) and 0.2 ml 1% H2O2. Twelve sections were chosen randomly from the left cortex and mounted in aquamount (G. U. M.) for light microscopic morphometry. Appropriate areas of the remaining sections were processed for electron microscopy. Tissues were post fixed for 90 minutes in 1% osmium tetroxide, dehydrated through graded alcohols, infiltrated with propylene oxide and embedded in Epon 812. The tissues were flat embedded, enabling the vessels to be cut transversely. Ultrathin sections were examined unstained or stained with 7% uranyl acetate in 0.5% lead citrate. An Hitachi H500 electron microscope was used at 75 KV.

4. Quantitative morphometry

i) Light microscopy: Ten cortical sections from each animal were photographed using a Zeiss photomicroscope and ×2.5 objective. 5 x 7 inch prints were made giving a final magnification of 51.8×. The area of cortex on each print was measured using a Zeiss MOP. Quantitative analysis of the vessel walls containing HRP were made by: a) measuring the length of the vessel and its branches using the MOP and b) placing a grid containing 0.25 cm squares over the print and counting the number of grid bars crossed by vessels containing HRP in their walls. Measurements were made by two independent observers who did not know the identity of the photomicrographs.

ii) Electron microscopy: The number of pinocytic vessels per μm² of arteriolar endothelial cytoplasm was determined in permeable cortical vessels of five animals infused with bradykinin (Group 2) and non-permeable vessels from the corresponding vascular segments of four control animals (Group 1). Permeable vessels from four animals pretreated with imidazole prior to bradykinin infusion (Group 3) were also examined. A low power electron micrograph was taken of each vessel examined to determine vessel diameter. A series of overlapping electron micrographs were taken around the circumference of the arterioles at 26,000 x magnification. The area of the endothelium was determined using a Zeiss MOP. Vesicles containing a definite unit membrane were counted within the endothelial cytoplasm. Vesicles at the luminal surface were counted only if they had a narrow neck and appeared to be pinching off from the surface.

RESULTS

Infusion of 0.9% NaCl containing HRP at a rate of 0.39 ml min⁻¹ (Group 1) did not alter the mean arterial pressure (MAP) significantly (Figure 1A). In animals receiving both HRP and bradykinin (Group 2) there was an immediate, transient blood pressure drop of approximately 25 mm Hg which lasted for about 30 seconds. This was followed by a gradual increase to near control levels by the end of the 3 minute infusion (see Figure 1A). The lowest MAP recorded for each animal did not fall below 80 mm Hg. Pretreatment with imidazole (Group 3). TFP (Group 4) or indomethacin (Group 5) did not significantly alter BP response to bradykinin and HRP infusion (Figure 1 B, C, and D). Blood gases were taken at the end of the infusions and animals with aPO2 less than 80 mm Hg or aPCO2 greater than 50 mm Hg were eliminated from the study. PH, PO2 and PCO2 did not differ among the groups.

Figure 1 — Change in mean arterial pressure during internal carotid artery infusion of bradykinin. Resting MAP of A, Group 1 (NaCl) = 115 ± 15 mm Hg; Group 2 (bradykinin) = 114 ± 7 mm Hg; B, Group 3 (Imidazole pretreatment, bradykinin) = 108 ± 14 mm Hg; C, Group 4 (TFP pretreatment, bradykinin) 119 ± 6 mm Hg; D, Group 5 (Indomethacin, bradykinin) = 107 ± 9 mm Hg. * significantly different from Group 1 p < 0.01 ** significantly different from Group 1 p < 0.001
L.M. Study

A representative slice from the left cortex of a rat in Group 1 receiving a NaCl infusion (Figure 2) shows no BBB disruption, whereas a rat in Group 2 (Figure 3) infused with bradykinin, displays extensive uptake of HRP into vessel walls. Sections from the right cerebral hemisphere rarely demonstrated the presence of HRP. There is a large involvement of very small arterioles in this model of BBB breakdown. This feature is seen more clearly in Figure 4 in which small arterioles have a high concentration of HRP. Infusion of bradykinin therefore produces significant BBB breakdown. The extent of this BBB breakdown is seen in Figure 5. A highly significant increase in the number of vessels containing HRP is found in animals infused with bradykinin (p < 0.001 measuring length of positive vessels and p < 0.0001 when the number of grid bars crossed by positive vessels are quantitated).

