Endothelial Dependent Relaxation Demonstrated

In Vivo in Cerebral Arterioles

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SUMMARY The endothelium of mouse pial arterioles was injured in situ with a light/dye technique. The response of the arterioles to acetylcholine or to bradykinin was compared before and after the injury. All vessels failed to dilate after injury. In fact the predominant response now became constriction. The injured vessels were still capable of dilating to papaverine. Uninjured vessels continued to dilate to acetylcholine or bradykinin. The data show that relaxation of pial arterioles to acetylcholine or bradykinin is dependent on a normal endothelium. This is in keeping with demonstrations by others that an endothelial dependent relaxing factor or factors is(are) the mediator of the dilation to either acetylcholine or bradykinin. The present demonstration of such endothelial dependence is important because in contrast with the bulk of the literature it deals with in vivo, rather than in vitro data, and with microcirculation rather than large vessels. It is also important because it concerns brain circulation. The data suggests that endothelial injury, known to occur in a wide variety of pathologic states, could enhance vasospastic potential by eliminating dilating influences and/or converting them to constricting forces.

SEVERAL YEARS AGO, Furchgott and coworkers demonstrated that the relaxation of large arteries to acetylcholine (ACh) was really mediated by some substance released from endothelium. The substance was called endothelial relaxing factor (EDRF). EDRF has now been demonstrated by many workers. Its chemical nature has not yet been identified, but it is extremely labile. Among candidates for EDRF have been free radicals, carbonyl compounds, products of lipoygenase activity, and substances produced by the action cytochrome P-450. In addition to ACh a number of vasoactive substances have also been found to trigger EDRF. Use of pharmacologic probes suggests that there may be more than one EDRF, and thus relaxation by two different endothelial dependent dilators may be mediated by two different EDRF's. However whether the actions of a vasoactive agent are mediated by EDRF depends upon the species of animal, and the vascular bed. Endothelial dependence was originally demonstrated in vitro by comparing responses of vessels with and without endothelium. The endothelium was totally removed by some mechanical abrasion. Some workers have questioned the physiologic significance of EDRF, at least insofar as ACh liberated from perivascular nerves, is concerned. They express doubt that ACh could diffuse all the way through adventitia and muscularis to initiate endothelial dependent relaxation. In addition they suggest that the ACh passing through the vessel wall would first trigger an effect mediated by muscarinic receptors on the muscle itself. This effect is constriction, not relaxation, and is observed in arteries from which endothelium has been removed. However ACh applied externally to smaller vessels (arterioles) in situ, definitely produces relaxation. Recently, endothelial injury of such arterioles in vivo has been shown to result in conversion of the relaxation to constriction. Such in vivo studies of microvessels and endothelial dependent relaxation are rare. Confirmation of such
data is extremely important since virtually all investigations concerning EDRF to date have occurred in vitro. Moreover, unlike the in vitro studies with larger vessels, the in vivo demonstration of EDRF in cerebral arterioles employed a technique which injured but did not remove large amounts of endothelium. We wish to report a study in which the endothelium of cerebral arterioles (pial arterioles) was also injured without causing denudation. Our data confirms the report of Kontos showing dependence of ACh induced relaxation upon a healthy endothelium in cerebral arterioles. In addition we show a similar dependence of the dilating response to bradykinin (BK). These are important observations because acetylcholine and other dilators with potential dependence upon an EDRF, are plentiful in brain and may be released in brain damage which is frequently accompanied by endothelial injury. Vasospasm may follow parenchymal injury and has heretofore been considered predominantly in terms of the presence or absence of constrictor substances. The present data suggest an additional possibility — namely that constriction may be accentuated by loss of dilating action from agents whose action depends upon an intact endothelium. Indeed dilators like ACh may not only lose their capacity to relax vessels after endothelial injury, but may then become constrictors in their own right.

Methods

Male mice, ICR strain (Dominion Labs, Dublin VA) were used. The preparation has been described in great detail. Briefly, a tracheostomy and craniotomy were performed in mice anesthetized with urethane. The dura was stripped and the pial vessels exposed and kept moist with a sulfamate of artificial CSF. flowing at 2 ml per minute at 37°C, and pH of 7.35. The mice were maintained at 37°C. The field was observed with a Leitz Ultrapak microscope using two different light sources at different times during an experiment.

