Interaction of Endothelium With Dilation Produced by Inhibitors of Cyclic Nucleotide Diesterases in Mouse Brain Arterioles In Vivo

William I. Rosenblum, MD; Takao Shimizu, MD; and Guy H. Nelson, MS

Background and Purpose: In vitro evidence gathered from extracerebral conductance vessels suggests interaction between the endothelium-derived relaxing factor for acetylcholine (EDRF<sub>ACH</sub>) and cyclic nucleotide action in vascular smooth muscle. The purpose of this study was to examine this interaction in vivo in pial arterioles. As had been done in vitro, we used phosphodiesterase inhibitors that elevate cyclic nucleotide levels in vascular smooth muscle.

Methods: Pial vessels of mice were observed with television microscopy. Diameter of the arterioles was monitored with an image-splitting technique. The responses to topically applied phosphodiesterase inhibitors were tested before and after focal endothelial injury or before and during application of N-guanidino-L-monomethyl arginine (L-NMMA). Both treatments are known to eliminate the endothelium-dependent response to acetylcholine in this preparation.

Results: Phosphodiesterase inhibitors diluted pial arterioles. This was true for phosphodiesterase inhibitors elevating levels of both adenosine 3’,5’-cyclic monophosphate (cAMP) and guanosine 3’,5’-cyclic monophosphate (cGMP) as well as for inhibitors thought to selectively affect either nucleotide. The dilation was inhibited by endothelial injury and by L-NMMA.

Conclusions: The data suggest that brain arterioles are diluted by both cAMP and cGMP but that this action is impaired if EDRF<sub>ACH</sub> levels are reduced. Since EDRF<sub>ACH</sub> elevates cGMP levels, these data support the hypothesis that the relaxing actions of cGMP and cAMP depend upon adequate basal levels of cGMP in vascular smooth muscle. This hypothesis, originally introduced in studies of extracerebral conductance arteries in vitro, can now be applied to brain resistance vessels in vivo. (Stroke 1993;24:266–270)

KEY WORDS • cerebral circulation • endothelium-derived relaxing factor • phosphodiesterase inhibitors • mice

Cyclic nucleotides are important regulators of vascular tone. Phosphodiesterase (PDE) inhibitors elevate nucleotide levels by preventing PDE from breaking down the nucleotides. Martin et al.<sup>1</sup> reported that PDE inhibitors relaxed rabbit aorta in vitro. The authors ascribed this relaxation to elevations of the concentration of guanosine 3’,5’-cyclic monophosphate (cGMP) in vascular smooth muscle (VSM). This conclusion was consonant with the known association between cGMP levels and relaxation.<sup>2</sup> Martin et al.<sup>1</sup> found that the relaxation was blocked if the aortic endothelium was removed. They knew that the endothelium released a relaxing factor (endothelium-derived relaxing factor, “EDRF”) that elevated cGMP levels in VSM. The authors postulated that endothelial removal, by eliminating “EDRF,” reduced cGMP concentrations

See Editorial Comment, page 270

in VSM to such a low level that subsequent elevation by a PDE inhibitor was insufficient to relax the aorta.

Since “EDRF” and cGMP are important regulators of cerebrovascular tone,<sup>3–5</sup> it was of interest to attempt to replicate the findings of Martin et al.<sup>1</sup> using cerebral blood vessels. Moreover, we thought it especially worthwhile to attempt this using an in vivo system and resistance vessels, rather than an in vitro system employing a conductance vessel as was the case in the studies of Martin et al.<sup>1</sup> We employed a well-studied method for injuring endothelium, and we also used inhibitors of “EDRF”-dependent responses.

Materials and Methods

The methods have been described in detail many times.<sup>3–6</sup> Briefly, male mice (ICR strain, Harlan Sprague Dawley, Inc., Indianapolis, Ind.) were anesthetized with urethane. A tracheostomy and craniotomy were performed. The dura was stripped, exposing the transparent arachnoid and the underlying vessels on the brain surface (pial vessels). The mice were maintained at 37°C. The craniotomy site was continuously suffused with Elliott’s solution<sup>8</sup> at pH 7.3–7.4. All drugs were given in this solution at a final pH that was the same as the pH of the regular suffusate. Diameters were moni-

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tored via a television microscope and an image-splitting device. Image-splitting techniques permit measurements of less than 0.50 μm.\textsuperscript{10,11} Only one arteriole was used per mouse. The arteriole was arbitrarily selected from those 25–45 μm in internal diameter. Changes in diameter were expressed as a percent of baseline.