Pretreatment with imidazole, TFP or indomethacin significantly reduced the number of leaking vessels (p < 0.05 for all groups), however it did not reduce the number to control levels. Groups 3, 4 and 5 all had significantly more vessels containing HRP than animals in Group 1 (p < 0.05 by both methods). The extensive BBB breakdown produced by bradykinin infusion can be reduced by the pharmacological action of imidazole, TFP or indomethacin.

E.M. Study

Four control animals infused with NaCl (Group 1), five animals infused with bradykinin (Group 2) and four animals infused with bradykinin following imidazole pretreatment (Group 3) were studied by EM. Arterioles were defined as vessels possessing smooth muscle cells in their walls.

The 27 arterioles from Group 1 rarely contained HRP, and only an occasional vesicle containing HRP was observed. There was no apparent morphological difference between the 33 permeable vessels from animals in Group 2 and the 22 studied in Group 3. HRP was present in the majority of pinocytotic vesicles and in the basement membrane, smooth muscle and adventitial cells surrounding the endothelium (Figure 6). Vesicles containing HRP were occasionally seen at the basement mem-

![Figure 2](image-url)  **Figure 2** — Mag. x 28.8. Group 1 (NaCl infusion). Note the absence of HRP from the cortex.

![Figure 3](image-url)  **Figure 3** — Mag. x 28.8. Group 2. (Bradykinin infusion) Numerous vessels contain and are surrounded by extravasated HRP.

![Figure 4](image-url)  **Figure 4** — Mag. x 140. Group 2. (Bradykinin infusion) The arteriole in centre field displays a typical distribution of HRP reaction product.

![Figure 5](image-url)  **Figure 5** — Quantitation of permeable vessels following bradykinin infusion. Method A. Measurement of the length of HRP positive vessels in microns per sq cm cortex using a Zeiss MOP. Method B. The number of grid bars crossed by HRP positive vessels. (For details, see methods).

- ***** signfiicantly different from Group 1 p < 0.001
- ** significantly different from Group 1 p < 0.005
- * significantly different from Group 1 p < 0.01
- + significantly different from Group 2 p < 0.01
- ++ significantly different from Group 2 p < 0.05

Pretreated Groups 3, 4, 5 were not significantly different from each other. Error bars denote S.D. number of animals is given in brackets.
brane depositing their contents into the subendothelial space (Figure 7). A similar observation was made by Nag et al in the acute hypertension model. Junctions between endothelial cells appeared intact in both Groups 2 and 3 (Figures 7, 8). Cerebral edema was present in rats infused with bradykinin, particularly surrounding the small arterioles (Figure 6) where astrocytic foot processes were frequently swollen.

The pinocytotic vesicles were counted in a large number of vessels in each group. Vessels were divided into groups according to their diameter; small (less than 10 μm); medium (10-18 μm); large (over 18 μm). The results are given in Table 1. The vessels examined in Group 1 contained very little HRP and only an occasional pinocytotic vesicle contained reaction product. The density of pinocytotic vesicles remained relatively constant, regardless of diameter, ranging from 4.5 to 7.3 vesicles per μm². Permeable vessels in Group 2 contained significantly more pinocytotic vesicles than Group 1. Small and medium vessels contained more pinocytotic vesicles per μm² (14.9 ± 5.0 and 15.0 ± 4.9) than vessels over 18 μm in diameter (9.2 ± 2.6). This represents a 3.3 fold increase (p < 0.001) in small arterioles, 2.1 fold (p < 0.002) in medium, and a 1.5 fold increase (p < 0.002) in large arterioles over equivalent vessels in Group 1. This increase in pinocytotic vesicle density is better demonstrated by Figure 9 in which the values observed in all the arterioles are shown.

