In Vivo Studies of Pial Vascular Permeability to Sodium Fluorescein: Absence of Alterations by Bradykinin, Histamine, Serotonin, or Arachidonic Acid

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We studied pial vessels in vivo in mice, examining under mercury light the permeability of these vessels to sodium fluorescein. Topical application of hypertonic solutions of NaCl caused leakage of fluorescein. However, putative chemical mediators of leakage, such as histamine, serotonin, arachidonic acid, and bradykinin, all failed to increase permeability to the dye. Apparent increases in permeability only accompanied endothelial damage caused by the dye + light combination, as indicated by production of local platelet aggregates. The technique is useful, provided inadvertent endothelial injury is recognized and avoided. The data in mice suggest that pial vessels may not participate in the permeability changes reportedly produced in parenchymal brain vessels by several of the mediators we tested. Therefore, studies of pial vascular permeability are not expected to provide reliable data concerning the actions of agents that might mediate cerebral edema. (Stroke 1987;18:1157–1159)

Few publications selectively describe the effects of putative mediators of cerebrovascular permeability on pial vessels. Such observations are important because these mediators may affect pial and parenchymal vessels differently. Arachidonate and bradykinin reportedly increase pial vascular permeability to sodium fluorescein, but with a tremendous interanimal variation in threshold dose, suggesting that extraneous factors might be at work in those studies. Moreover, not everyone finds that arachidonate increases permeability of brain vessels. We reevaluated the effects of arachidonate and bradykinin and tested other agents as well.

Materials and Methods

Male mice, ICR strain (Dominion Labs, Dublin, Virginia) were anesthetized with urethane, prepared, and maintained as previously reported for in vivo microscopic study of pial vessels. Observations were made with a Leitz Utrpapak microscope (Rockleigh, N.J.) using incident light from a 200-W mercury lamp heavily filtered with a KG-1 heat filter and a BG-12 blue filter. Observations were made with 10 x wide-field eyepieces and an 11 x Ultpapak objective with a dipping cone. The space between the cone and the brain surface was filled with artificial cerebrospinal fluid at pH 7.35, which was periodically replaced with fluid containing putative mediators of permeability.

Histamine phosphate, serotonin creatinine sulfate, bradykinin triacetate (Sigma Chemical Co, St. Louis, Mo.), or sodium arachidonate (Nuchek, Elysian, Minn.; 99% pure) were made up in fresh solutions of artificial cerebrospinal fluid each day; the pH was adjusted to 7.35 by altering its CO2 content as necessary. Hypertonic solutions were made by dissolving NaCl in deionized water with no effort made to control pH. Sodium fluorescein, mol wt 326, was made up as a 2% solution in saline. The leakage of dye was evaluated by counting the leaking spots in a preselected microscopic field containing arterioles and venules. Solutions to be tested for their ability to produce leaks were left in place at the craniotomy site for either 5 or 30 minutes. After this time, we injected the 2% sodium fluorescein, 0.2 ml/25 g body wt, via tail vein and counted leaks in the microscopic field 15 seconds later.

A variable number of control mice displayed leaks, apparently due to operative trauma. In 2 studies, 1 with bradykinin and 1 with arachidonate, such mice were weeded out by injecting a very small preliminary dose of 2% sodium fluorescein (0.05 ml/25 g rather than 0.2 ml/25 g). This injection was given before applying arachidonate or bradykinin. Only mice that showed no leaks of the test dose of dye were used in further studies. In these studies, the test drug was then applied 30 minutes after the preliminary injection of fluorescein and left in place for 30 minutes. During this time, fluorescence from the low-dose preliminary injection of sodium fluorescein faded to very low levels. Consequently, the regular indicator dose of sodium fluorescein (0.2 ml/25 g) was injected 30 minutes after the onset of exposure to arachidonate or bradykinin, and the leaks were counted 15 seconds later as usual.

Results

Studies with Hypertonic NaCl

As a positive control demonstrating our ability to detect leaks, we applied hypertonic NaCl to the crani-
otony site in 4 groups of 10 mice each. Each group received a different concentration of NaCl for 5 minutes. Table 1 shows that the total number of mice with leaks in each group decreased with declining concentration of NaCl. Normotonic NaCl caused minor leakage in only 2 of 10 mice. The amount of leakage at the high dose was massive, with 99 separate leaks counted as opposed to only 3 leaks counted with 0.9% NaCl. Since 0.9% NaCl is isotonic, these mice may be considered controls, and the small number of leaks in this group indicates that some leakage may result from surgical trauma rather than from the nature of the superfused solution.

