In Vivo Effect of Methylene Blue on Endothelium-Dependent and Endothelium-Independent Dilations of Brain Microvessels in Mice

Manabu Watanabe, William I. Rosenblum, and Guy H. Nelson

Arterioles on the surface of the mouse brain were observed by in vivo TV microscopy. Four dilators were topically applied to relax the vessels in vivo. Two of the dilators were acetylcholine and bradykinin, whose action in this vascular bed is dependent upon production of endothelium-dependent relaxing factors. The other two dilators were sodium nitroprusside and 8-bromo-cGMP, whose action is not endothelium dependent. The dilations by acetylcholine, bradykinin, and nitroprusside were significantly depressed by 10^{-4} M methylene blue applied topically for 7 minutes prior to application of the dilators. The inhibitory effect was reversible, was greatest against acetylcholine, and was least against nitroprusside. These data parallel reports of methylene blue's action against these dilators when applied to large blood vessels in vitro. Our data appear to be the first microvascular data and the first in vivo data showing this effect. The data thus suggest that the mechanisms underlying dilation of cerebral arterioles and large extracerebral vessels are similar. The literature accounts for the effect of methylene blue on the basis of its action as an inhibitor of guanylate cyclase. Our data, including the failure of methylene blue to alter dilation by 8-bromo-cGMP, are in keeping with this hypothesis and with current beliefs that guanylate cyclase and cGMP have a central role in vasodilation. The data do not rule out the possibility that methylene blue has an additional action in the case of acetylcholine and inactivates the endothelium-dependent relaxing factor for that dilator. (Circulation Research 1988;62:86-90)

It is now known that many humoral agents or neurotransmitters capable of dilating blood vessels do so by causing endothelial cells to release the actual dilating agent. The substance released by the endothelial cells has been called endothelium-dependent relaxing factor (EDRF). It is also known that there is more than one EDRF and that different endothelium-dependent dilators may release different EDRFs, depending on the vascular bed and animal species. Even within a single species, a given agent may produce relaxation in different beds by different means. The chemical nature of EDRFs is under active investigation, and a variety of free radicals have been implicated.

Because of the diverse nature of the relaxation process in different vessels and species, it was important to demonstrate the existence of endothelium-dependent relaxation in the microcirculation. A brief report suggested the presence of EDRF in mesenteric arterioles. Data later appeared showing EDRF in small mesenteric arteries (200 \mu m i.d.) in vitro. Subsequently, by studying vessels less than 100 \mu m in size and by using two different techniques for injuring microvascular endothelium in vivo, Rosenblum showed that such injury did indeed eliminate relaxations by acetylcholine (ACh) and bradykinin (BK) while leaving intact relaxations to nitroprusside or papaverine, agents known to work independently of an EDRF.

Rosenblum's studies dealt with arterioles on the cerebral surface (pial arterioles). These vessels are of interest because they participate in control of cerebral blood flow and because they respond in many ways like the general cerebral circulation. Investigations of EDRF and its mechanisms of action in cerebral circulation are of interest because of the potential for endothelial injury in these vessels, in a variety of pathologic states, and because of the potentially catastrophic effects of limited vasodilation in an organ where oxygen supply is precariously balanced against demand. The EDRF for ACh in pial arterioles is unknown but appears to be different from that for BK. The EDRF for BK appears to be either a hydroxyl radical or to be dependent upon generation of that radical by BK.

In vitro studies of large vessels have shown that methylene blue inhibits both the endothelium-dependent responses to ACh and also the endothelium-independent relaxations produced by so-called nitro-dilators like nitroprusside. These results have been interpreted as supporting the hypothesis that guanylate cyclase and cyclic guanosine monophosphate...
(cGMP) mediate these relaxations because methylene blue inhibits the cyclase as well as the dilation.\textsuperscript{14-20} The following study tests the effect of methylene blue on pial arteriolar responses. Our purpose was, first, to see whether we could demonstrate results like those reported in vitro for large arteries. This is of interest apart from the cGMP hypothesis because of the diversity of vascular responses between beds and species described above and because some investigations of extracerebral microvasculature have failed to demonstrate an inhibitory effect of methylene blue on responses to ACh or sodium nitrite.\textsuperscript{21} Secondly, our data are of interest because of their relevance to the status of the cGMP hypothesis in the cerebral microcirculation.