A regression line has been plotted for each group studied. The regression line for Group 1 has a slight positive slope of +0.0344 showing a tendency for the number of pinocytotic vesicles per μm² to increase with vessel diameter. Group 2 has a negative slope of −0.2911 demonstrating the increased density of pinocytotic vesicles in the small arterioles in this model of BBB breakdown. This line is significantly different to that of Group 1 (p < 0.001).

Imidazole pretreatment (Group 3) did not prevent the increase in density of pinocytotic vesicles compared to bradykinin infusion alone (Group 2 Table 1). There was a 2.7 fold increase in the small vessels (p < 0.02), 1.6 fold (p < 0.02) in medium vessels and a 1.4 fold (p < 0.002) increase in the large arterioles over vessel density in Group 1. The slope of the regression line is −0.2210; slightly less than that of Group 2 but not significantly different. Regression lines from Groups 1 and 3 are significantly different at p < 0.001 level.

![Figure 6 — Small arteriole from Group 2. Mag. × 9,300. Note the swelling of surrounding astrocytes and the large accumulation of HRP reaction product in the vessel wall.](image1)

![Figure 7 — Mag. × 44,000. Permeable arteriole from Group 2 containing several positive pinocytotic vesicles and an intact tight junction.](image2)

![Figure 8 — Mag. × 45,200. Group 3. Arteriole in the initial stage of becoming permeable. Note the HRP-containing vesicle 'dumping' reaction product into the subendothelial space (arrow).](image3)

![Figure 9 — Density of pinocytotic vesicles in arteriolar endothelium](image4)

<table>
<thead>
<tr>
<th></th>
<th>Group 1 (NaCl)</th>
<th>Group 2 (Bradykinin)</th>
<th>Group 3 (Imidazole pretreatment + bradykinin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slope</td>
<td>0.0344 ± 0.1172</td>
<td>−0.2911 ± 0.1492</td>
<td>−0.2210 ± 0.1766</td>
</tr>
<tr>
<td>r</td>
<td>−0.365 (27)</td>
<td>−0.5656 (33)</td>
<td>−0.5021 (22)</td>
</tr>
</tbody>
</table>

Numbers of vessels studied is given in brackets.
+ correlation coefficient
* significantly different from Group 1 where p < 0.001
Group 2 is not significantly different from Group 3

References:
1. Nag et al.
2. Imidazole pretreatment.

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In both groups infused with bradykinin, the smaller vessels demonstrated a large increase in pinocytotic vesicles. This increase was examined more closely (Table 1) by comparing the numbers of vesicles containing HRP reaction product (positive vesicles) and the remaining vesicles (negative vesicles). Vessels in Group 1 contained the occasional positive vesicle: the number was greatest in the small arterioles (0.26/μm² compared to 0.03 and 0.04/μm² in larger vessels). This represents 6% of the vesicles in small vessels and less than 1% of vesicles in larger vessels.

In bradykinin infused rats (Groups 2 and 3) the number of negative pinocytotic vesicles remained the same, regardless of the size of the vessel (2.4 - 3.0 vesicles/μm²). It is therefore the number of HRP-containing vesicles that was increased, and in small vessels these made up 80% of the pinocytotic vesicles in both Groups 2 and 3. This figure was reduced to 73% (Group 2) and 66% (Group 3) in the large vessels.

The density of negative vesicles in the medium and larger vessels of Groups 2 and 3 was reduced by 60% compared with Group 1 (p < 0.001). Small vessels showed an insignificant decrease in negative pinocytotic vesicles.

In conclusion, both LM and EM observations demonstrate that the smaller vessels play an important part in BBB breakdown caused by bradykinin infusion. Imidazole pretreatment reduced the number of permeable vessels, but had no effect on the increased number of pinocytotic vesicles formed in these vessels.

**DISCUSSION**

This model of BBB disruption in which bradykinin is infused via the internal carotid artery consistently lowered mean aortic blood pressure to a small extent. This observation agrees with the general peripheral vessel dilatation caused by bradykinin.8,9 Whalley and Wahl10 and Wahl and co-workers11 found that topical application of bradykinin (0.1-100 μM) to cat pial arteries in situ caused a significant dilatation. The same authors observed a similar result with isolated cat and dog middle cerebral arteries; the maximum dilatation occurring with 10-8M bradykinin.17,18 In contrast, a pressor action has been observed after bradykinin injection into the lateral ventricle of rats.11,13 It is uncertain whether this pressor effect is linked to renin release or angiotensin II and may be quite unrelated to the vasodilatory action of bradykinin.

