Endothelium Dependence of Dilation of Pial Arterioles in Mouse Brain by Calcium Ionophore

William I. Rosenblum, MD, and Guy H. Nelson, MS

Previous studies have shown that local selective in situ injury of pial arteriolar endothelium eliminates the dilations produced by acetylcholine or bradykinin. One means of producing such injury employs a helium-neon laser in the presence of intravascular Evans blue. Since the endothelium-dependent dilations produced by acetylcholine or bradykinin may be initiated by interaction with endothelial surface receptors, it is possible that the light simply inactivates or destroys these receptors. We used calcium ionophore A-23187, another dilating agent known from in vitro studies of large arteries to be endothelium-dependent, which moves calcium into endothelial cells rather than interacting with surface receptors. Our data in 10 mice show that before injury, $10^{-5}$ M A-23187 dilated arterioles to $109\pm2\%$ of control diameter. After selective endothelial injury by helium-neon laser, dilation was essentially abolished ($101\pm1\%$ of baseline diameter; $p<0.01$, Wilcoxon test). Undamaged sites along the arteriole still dilated to A-23187. Our data indicate that the laser must do more than inactivate surface receptors and are the first in vivo microvascular (vessels of <100 $\mu$m diameter) data showing endothelium dependence of the response to A-23187. (Stroke 1988;19:1379-1382)

In vitro studies of large extracerebral blood vessels have shown that many vasodilators relax these vessels not by acting directly on vascular smooth muscle but by causing endothelial cells to release a dilating substance or substances. Among these dilators are several that may alter cerebrovascular tone because they can reach cerebral blood vessels after release by perivascular nerves or after release by brain tissue itself (e.g., acetylcholine, bradykinin). We have shown that acetylcholine and bradykinin cause relaxation of pial arterioles, which was abolished by endothelial injury, and suggested that pial arterioles shared the phenomenon of endothelium-dependent relaxation with the larger extracerebral arteries in which the phenomenon had first been demonstrated. Our studies not only suggested the existence of endothelium-dependent relaxation in brain vessels but also appeared to demonstrate the phenomenon in the microcirculation (vessels of <100 $\mu$m diameter) in vivo. We selectively injured endothelial cells by exposing them to light from a mercury lamp or a helium-neon (HeNe) laser. Light was injurious only in the presence of a circulating dye, sodium fluorescein with the mercury lamp and Evans blue dye with the laser.

Essential to our interpretation of the data was the fact that light/dye injury failed to impair microvessel responses to nitroprusside or papaverine, substances with dilating actions independent of endothelium and receptors, reflecting integrity of the dilating machinery in vascular smooth muscle. However, acetylcholine and bradykinin initiate responses by activating receptors. It is possible that our selective disruption of responses to acetylcholine and bradykinin simply reflected destruction of receptors; moreover, these receptors could conceivably be located on vascular smooth muscle rather than on endothelium. In that case, even data showing normal responses to nitroprusside or papaverine need not support the existence of endothelium-dependent responses. Instead, our earlier data might simply reflect a selective destruction by light/dye of receptors regardless of location. To further demonstrate that injury caused by laser/Evans blue dye can interfere with endothelium-dependent responses and does so by a mechanism other than destruction of receptors, the following study was performed.

We used calcium ionophore A-23187 as the endothelium-dependent relaxing agent. Its action has been shown to be endothelium-dependent in...
large arteries in vitro by facilitating the entry of calcium into endothelial cells. The elevated internal calcium concentration triggers production and/or release of an endothelium-dependent relaxing factor (EDRF) that causes relaxation of underlying vascular smooth muscle. Relaxation caused by A-23187 does not depend on interaction with a receptor. Therefore, elimination of the response to A-23187 would show that injury did more than just destroy receptors. Our data show that HeNe laser/Evans blue dye injury abolishes relaxation caused by A-23187 and is the first to demonstrate in the microcirculation in vivo the endothelium-dependent nature of the response to A-23187.