Materials and Methods

Male mice, ICR strain (Dominion Labs Virginia, Dublin, Va.) were anesthetized with urethane, and the pial arterioles were exposed by craniotomy as previously described.\textsuperscript{22-24} The dose of urethane required by the ICR mouse is at least 1.5 g/kg i.p. If this dose did not result in absence of twitching, a single additional dose was given during the 30-minute equilibration period before the experiment. The total maximal dose was 2.0 g/kg. The body temperature was maintained at 37°C. The cerebral surface was continuously suffused at 1 ml/min with artificial cerebrospinal fluid\textsuperscript{23} (CSF) at pH 7.35.\textsuperscript{23} This was the mean pH both at the point where the suffusate first contacted the craniotomy site and in the suffusate collected as it exited from the opposite side of the craniotomy site, with the standard error of measurement less than 0.01. The suffusate was equilibrated with 6.5% O\textsubscript{2}. An arteriole 30-50 \(\mu\)m in diameter was arbitrarily selected for observation from the opposite side of the craniotomy site, with the image splitter, TV camera, and monitor, as described by Baez.\textsuperscript{26} The magnification on the monitor was 759\times. The image splitting technique permits changes of less than 0.5 \(\mu\)m to be detected.\textsuperscript{26,27} Output from the image splitter was recorded on a strip chart to give a permanent record of the responses.

All drugs were obtained from Sigma Chemical Co., St. Louis, Mo., and were applied to the cerebral surface in the artificial CSF at pH 7.35. Acetylcholine chloride (4 \(\times\) 10\textsuperscript{-4} M final concentration), bradykinin triacetate (6.5 \(\times\) 10\textsuperscript{-3} M final concentration), or sodium nitroprusside (10\textsuperscript{-4} M final concentration) was applied in a 1-ml bolus for 60 seconds. In some studies, cGMP or the cGMP analogue 8-bromo-cGMP (10\textsuperscript{-3} M) (Sigma) was used instead. The maximal change in diameter produced by the bolus was used as the basis for calculating dimensions as a percent of the baseline diameter. The doses used were selected because they produced dilations of approximately equal magnitude.

After testing the dilation, suffusion with artificial CSF continued for 8 minutes during which diameter returned to baseline levels. Then methylene blue chloride (Sigma) was used in attempts to block ACh, BK, nitroprusside, or 8-bromo-cGMP. The suffusing solution was switched to an artificial CSF containing methylene blue in concentrations of 10\textsuperscript{-4} or 10\textsuperscript{-3} M, and the cerebral surface was continuously suffused with this solution for 7 minutes. The bolus of dilator was applied again, and the response was monitored in the presence of the continuously flowing CSF containing the methylene blue. The latter was then washed out with a 15-minute suffusion of artificial CSF, and the response to the dilator (ACh, BK, nitroprusside, or 8-bromo-cGMP) was tested again. Thus, in each mouse, the response in the presence of methylene blue could be compared with the control responses 15 minutes earlier and with the recovery responses 15 minutes later using the Wilcoxon matched pairs signed ranks test.\textsuperscript{28} Only one dilator was tested per mouse, so separate groups of 10 mice each were used in individual studies of methylene blue's effect on each dilator.

At the end of each experiment, 100 \(\mu\)l of blood from the carotid artery was obtained for measurements of \(\text{O}_2\), \(\text{CO}_2\), and pH. These did not differ from study to study and are presented to indicate the general condition of the mice. Overall mean ± SD values were 107 ± 9 mm Hg, 35 ± 4 mm Hg, and 7.35 ± 0.04 pH units, respectively. These values will not be referred to again.

Results

Methylene Blue Inhibited Responses to Acetylcholine, Bradykinin, and Sodium Nitroprusside

Significant, reversible inhibition of both ACh and BK was produced by 10\textsuperscript{-4} M methylene blue. This is shown in the first 2 lines of Table 1. The greater effect of methylene blue on ACh reflects the fact that in the presence of methylene blue, more constrictions were observed with ACh than with BK. This is shown by the mean response to ACh during exposure to methylene blue. This response is a constriction. Thus, the greater effect of methylene blue on the response to ACh may not reflect greater inhibition of the dilating mechanism(s) to ACh but rather the presence of pronounced contractile responses to ACh in the absence of a functional dilating mechanism. On the other hand, recovery from methylene blue was less complete with ACh than with BK, suggesting that the inhibitory effect on ACh might be more potent than on the other agonists. The table also shows that 10\textsuperscript{-4} M methylene blue inhibited the response to nitroprusside.

In each of the studies presented in Table 1, methylene blue itself had no effect on diameter, so within each study, the diameters shown in the table were the same prior to each of the three tests of the dilator in question. A lower concentration of methylene blue, 10\textsuperscript{-3} M, was tested against ACh and showed a trend toward inhibition that was not statistically significant. In the absence of methylene blue, ACh increased diameter to 114 ± 7% of control, while in the presence of methylene blue, ACh increased diameter to only 106 ± 10% of control (\(n = 10\) pairs; \(p > 0.05\)). This lower concentration of methylene blue had absolutely no influence on dilation by nitroprusside. The diameter was in-
increased to 118 ± 4% of control in the absence of methylene blue and to 120 ± 7% in the presence of methylene blue.