Indomethacin pretreatment reduces the fall in blood pressure caused by prostaglandin release22 during renal infusion of bradykinin.23 However, prostaglandins are not the sole mediators of arterial relaxation by bradykinin24 additional compounds such as “endothelium releasing factor”24 and lipoxigenases25,26 are thought to mediate arterial relaxation. It is not surprising that indomethacin, a prostaglandin inhibitor, failed to reduce the drop in blood pressure caused by intracarotid bradykinin infusion.

The breakdown of the BBB by bradykinin infusion was extensive. Arterial hypotension may play a small part in this disruption, but it cannot be entirely responsible because the three drugs used in our experiments significantly reduced the extent of BBB damage but did not alter the pattern of MAP change. Bradykinin increases microvascular permeability.27 in the brain, a similar pattern is observed in which the small arterioles appear to be most affected. Acute hypertension causes a different pattern of BBB breakdown, in which larger arterioles 100-200 nm from the cortical surface are most heavily involved.2 This change in pattern is unlikely to be due to different circulation times of the tracer HRP, since both models show mainly discrete lesions in which the HRP is restricted to vessel walls and has not had sufficient time to leak into the parenchyma.

Unterberg and co-workers16,10 found that topical application of bradykinin to pial and cortical vessels caused selective opening of the blood brain barrier. In later studies they found that intraventricular infusion of bradykinin causes cerebral edema.15 These observations are consistent with the swollen astrocytic foot processes surrounding leaking vessels present in our model.

All three drugs used to treat the rats before bradykinin infusion significantly reduced the number of leaking vessels. Indo-

### Table 1: Density of pinocytotic vesicles with and without HRP, per μm² of endothelial cytoplasm

<table>
<thead>
<tr>
<th></th>
<th>Small (diameter &lt; 10 μm)</th>
<th>Medium (diameter 10-18 μm)</th>
<th>Large (diameter &gt; 18 μm)</th>
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<tbody>
<tr>
<td></td>
<td>Mean S.D. #vessels</td>
<td>Mean S.D. #vessels</td>
<td>Mean S.D. #vessels</td>
</tr>
<tr>
<td><strong>Group 1 (NaCl)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-ve vesicles</td>
<td>4.22 1.7 (5)</td>
<td>6.96 1.96 (9)</td>
<td>5.98 1.66 (13)</td>
</tr>
<tr>
<td>+ve vesicles</td>
<td>0.26 0.24 (5)</td>
<td>0.04 0.06 (9)</td>
<td>0.03 0.04 (13)</td>
</tr>
<tr>
<td>Total</td>
<td>4.48 1.80</td>
<td>7.0 1.99</td>
<td>6.01 1.69</td>
</tr>
<tr>
<td>% +ve vesicles</td>
<td>5.8</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Group 2 (Bradykinin)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-ve vesicles</td>
<td>2.96 0.93 (10)</td>
<td>2.59 *** 0.90 (8)</td>
<td>2.51 *** 0.73 (15)</td>
</tr>
<tr>
<td>+ve vesicles</td>
<td>11.92 *** 5.06</td>
<td>12.37 *** 5.33</td>
<td>6.70 *** 2.26</td>
</tr>
<tr>
<td>Total</td>
<td>14.88 *** 5.03</td>
<td>14.96 ** 4.90</td>
<td>9.21 ** 2.58</td>
</tr>
<tr>
<td>% +ve vesicles</td>
<td>80.1</td>
<td>82.7</td>
<td>72.7</td>
</tr>
<tr>
<td><strong>Group 3 (Imidazole pretreat. and Bradykinin)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-ve vesicles</td>
<td>2.41 1.34 (4)</td>
<td>2.73 *** 1.13 (8)</td>
<td>2.73 *** 0.89 (10)</td>
</tr>
<tr>
<td>+ve vesicles</td>
<td>9.68 *** 4.06</td>
<td>8.21 *** 2.66</td>
<td>5.34 *** 1.46</td>
</tr>
<tr>
<td>Total</td>
<td>12.09 * 5.00</td>
<td>10.94 * 3.00</td>
<td>8.13 * 1.83</td>
</tr>
<tr>
<td>% +ve vesicles</td>
<td>80.1</td>
<td>75.0</td>
<td>65.7</td>
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</table>