**Materials and Methods**

Male mice, ICR strain (Dominion Labs, Dublin, Virginia) were anesthetized with urethane, and the pial arterioles were exposed by craniotomy. Body temperature was maintained at 37°C. The cerebral surface was continuously suffused at 1 ml/min with mock cerebrospinal fluid (CSF) at pH 7.35. The flow of mock CSF was so rapid and the volume of the open craniotomy well over the brain was so small (<0.05 ml) that the pH of the effluent from the edge of the well was the same as that of the mock CSF. The pH was rigorously maintained constant throughout the experiment since dilution is produced by a decrease and constriction by an increase in local pH. Indeed, alterations in arterial CO₂ concentration bring about changes in cerebrovascular diameter only by causing a change in local pH. In mice it is impossible to serially sample for arterial gas content or pH; therefore, 100-μl samples of blood obtained from the carotid artery at the end of each experiment were analyzed with a Radiometer ultramicro blood gas analyzer (Cleveland, Ohio). The mean±SD values were 31±5 mm Hg for CO₂, 121±5 mm Hg for O₂, and 7.33±0.05 for pH. Our success in maintaining local pH throughout the experiment and in avoiding systematic changes in arterial CO₂, O₂, pH, or other systemic variables that might affect diameter is attested to by the fact that baseline arteriole diameter changed by 5% during a given experiment.

Arterioles 29–35 μm in diameter were observed through a Leitz microscope (Rockleigh, New Jersey) with infinity-corrected objectives. The microscopic field was illuminated from the side by a halogen lamp and fiber optic guide. The microscope was fitted with the objective lens turret of a Leitz metallurgic illuminator, which was disconnected from the turret and not used. Removal of the illuminator exposed a side port on the turret at right angles to the objective. The beam of a 6-mW HeNe laser (Spectra Physics, Mountainview, California) was directed through the side port down through the objective by the optics within the turret. Thus, the laser beam, 36 μm in diameter with a ×10 objective, epi-illuminated the target and appeared as a red dot. The arteriole was sensitized to the light by a 1 ml/100 g body wt i.v. injection of a 2% Evans blue solution in normal saline 30 minutes before the start of the study. Evans blue rapidly binds to albumin and continues to circulate.

We used television microscopy to monitor the vascular bed and measured diameters using a Baez image-shearing device at ×750. Diameters were measured continuously, and the diameter after administration of A-23187 was expressed as percent of baseline. Output from the image splitter was recorded on a strip chart for a permanent record of the responses. We used an image-intensified silicon television tube and camera (Model 66, with a 737058-01 silicon tube, Dage MTI, Michigan City, Indiana).

Because increased toxicity of the light was noted where the mock CSF crossing the craniotomy site was 37°C, the temperature of the suffusate was reduced to 24°C. At 37°C many arterioles displayed narrowing at the site of light damage; at 24°C narrowing occurred in only 10% of the arterioles, and these mice were discarded without further study. This observation of greater toxicity at higher temperature may support the opinion of those who originally devised the laser/Evans blue dye technique that endothelial damage was heat-mediated (laser burn with light absorbed and heat generated by the dye).

Exposure to the light lasted only 20 seconds, assuring such minimal injury that platelet aggregates were usually not produced. When platelet thrombi were seen at the site of injury, these mice were discarded without further study.

We used 10 mice. After craniotomy, the flow of mock CSF was started and Evans blue dye was injected. A single arteriole was arbitrarily selected for monitoring in each mouse. A-23187 (Sigma Co., St. Louis, Missouri) was prepared as a 10⁻² M stock solution in dimethyl sulfoxide and frozen. Each day, 1 ml stock solution was added to 1 ml mock CSF to give a final concentration of 10⁻³ M at pH 7.35 and was suffused across the exposed vessels at 24°C for 60 seconds in place of the ordinary suffusion of mock CSF. A-23187 was applied three times in each mouse, the first application 5 minutes before light exposure, the second 15 minutes after light injury, and the third 15 minutes later. Responses to the first and second applications were monitored at the same site on the arteriole and response to the third was monitored 100 μm from the original site. Response to the third application served as a check on the responsiveness of the preparation. Between applications, mock CSF washed away A-23187 and restored baseline diameters.

Response to the first application was compared with those of the second and third applications using the Wilcoxon matched pairs signed ranks test.