**Studies with Histamine or Serotonin**

Preliminary studies using 1.8, 18, or 36 μg/ml histamine for 5-minute applications showed leaks in 1 of 3 mice at each dosage. Five additional mice were then tested with 36 μg/ml and compared with 5 untreated controls. Only 2 of the treated and 1 of the control mice leaked. The lack of a dose effect and the similar degree of leakage in treated and control mice led us to conclude that the leakage was probably related to otherwise undetectable trauma rather than to histamine.

For similar reasons, we concluded that serotonin produced no leaks. Three groups of 3 mice each received 5, 10, and 40 μg/ml serotonin, respectively, for 5 minutes. In each group, only 1 mouse leaked, as did 1 mouse of each group of 3 controls paired with the experimental mice.

**Studies with Sodium Arachidonate**

Arachidonate was continuously in contact with the pial surface for 30 minutes before testing for leaks. Since we found a variable but small percent of control mice with leaks in the preceding studies, we screened out leaking mice by giving a small preliminary dose of sodium fluorescein before beginning the experiment (see "Materials and Methods"). However, in an occasional mouse even this small dose of fluorescein triggered endothelial damage and platelet aggregation in pial vessels exposed to mercury light for as long as 30 minutes. These few mice were also discarded, and nontraumatized mice replaced them. The craniotomy site of 1 group of 10 mice received 100 μg/ml sodium arachidonate for 30 minutes. Another group of 5 mice received 1,400 μg/ml, an extraordinarily high concentration stated to break the blood-brain barrier in some mice and to overstimulate the brain in its perfused solution.

| Table 1. Effect of Hypertonic NaCl on Permeability of Mouse Pial Vessels to Fluorescein |
|----------------------------------|------------------|------------------|------------------|------------------|
| NaCl                             | Leaking/nonleaking |
|                                  | 4.8% | 3.2% | 1.6% | 0.9% |
| Hypertonic solutions of NaCl caused leakage of dye from mouse pial vessels, with declining effect as tonicity decreased. Normotonic NaCl caused minor leakage in only 2 of 10 mice, probably reflecting operative trauma.

Since 0.9% NaCl is isotonic, these mice may be considered controls, and the small number of leaks in this group indicates that some leakage may result from surgical trauma rather than from the nature of the superfused solution.

Table 2. Effect of Sodium Arachidonate on Permeability of Mouse Pial Vessels to Fluorescein

<table>
<thead>
<tr>
<th>Sodium arachidonate</th>
<th>Leaking/nonleaking</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 μg/ml</td>
<td>0/10</td>
</tr>
<tr>
<td>1,500 μg/ml</td>
<td>0/5</td>
</tr>
</tbody>
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In these groups of mice, traumatized animals leaking dye, were identified prior to the start of the experiment and were replaced by nontraumatized mice. Sodium arachidonate, applied for 30 minutes, even in exceedingly high dose, failed to elicit leaks.

Of fluorescein was then injected. No mice in either the low- or high-dose group leaked (Table 2).

**Studies with Bradykinin Triacetate**

A 30-minute exposure of bradykinin triacetate was used because this was reported to be effective in the literature. A preliminary 2-dose study was performed in which we made no effort to weed out mice that might leak as a result of surgical trauma. Three mice received 30 μg/ml bradykinin triacetate, 10 received 150 μg/ml, and 10 served as controls. Essentially the same proportion of mice (20-33%) leaked in each group. Nevertheless, we decided to study many more mice at the higher dose of bradykinin triacetate.