* Significantly different from Group 1 where p < 0.02
** Significantly different from Group 1 where p < 0.002
*** Significantly different from Group 1 where p < 0.001
methacin probably acts by preventing cerebral prostaglandin formation. It may also diminish the increase in CBF stimulated by prostaglandin formation.

TFP inhibits several calmodulin regulated enzymes which are involved in membrane transport including adenylate cyclase and calcium ATPase activity. This inhibition may enable TFP to stabilize cell membranes in culture and reduce phagocytosis. TFP reduces BBB disruption in acute hypertension and following a cold lesion; suppression of pinocytosis was suggested as a possible mechanism in both models. Calmodulin activates phospholipase A2, an enzyme involved in prostaglandin production and TFP can inhibit prostaglandin synthesis stimulated by bradykinin in aortic endothelial cells. Therefore, TFP may suppress prostaglandin production, and reduce pinocytosis.

Imidazole inhibits the increase in intraocular pressure induced by prostaglandin and may therefore suppress the action of bradykinin. It activates phosphodiesterase which converts cAMP to 5'-AMP. Westergaard and Joë suggested that cAMP regulates pinocytosis in cerebral endothelium. Imidazole may reduce tissue levels of cAMP, and in turn reduce pinocytosis. This theory was suggested as a mechanism for imidazole action in reducing albumin extravasation during acute hypertension by Johansson and following a cold lesion. Imidazole may have a similar action in our model, since bradykinin has been reported to increase levels of cAMP in tissue.

We found a great increase in the numbers of pinocytic vesicles in vessels containing HRP. The total number of vesicles noted in the present study for vessels over 18 μm in diameter agrees closely to that obtained by Nag and co-workers for both permeable (9.2 compared to 9.9/μm²) and control vessels (6.0 compared to 5.1 vesicles/μm²). However, the small permeable vessels had a much higher density of vesicles (14.9/μm²) which represents a 50% increase over large permeable vessels and almost a 3 fold increase over small control vessels. The number of negative vesicles in these vessels remained at 3.0/μm², of which some are probably invaginations of the plasma membrane, and not true pinocytotic vesicles. Thus over 80% of the vesicles in small leaking arterioles appear to be transport- ing material across the BBB. This figure is reduced slightly in larger vessels. All tight junctions observed in this study appeared intact so it is unlikely that HRP is reaching the subendothelial space via endothelial junctions as suggested by Huttner et al.

Imidazole reduced the length of leaking vessels; the methods used did not measure the actual number of vessels involved. However, when the permeable sections of these vessels were examined by EM, the density of positive vesicles was not reduced. This suggests that when a vessel becomes permeable to HRP in this model, it is an all-or-nothing mechanism. Imidazole appears to be acting by preventing the arterioles from becoming permeable, not in reducing the rate at which they transport material.

The dose of indomethacin and TFP per kg used in these experiments is approximately five times greater than the recommended clinical dose. Since rats require higher drug levels to achieve a similar response to that seen in large animals and humans, the effects we observed could well take place in a clinical situation. It is possible that indomethacin or trifluoperazine could be used in the treatment of cerebral edema due to hypertension, or in other forms of cerebral edema; studies now underway will address this problem.

To conclude, bradykinin infusion produced extensive BBB breakdown which was significantly reduced by pretreatment with imidazole, TFP or indomethacin. There was extensive involvement of the small arterioles which contained the highest numbers of positive vesicles. Imidazole, though reducing the number of leaking vessels, did not prevent the increase in positive vesicles.

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