**Results**

Our results (Table 1) show that A-23187 increased the mean±SD diameter of the arterioles by 9±2% before injury but by only 1±1% 15 minutes after
Dilation by Calcium Ionophore 1381

Table 1. Injury Caused by Helium-Neon Laser Light/Evans Blue Dye Eliminates Dilation of 10 Mouse Pial Arterioles by Calcium Ionophore A-23187

<table>
<thead>
<tr>
<th></th>
<th>Before injury</th>
<th>15 min</th>
<th>100 µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control diameter (µm)</td>
<td>34±2</td>
<td>35±2</td>
<td>35±2</td>
</tr>
<tr>
<td>Response (% of control)</td>
<td>109±2</td>
<td>101±1*</td>
<td>106±2</td>
</tr>
</tbody>
</table>

Data are mean±SD.
*p<0.01 (Wilcoxon test for matched pairs), different from before and 100 µm.

injury (p<0.01); in every mouse the postinjury response was less than the preinjury response. Fifteen minutes after injury, A-23187 increased the arteriole diameter by 6±2% over baseline at an uninjured site 100 µm from the injury. This response was significantly greater than that at the injured site (p<0.01, Wilcoxon test). In every mouse the response at the uninjured site was greater than that at the injury. Thus, the loss of response at the injured site could not be due to general loss of responsiveness in the preparation caused by some time-dependent shift in an uncontrolled systemic variable.

Discussion

The response of mouse arterioles to A-23187 was virtually abolished by an injury known to damage endothelium and to abolish responses to acetylcholine and bradykinin while leaving the response to nitroprusside intact. Our results are the first to show the endothelium-dependent nature of this response in the microcirculation in vivo. Our results parallel those in large arteries showing that responses to acetylcholine, bradykinin, and A-23187 depend on an intact endothelium while the response to nitroprusside does not. The normal response to nitroprusside attests to the fact that vascular smooth muscle is not injured by the noxious stimulus.

Since the actions of A-23187 depend on endothelium, they are believed to depend on release by the endothelium of a vasodilator (EDRF) the chemical identity or identities of which is a subject of active investigation. In bioassays, A-23187 as well as acetylcholine or bradykinin can cause release of EDRF(s) in vitro, with downstream relaxation of a target vessel from which the endothelium has been removed.

A-23187 causes an EDRF to be synthesized by and/or released from the endothelium. It does so by interacting with a receptor but by directly increasing calcium entry into cells. Acetylcholine and bradykinin interact with endothelial receptors (muscarinic in the case of acetylcholine). This receptor interaction initiates the sequence of events that leads to EDRF release. Laser/Evans blue inhibits endothelium-dependent dilation whether the latter is receptor-mediated or not.

If laser/Evans blue failed to inhibit constriction by A-23187 we would have been forced to reconsider the interpretation of our original data obtained with acetylcholine and bradykinin. When laser/Evans blue eliminated dilation by acetylcholine and bradykinin, we concluded that their responses were endothelium-mediated. If, in this study, laser/Evans blue had not also inhibited dilation by A-23187, laser/Evans blue could have merely inactivated receptors rather than interfered with EDRF synthesis/release or inactivated EDRF itself. In such a case, our earlier data with laser/Evans blue could no longer be used as evidence that the actions of acetylcholine or bradykinin were mediated by EDRF(s) released from endothelial cells in pial arterioles. In mouse pial arterioles, loss of response to acetylcholine and bradykinin with preservation of response to nitroprusside might simply have reflected receptor dependence of the former two and receptor independence of the latter; the receptors could have been on the vascular smooth muscle rather than on the endothelium. Data from other species and other vascular beds, largely gathered in vitro and from conductance vessels, would not necessarily apply to these cerebral arterioles since marked differences between species and beds have been reported. However, our data with A-23187 supports the original interpretation of the laser/Evans blue effect since it shows that laser/Evans blue inhibits receptor-independent dilation by an agent known to release EDRF. Thus, laser/Evans blue does not merely inactivate or destroy receptors. Moreover, since the response to nitroprusside, an endothelium-independent dilator, remains intact, laser/Evans blue must selectively interfere with endothelium-dependent mechanisms and not injure smooth muscle. Additional data are required to explain how endothelial damage by laser/Evans blue interferes with endothelium-dependent responses; synthesis or release of EDRF(s) may be impaired. However, electron microscopy shows that when endothelium-dependent responses are eliminated, morphologic alterations of endothelial cells is slight. Consequently, we must also consider the possibility that EDRF(s) are still synthesized and released following injury but are somehow inactivated by laser/Evans blue.

References


**KEY WORDS** • endothelium, vascular • microcirculation • mice
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