Prior to the initiation of endothelial damage a 200 Watt mercury lamp with heat and UV filters, and a green filter was used. The light from this lamp and filters is innocuous and is the light used by us for many years when studying responses of pial arterioles (e.g. ). However if 0.2 ml of 2% sodium fluorescein is injected via tail vein, the combination of light and dye damages the endothelium. This technique has been completely described in a number of reports (e.g. ). The aggregates will fluoresce if a BG-12 (blue) filter is substituted for the green, and if appropriate barrier filters are used. Endothelial damage occurs first in venules, later in arterioles, in the illuminated field. Ultrastructural studies show that the damage is initially manifest only by endothelial lucencies and vacuoles, and progresses to denudation only if the noxious illumination persists. No damage is ever seen in the smooth muscle. Platelet aggregation begins immediately after the initial appearance of the endothelial lucencies and vacuoles. By reducing the intensity of the noxious light at the focal plane one can greatly attenuate the onset of damage and aggregation. If one interrupts the mercury light immediately after aggregates appear in the venules, no aggregates will ever appear in the arterioles. We utilized this maneuver to investigate the effects of endothelial injury in the arterioles, without the complicating factor of platelet aggregation in the arteriole. In order to prevent endothelial injury from progressing and aggregates from developing in the arteriole we switched, at this point in each experiment, to a 100 Watt halogen lamp, which illuminated the field via a fibroptic light guide. A blue filter was interposed in the light path, and between periods of observation, the halogen lamp itself was blacked out.

One further manipulation of the optical system was performed. The initial measurements of diameter and response to vasoactive agents were made with a 6.5 X Ultrapak objective. However the damage was produced with an 11 X objective and immersion attachment in place. The higher magnification focuses the light on a smaller spot with resultant increase in light intensity. With this system, platelet aggregates began to form after approximately 6 minutes of continuous exposure to mercury light following injection of sodium fluorescein. After 6 minutes the 11 X objective was replaced with the 6.5 X objective and the halogen lamp replaced the mercury.

The responses to vasoactive agents were monitored through the Ultrapak microscope via its trinocular tube and a TV camera and monitor using the Baez image splitter. Approximately 15 minutes elapsed between each determination of microvascular response. The 6 minute period of exposure to noxious light/dye was inserted into the 15 minute period between applications of the vasoactive agent. The vasoactive agent was administered as a 1 ml bolus over a 30 second period in artificial CSF at pH 7.35 and 37°C. The changes in diameter were continuously recorded with the image splitter and the maximal change was used in all calculations and tables.

The agents used were acetylcholine chloride (ACh) and bradykinin triacetate (BK) (80 µg/ml of each). Papaverine HCl was also used (320 µg/ml). The intention was to search for a qualitative difference in response to either ACh or BK following endothelial injury (i.e. to see whether dilation was eliminated or converted to constriction). Papaverine served as a check against “non specific” elimination of dilator responses. Papaverine was chosen as a control because relaxation to papaverine is independent of endothelial damage and EDRF.

In each craniotomy site only a single arteriole was chosen for monitoring response to the dilator. This selection was arbitrary, the only criterion being the size of the arteriole (30-45µ I.D.).

Results

Table 1 shows that prior to endothelial damage ACh relaxed the arterioles to 117 ± 4% (M ± SD) of their resting diameter. Following damage of endothelium ACh constricted the arterioles to 91 ± 6% of their
resting diameter. By “resting diameter” we mean the diameter immediately preceding application of ACh. This diameter was constant for at least 3 minutes of baseline measurements preceding each application of ACh. Following endothelial damage ACh failed to dilate any of the 12 arterioles. Indeed 10 of the 12 constricted.

A fifteen minute period was allowed for recovery from the ACh. Papaverine (320 μg/ml) was then applied. Each of the 12 arterioles was dilated by the papaverine. The mean increase in diameter was 20 ± 7%. Thus capacity to dilate was still present after endothelial injury and had not been eliminated by deterioration of the preparation or by some nonspecific event occurring during the course of the experiment.

As an additional check against nonspecific alterations in the preparation we took four of the mice and followed the measurement of response to papaverine with still another measurement of response to ACh. But this time we selected an arteriole that had been outside the microscopic field illuminated by the mercury light. These arterioles should not have suffered endothelial injury and should have displayed normal responses to ACh. This prediction was confirmed. The arterioles were relaxed to 113 ± 3% of their resting diameter by the ACh.

Table 2 displays the response to BK. These mice were different animals from those used to study ACh. Prior to endothelial damage BK dilated the arterioles to 116 ± 3% (M ± SD) of resting diameter. Following damage each of these five arterioles was constricted by BK. The constriction reduced arteriole diameter to 87 ± 4% of baseline.

Papaverine was still able to dilate each vessel, the overall dilation reaching 117 ± 6% of resting diameter. Examination of an undamaged arteriole in each group of mice showed a normal response to BK (relaxation to 115 ± 6% of resting diameter).