To injure the endothelium, a light/dye technique was employed that uses a HeNe laser and Evans blue injected intravenously.\textsuperscript{3,4} The dye acts as an energy-absorbing, heat-generating target. The laser beam is focused through the objective of the microscope\textsuperscript{3} and was 36 μm in diameter. We have published many papers that used this technique to demonstrate endothelium-dependent responses.\textsuperscript{3,4,7,12,13}

**Drugs Used**

\textit{N}-Guanidino-1-monomethyl arginine (L-NMMA) was obtained from Sigma Chemical Co., St. Louis, Mo. This drug inhibits “EDRF”-dependent responses either by inhibiting the enzyme synthesizing “EDRF”\textsuperscript{14} or by inhibiting a sequence of events that inactivate “EDRF.”\textsuperscript{14} Stock solutions were made in deionized water and further diluted in Elliott’s solution. Inhibitors of PDE were obtained from the sources indicated below. The selectivity shown pertains to the doses we used and is based on the literature that describes the selective action of these inhibitors on either the PDE for cGMP or the PDE for adenosine 3’,5’-cyclic monophosphate (cAMP). There is no literature dealing with their selectivities in cerebral blood vessels. Therefore, we used the literature dealing with other test objects. By selecting these doses, which are low and produce small dilations, we minimize the chance that the inhibitors are working via some other, alternative, pathway. 2-o-Propanoxyphenyl-N-azapurin-6-one (M+B 22948), an inhibitor of PDE with a selective action on cGMP,\textsuperscript{15} was a gift from the manufacturer (May and Baker, Dagenham, UK).

The following inhibitors were obtained from Research Biochemicals Inc., Natick, Mass.: dipyridamole, which at the dose used is a selective inhibitor of the PDEs with a predominant action against cGMP\textsuperscript{15}; isobutylmethylxanthine (IBMX), which inhibits both the PDE directed against cGMP and the PDE directed against cAMP\textsuperscript{15}; and 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro-201724), which inhibits the PDE directed against cAMP.\textsuperscript{15} Stock solutions of the PDE inhibitors were made in ethanol. Subsequent dilutions were made in Elliott’s solution. The final concentration of the vehicle in Elliott’s solution never exceeded 0.01%. This concentration of ethanol has no effect on our preparations.

**Experimental Design**

Only one PDE inhibitor was tested per mouse. There were 10 mice in each group, each mouse serving as its own control. The doses selected and the durations of exposure were determined by tests of dose responses and by determining which doses and/or durations produced relaxations that recovered during 5 minutes of washout. Dipyridamole was given for 2 minutes and the other PDE inhibitors for 1 minute. In the laser/dye studies the PDE inhibitor was given 5 minutes before and 10 minutes after injuring the endothelium. A third challenge with the drug was made 15 minutes later, but the diameter change was monitored at a site 100 μm away from the site monitored during the first two challenges. The response at the distant site established that inhibition of the response at the site of endothelial injury by laser/dye treatment was not merely due to fatigue of the preparation. The distant site responded in every case. To simplify presentation of the results, these data from the distant site are not given.

When L-NMMA was used, a concentration of 10 -6 M was suffused for 10 minutes. It did not produce a change in diameter. To simplify presentation of the results, these negative data are not given, but a similar result was previously reported.\textsuperscript{4} The response to a PDE inhibitor with no L-NMMA treatment was compared with the response to a PDE inhibitor with L-NMMA treatment. For each PDE inhibitor, 10 mice were tested, first without and then with L-NMMA. Ten additional mice were first tested after treatment with L-NMMA and again 15 minutes after washout of the L-NMMA. Results were always the same whether the L-NMMA-treated response occurred first or last. Therefore, the data are always combined so \( n = 20 \) in each study.

**Statistics**

Since each arteriole served as its own control and the data were expressed as percent change in diameter, the responses before and after treatment were compared using the Wilcoxon matched pairs test.\textsuperscript{16} A treatment effect was considered significant when the null hypothesis was rejected at the 0.05 level.