In the larger study, we screened out mice with traumatic leaks (see "Materials and Methods"); 150 μg/ml bradykinin triacetate was then applied for 30 minutes to each of 20 mice, which were compared with 20 controls (Table 3). Seven experimental and 5 control mice leaked — a difference very far from statistical significance, showing that bradykinin was not the cause of leakage. However, platelet aggregates were seen in a number of mice in each group — resulting from the long exposure to fluorescein dye plus mercury light. These few mice were also discarded prior to final observation in the arachidonate study but were not discarded in this study because we wished to see whether the endothelial damage causing these aggregates predisposed the vessels to leaks. When we analyzed the data this way, we found that of the 7 leaking mice treated with bradykinin, 6 had aggregates; of the 5 leaking control mice, 4 had aggregates. The association of leaks with aggregates was significant at the 0.05 level (Fisher's test of exact probability).

Since there was an association between leakage and platelet aggregation, we performed another study in which the confounding factor of damage by light + dye was eliminated. As before, we screened out mice with

| Table 3. Effect of 150 μg/ml Bradykinin Triacetate on Permeability of Mouse Pial Vessels to Fluorescein |
|-------------------------------------------------|------------------|------------------|------------------|------------------|
| With aggregates                                 | Without aggregates |
| With leaks                                     | Without leaks    | With leaks       | Without leaks    |
| Control (n = 20)                                | 4                | 1                | 1                | 14               |
| Bradykinin (n = 20)                             | 6                | 4                | 1                | 9                |

In 10 of the 12 leaking mice, endothelial injury sufficient to produce platelet aggregation had been produced by light + dye; association of aggregation and leakage was significant at p < 0.05, Fisher's test.
surgical trauma by giving a small initial dose of fluorescein, but to prevent any endothelial injury induced by the dye combined with the light over the next hour, we placed neutral density filters in the light path. Bradykinin triacetate (150 μg/ml for 30 minutes) then produced no leaks in 10 mice.

Discussion

At the doses used, neither serotonin, histamine, sodium arachidonate, nor bradykinin caused pial arterioles or venules to leak sodium fluorescein. In contrast, our data with NaCl confirms classical studies showing leakage, especially from venules, following local application of hypertonic solutions. The negative results with histamine also agree with studies that monitored parenchymal or pial vessels, though contrary reports also exist.

The negative results with arachidonate and bradykinin conflict with reports in cats, in which fluorescein was also used to indicate leakage from pial vessels. We have shown in mice that these concentrations of sodium arachidonate or bradykinin produce vascular constriction or dilation, consequently. Respectively, we know that topically applied drugs reach the pial arterioles. Since relaxation by bradykinin is mediated by a factor released only when bradykinin reaches the endothelium, we also know that our failure to alter permeability was not due to a failure of the drug to reach the endothelium. Species differences might explain why the permeability of cat pial vessels was altered by arachidonate and bradykinin whereas the permeability of mouse pial vessels was not.

However, we should also consider the possibility that published results with bradykinin and arachidonate were caused by damaging pial vessels with light in the presence of intravascular sodium fluorescein. One must be careful that the excitation of fluorescein by mercury light does not induce endothelial injury, which is manifest by local aggregation of platelets. These platelet masses fluoresce and can look exactly like the clumpy material seen along the venules in Figure 5a and b of Unterberg et al. Other evidence of endothelial damage would be loss of endothelium-dependent relaxation of arterioles by bradykinin. In fact, the arterioles in Figure 5 of Unterberg et al did not respond to bradykinin. In view of 2 separate indications of endothelial trauma in the paper of Unterberg et al, it may be that leakage was produced because arachidonate or bradykinin acted on injured rather than normal vessels. This suggestion is valid even in cases in which fluorescent platelet aggregates cannot be recognized in the figures since the aggregates may have embolized and left the field. In support of the suggestion relating endothelial trauma to dye leaks was our own finding of a significant relation between apparent dye leakage and recognizable platelet aggregation in our bradykinin studies.

Once the effects of surgical trauma and damage by light + dye were taken into account, we failed to find that histamine, serotonin, arachidonate, or bradykinin break the barrier of the pial vessels to small molecules like sodium fluorescein. Nevertheless, one or more of these agents may mediate edema formation in disease states, with barrier breakdown occurring in parenchymal vessels. Based on our studies, we suggest that pial vessels are not suitable for studying the effects on permeability of mediators potentially important for the production of cerebral edema.

References

In vivo studies of pial vascular permeability to sodium fluorescein: absence of alterations by bradykinin, histamine, serotonin, or arachidonic acid.

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