Discussion

We have shown in this paper that acetylcholine (ACh) and bradykinin (BK) will not relax pial arterioles in vivo, if the endothelium of these arterioles has been injured. Earlier studies showed that endothelium injured with our light/dye technique displays only minimal ultrastructural evidence of damage, prior to onset of platelet aggregation at the injured site. This is the set of circumstances which existed during the present study. It would appear that the endothelial dependent relaxing factor(s) required for relaxation by ACh or BK can be eliminated without denuding endothelium.

Heretofore endothelial denudation has been the rule when EDRF has been eliminated especially in the usual in vitro demonstration of EDRF but even in the rare in vivo demonstration of EDRF. The only prior exception to this use of denudation has been the work of Kontos et al also using pial arterioles. They used cats and showed that acute elevations of blood pressure eliminated the relaxation of pial arterioles by ACh. They had previously shown that the acute pressure elevation produced endothelial lesions which resemble those produced by our light/dye technique, and did not result in denudation.

Kontos et al only investigated the effect of endothelial injury on the response to ACh. We have not only confirmed the reversal of that response after injury of pial arterioles, but have extended this finding to BK as well. With respect to our own technique, we are unable to say whether the endothelium has been irreversibly injured in spite of the minimal ultrastructural damage. It may be that examination of endothelium at longer intervals after insult would reveal cell death signifying irreversible injury during the original exposure to light/dye.

Kontos et al have presented pharmacologic data indicating that, in pial arterioles of the cat, the EDRF for ACh is different from the EDRF for BK. Our study was not designed to investigate the nature of either EDRF.

Kontos et al were able to eliminate the dilation to BK by applying free radical scavengers to the vessels. Under such circumstances the vessels were unresponsive to BK, neither dilating nor constricting. In our study, the response to BK became one of constriction following endothelial injury. Vasoconstriction by BK is unusual but not unknown. For example, BK constricts canine cerebral arterioles in vitro.

Both our data and that of Kontos et al shows conversion of the response to ACh dilation to constriction following endothelial injury of pial arterioles. The constriction is thought to depend upon muscarinic receptors on vascular smooth muscle.

Our data permit an expansion of the concepts concerning cerebral vasospasm and regulation of cerebral blood flow in pathologic states. Ischemia, subarachnoid hemorrhage, head trauma, and hypertension may each be associated with endothelial injury. Vasocon-

### Table 1: Responses of Pial Arterioles to Acetylcholine Are Reversed after Endothelial Injury

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<th>Before Injury</th>
<th>After Injury</th>
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<tr>
<td>Baseline diameter (μ)</td>
<td>36 ± 4</td>
<td>35 ± 8</td>
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<tr>
<td>Response to ACh*</td>
<td>+17 ± 4t</td>
<td>+9 ± 6</td>
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All values are mean ± SD. N = 12 mice.

*80 μg/ml. Response expressed as a % of baseline diameter. t (+) = dilation; (—) = constriction. Prior to injury ACh dilated arterioles to 117 ± 4% of baseline. After injury ACh constricted arterioles to 91 ± 6% of baseline; p < 0.001, paired t test.

### Table 2: Responses of Pial Arterioles to Bradykinin Are Reversed after Endothelial Injury

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<th>Before Injury</th>
<th>After Injury</th>
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<tbody>
<tr>
<td>Baseline diameter (μ)</td>
<td>36 ± 4</td>
<td>35 ± 9</td>
</tr>
<tr>
<td>Response to BK*</td>
<td>+16 ± 3t</td>
<td>-13 ± 4</td>
</tr>
</tbody>
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All values are mean ± SD. N = 5 mice.

*80 μg/ml. Response expressed as a % of baseline diameter. t (+) = dilation; (—) = constriction. Prior to injury BK dilated arterioles to 116 ± 3% of baseline. After injury BK constricted arterioles to 87 ± 4% of baseline; p < 0.001, paired t test.
striction could be enhanced in all these states by endogenous agents like ACh whose normal effect is relaxation but whose action will be a constricting one if endothelium is injured. Moreover, even a failure to exert a relaxing influence may be sufficient to enhance constriction in the presence of other constricting substances. Enhanced vasoconstriction, even if it falls short of "spasm" may be an unwanted response in the injured brain. Heretofore such responses and especially "spasm" have been viewed as consequences of an outpouring of vasoconstrictors. Clearly this view must be modified to consider the consequences of lost relaxing influences in brains with endothelial injury.

References
Endothelial dependent relaxation demonstrated in vivo in cerebral arterioles.
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