**Results**

Each PDE inhibitor dilated pial arterioles immediately and in a dose-dependent manner. However, the higher the dose the longer it took for recovery to occur. Doses higher than those shown here might produce larger relaxations but with recovery periods too long for repeated study of the same preparation. Consequently, experiments studying the responses before and after a treatment used low, single doses of PDE inhibitor with a predictably small response. In the case of dipyridamole, only one tested dose gave relaxations that recovered in 5 minutes. This dose was 10 -7 M given for 2 minutes. The other PDE inhibitors produced dose-related, readily reversible dilations when given for 1 minute at doses between 10 -8 and 10 -8 M (Table 1). The responses to the low dose of each drug in Table 1 were small, and there were only three mice in each group. However, the responses to the low dose of each inhibitor proved reliable, as shown when the same low doses were tested in later groups of mice. This is shown in Table 2, where mean response to the drug is slightly greater than in earlier tests of the same dose shown in Table 1.

Endothelial damage blocked dilation by three of the PDE inhibitors (Table 2). Two of these were dipyridamole and M+B 22948, both said to have selective action against the PDE for cGMP.\textsuperscript{15} Endothelial damage also blocked dilation by IBMX (Table 2), the PDE inhibitor that inhibits PDEs for both cGMP and cAMP.\textsuperscript{2} Ro-201724, with selective action against the PDE for cAMP,\textsuperscript{2} acted peculiarly in studies of endothelial injury. Before damage 1.8×10 -6 M relaxed the arterioles by 7±2\% (mean±SD). After endothelial damage the drug still elicited dilation, albeit less (5±2\%). However, in seven of the 11 tested vessels this relaxation after endothelial injury was not reversible. Fifteen minutes
later at a noninjured site, a reversible relaxation was produced. In short, the dilating action of the selective inhibitor of cAMP PDE was apparently less impaired than that of the other PDE inhibitors by endothelial injury. However, irreversible relaxation is difficult to interpret since it may represent some strange interaction of the drug with the injured endothelium. Consequently, the next set of experiments was performed. These used L-NMMA instead of injury to interfere with “EDRF”-dependent phenomena.

L-NMMA at 10⁻⁶ M did not alter the diameter of pial arterioles, but it did inhibit the dilation produced by M+B 22948, a selective cGMP PDE inhibitor, and the dilation produced by Ro-201724, the selective cAMP PDE inhibitor (Table 3). N-Guanidino-D-monomethyl arginine at 10⁻⁶ M had no effect (data not shown).

**Discussion**

The new data in this report demonstrate two important findings: 1) brain arterioles in vivo are dilated by inhibitors selective for either cAMP PDE or cGMP PDE, and 2) this dilation was inhibited by endothelial injury or by L-NMMA.

With respect to our methods, the laser/dye technique as used here on mouse pial arterioles has no effect on a variety of agonists, both dilators and constrictors. This is true for sodium nitroprusside,³⁴ cAMP analogue,⁵ prostacyclin,⁶ and uridine triphosphate.¹³ Consequently, we are confident that the elimination of responses to the PDE inhibitors in the present study was caused by injury to the endothelium and not to VSM. Moreover, L-NMMA in the present animal model and at the concentration used here fails to affect dilation by either bradykinin or prostacyclin.⁴⁷ Thus, the action of L-NMMA is not due to some action on VSM and is, in fact, selective only for the “EDRF” for acetylcholine and not for the “EDRF” for bradykinin, which appears to be an oxygen-centered free radical in the pial circulation.¹⁸ We used a low concentration of L-NMMA that had no effect on pial arteriole diameter. Higher concentrations cause constriction,⁴ as would be expected if “EDRF” levels are reduced by L-NMMA. We used the lower dose because this avoids the problems of data interpretation that are introduced when an inhibiting drug also changes basal tone or diameter. The low dose of L-NMMA inhibits dilation by acetylcholine even though basal diameter is not affected.⁴ This indicates, as do the present data, that as we progress down the dose–response curve for “EDRF” given decrements in the “EDRF” concentration produce increasingly smaller changes in diameter. This is a well-known characteristic of dose–response curves generally.

M+B 22948 is a selective blocker of the PDE for cGMP.¹⁵ Dipyridamole may have a variety of effects, but at the dose used here it is reported to be a selective inhibitor of the PDE for cGMP.¹⁵ The use of dipyridamole was redundant since M+B 22948 inhibits the same PDE. Ro-201724 is a selective blocker of the PDE for cAMP.¹⁵ IBMX inhibits both classes of PDE.¹⁵ The fact that dilation was produced by dipyridamole, M+B 22948, Ro-201724, and IBMX supports our previous data showing that cGMP or its analogue and cAMP or its analogue each produce relaxation of these arterioles.¹⁵ The small magnitude of the relaxations is immaterial to our conclusions, which are simply that either cGMP or cAMP can mediate relaxation following arrival of an appropriate agonist.

The data, taken as a whole, support the suggestion of Martin et al¹⁴ that endothelial injury reduces basal levels of cGMP within VSM and that subsequent increases of cGMP levels in VSM do not relax, presumably because the basal cGMP level to which they are added is too low. Removal of the endothelium by Martin et al¹⁴ or injury to the endothelium in our study would reduce the basal level of cGMP in VSM because it is set by the basal release of “EDRF” from an intact, normal endothelium. The conclusion of Martin et al¹⁴ also explains earlier data of ours, which showed that endothelial injury reduced the response of pial arterioles to a cGMP analogue.¹⁷ The new data from the L-NMMA experiments are similarly explained; L-NMMA reduced “EDRF” levels, and this reduced basal cGMP levels in

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**Table 1. Phosphodiesterase Inhibitors Dilate Mouse Brain Arterioles**

<table>
<thead>
<tr>
<th>Drug</th>
<th>n</th>
<th>Dose (M)</th>
<th>Diameter (μm)</th>
<th>Increase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isobutylmethylxanthine</td>
<td>3</td>
<td>2.3x10⁻⁴</td>
<td>32±4</td>
<td>3±2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.3x10⁻³</td>
<td>...</td>
<td>8±4</td>
</tr>
<tr>
<td>M+B 22948</td>
<td>3</td>
<td>1.1x10⁻⁷</td>
<td>32±3</td>
<td>4±3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.1x10⁻⁸</td>
<td>...</td>
<td>10±3</td>
</tr>
<tr>
<td>Ro-201724</td>
<td>3</td>
<td>1.8x10⁻⁷</td>
<td>37±2</td>
<td>2±2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.8x10⁻⁸</td>
<td>...</td>
<td>6±1</td>
</tr>
</tbody>
</table>

Values are mean±SD.

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**Table 2. Endothelial Damage in Mouse Brain Arterioles Blocks Dilation by Phosphodiesterase Inhibitors**

<table>
<thead>
<tr>
<th>Drug</th>
<th>n</th>
<th>Diameter (μm)</th>
<th>Before damage</th>
<th>After damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dipyridamole (10⁻⁷ M)</td>
<td>6</td>
<td>34±4</td>
<td>7±2</td>
<td>1±1*</td>
</tr>
<tr>
<td>M+B 22948 (1.1x10⁻⁷ M)</td>
<td>6</td>
<td>36±3</td>
<td>6±2</td>
<td>0±0*</td>
</tr>
<tr>
<td>Isobutylmethylxanthine (2.3x10⁻⁸ M)</td>
<td>6</td>
<td>34±3</td>
<td>5±2</td>
<td>0±0*</td>
</tr>
</tbody>
</table>

Values are mean±SD.

* p<0.01 compared with response before endothelial injury.
VSM. When a PDE inhibitor was added, the consequent increase of cyclic nucleotide concentrations was insufficient to produce relaxation because the increase was superimposed on a low basal level of cGMP.

The fact that dilation by all classes of PDE inhibitor was inhibited in these studies indicates that basal "EDRF" levels and the resulting basal cGMP levels in VSM are necessary not only for normal relaxation by subsequent elevations of the cGMP level but also for normal relaxation by subsequent elevations of the cAMP level. This can be explained by data indicating an additive or synergistic effect on tone by cGMP and cAMP. While L-NMMA blocked dilation by Ro 201724, a selective inhibitor of cAMP PDE, endothelial injury was far less effective as an inhibitor of that dilation. We cannot explain this discrepancy. However, the postinjury response to Ro-201724 was not reversible, making its interpretation extremely difficult. We do not feel that this peculiar result should negate the conclusion that fits the rest of the data, namely, that when either cGMP or cAMP levels in VSM are elevated, an expected relaxation may not occur if basal levels of cGMP have been reduced by reductions in "EDRF" concentrations resulting from endothelial injury or L-NMMA.

We cannot explain why Martin et al17 failed to impair dilation with isoproterenol when they removed aortic endothelium. Presumably isoproterenol is cAMP-dependent, and our new data indicate that responses to cAMP are also impaired if basal cGMP levels are reduced. Perhaps Martin et al17 produced much greater increases in the cAMP concentration than were produced in our study. A larger increase in the cAMP level might produce relaxation even with a decrease in the background cGMP level. Our new data do not explain why Souven et al21 failed to observe an effect of cGMP PDE inhibitors on cGMP levels in VSM unless endothelium was present. Our new data are in keeping with the conclusion of Kuhn et al,22 who concluded that neither cAMP nor cGMP play a role in "EDRF" release.

However, our new data are not easy to reconcile with some earlier data from our laboratory.17 If the action of all classes of PDE inhibitors is blocked by endothelial injury or by L-NMMA and if cGMP and cAMP have similar effects on pial arteriole diameter, how can we explain the fact that endothelial injury inhibited dilation by a cGMP analogue without inhibiting dilation by a cAMP analogue?17 We originally speculated that endothelial cGMP but not cAMP played a role in the synthesis/release of "EDRF" by endothelium. We could then explain that endothelial injury would selectively impair dilation by an analogue of cGMP but not an analogue of cAMP. We would then predict that the same injury would selectively impair inhibitors of cGMP PDE. But the present data show that both PDE inhibitors are impaired by endothelial injury or by interfering with "EDRF." Consequently, our original hypothesis of a role for cGMP in the endothelial injury/release of "EDRF" is not supported. To explain our earlier data17 we can only speculate that in the latter experiments equimolar concentrations of cGMP and cAMP analogues produced different increases in levels of these respective nucleotides within VSM. We must suggest that a greater rise in the cAMP concentration was produced and that this rise produced by the cAMP analogue exceeded that produced by any of the PDE inhibitors used in the present experiments. Then the rise in the cAMP level produced in the earlier study was sufficient to produce relaxation in spite of endothelial injury and a fall in basal cGMP levels in VSM while the rise in the cAMP level produced by the PDE inhibitors in the present study was insufficient to produce relaxation in the absence of normal basal levels of cGMP. This reconciliation of the past and present data seems reasonable but must, of course, be considered tentative since levels of nucleotides within the microvascular VSM were not measured in either study.

In any case, the present data demonstrate in vivo in resistance vessels of the brain that endothelial damage and L-NMMA treatment impair the regulation of tone by cyclic nucleotides. This impairment is presumably based on a reduction in basal levels of "EDRF" within VSM.

References

1. Martin W, Furchgott RF, Villani GM, Jothianandan D: Phosphodiesterase inhibitors induce endothelium-dependent relaxation of rat and rabbit aorta by potentiating the effects of spontaneously released endothelium derived relaxing factor. J Pharmacol Exp Ther 1986;237:539–547

Table 3. L-NMMA (10^-4 M) Blocks Relaxation of Mouse Brain Arterioles by Inhibitors of Phosphodiesterase for Either Guanosine 3',5'-Cyclic Monophosphate or Adenosine 3',5'-Cyclic Monophosphate

<table>
<thead>
<tr>
<th>Drug</th>
<th>n</th>
<th>Diameter (μm)</th>
<th>Before L-NMMA</th>
<th>After L-NMMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ro-201724 (1.8x10^-6 M)</td>
<td>20</td>
<td>37±2</td>
<td>5±3</td>
<td>1±1*</td>
</tr>
<tr>
<td>M+B 22948 (1.1x10^-7 M)</td>
<td>20</td>
<td>33±2</td>
<td>5±2</td>
<td>1±1*</td>
</tr>
</tbody>
</table>

L-NMMA, N-guanidino-L-monomethyl arginine. Values are mean±SD.
*p<0.01 compared with response before L-NMMA.
Cyclic nucleotide phosphodiesterases appear to modulate many physiological responses in various cell types and tissues, including platelet aggregation and vascular relaxation.\(^1\)\(^-\)\(^3\) Phosphodiesterase inhibitors elevate cyclic nucleotide levels by preventing phosphodiesterase from cleaving the specific nucleotide. Thus, the selective use of phosphodiesterase inhibitors can provide valuable insights into investigating the role of phosphodiesterase in physiological responses.

The present studies by Rosenblum et al examine in vivo responses of cerebral (pial) arterioles in mice to topical application of phosphodiesterase inhibitors. In addition, Rosenblum et al examined the role of the endothelium, and endothelium-derived relaxing factor, in dilatation of pial arterioles to application of phosphodiesterase inhibitors. The authors found that phosphodiesterase inhibitors, which produced elevations in the levels of cAMP and cGMP, produced modest dilatation of pial arterioles. More importantly, dilatation of pial arterioles in response to phosphodiesterase inhibition could be attenuated by injury to the endothelium or by application of an enzymatic inhibitor of nitric oxide, L-NMMA. Thus, the present studies by Rosenblum et al demonstrate that the actions of cGMP and cAMP on dilatation of cerebral arterioles depend on basal levels of cGMP in vascular smooth muscle.

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