Methylene Blue and Response to 8-Bromo-cGMP

The last line in Table 1 shows a failure of methylene blue to alter the dilation produced by 10⁻³ M 8-bromo-cGMP.

It was also of interest to see whether an identical concentration of cGMP itself, rather than the 8-bromo-analogue, would cause dilation because the analogue is known to be much more active than cGMP itself.²⁹ We failed to obtain significant dilation from 10⁻³ M cGMP (104 ± 6% of control, not significantly different from 100%). Consequently, we had no need to test methylene blue against cGMP.

Discussion

Similarities of Our In Vivo Microvascular Data to the In Vitro Literature Concerning Large Vessels

The data show an inhibitory effect of 10⁻⁴ M methylene blue on the dilations produced by ACh, BK, and nitroprusside. The effect is less marked against the nitroprusside. Higher concentrations of methylene blue (e.g., 10⁻³ M) were not used because they discolored the brain and made it too difficult to monitor diameter.

These data resemble those gathered by others during in vitro observations of large extracranial vessels, except that we had to use 10⁻⁴ M rather than 10⁻³ M methylene blue. Thus, Gruetter et al²⁸ used 10⁻⁵ M methylene blue to inhibit the relaxation of bovine coronary artery induced by nitroprusside, Holzmann²⁹ used 5 × 10⁻⁵ M methylene blue to inhibit the relaxation of bovine coronary artery induced by ACh, Martin et al²⁸ used 10⁻³ M methylene blue to inhibit the relaxation of aorta induced by acetylcholine, and Ignarro et al³⁰ used 10⁻³ M methylene blue to abolish relaxation of intrapulmonary artery produced by BK.

The fact that we had to use a somewhat higher concentration of methylene blue than that used in the in vivo literature concerning large vessels may simply reflect a time-dependent phenomenon. For example, Martin et al²⁸ reported larger and increasingly irreversible effects of methylene blue as incubation time with aorta was increased. Because methylene blue is a well-known vital stain for neural tissue,³⁰ it may even be that a "steal" of methylene blue by underlying brain created the need for higher concentrations of dye. Data we did not present showed less inhibition by dye if it was applied for only 1 minute, than if for 7 minutes. Others have offered preliminary data showing no effect of methylene blue in the microcirculation, even against ACh or nitro compounds.³¹ The latter data come from a noncerebral bed and could simply reflect differences in vessels or species when compared with our own data. In addition, attempts to use concentrations higher than 10⁻³ M were not reported.

The failure of methylene blue to alter baseline arteriolar diameter is in contrast to in vitro data reporting constriction by the dye.¹⁹,²⁰ We have no explanation for this; however, with this one exception, the major point to be made from our microvascular data, restricted of course to pial arterioles, is the similarity of our findings to those in the literature concerning in vitro studies of large vessels. This suggests that the same mechanism operates in the pial arterioles in vivo and in large extracerebral vessels in vitro to produce relaxation by ACh, BK, or nitroprusside. The identification of the mechanism(s) must be discussed separately. Chief among these is a guanylate cyclase-cGMP-dependent mechanism implicated in relaxation of many, but not all, smooth muscles.¹³ The

<table>
<thead>
<tr>
<th>Dilator</th>
<th>Before 10⁻⁴ MB</th>
<th>During 10⁻⁴ MB</th>
<th>After 10⁻⁴ MB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine CI 4 × 10⁻⁴ M (n = 10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diameter</td>
<td>34 ± 2</td>
<td>35 ± 4</td>
<td>34 ± 3</td>
</tr>
<tr>
<td>Response (% diameter)</td>
<td>114 ± 4</td>
<td>89 ± 6*</td>
<td>108 ± 10*</td>
</tr>
<tr>
<td>Bradykinin triacetate 7 × 10⁻⁵ M (n = 10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diameter</td>
<td>32 ± 3</td>
<td>32 ± 2</td>
<td>32 ± 2</td>
</tr>
<tr>
<td>Response (% diameter)</td>
<td>111 ± 3</td>
<td>103 ± 11†</td>
<td>113 ± 5†</td>
</tr>
<tr>
<td>Sodium nitroprusside 10⁻⁴ M (n = 10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diameter</td>
<td>34 ± 2</td>
<td>34 ± 3</td>
<td>34 ± 3</td>
</tr>
<tr>
<td>Response (% diameter)</td>
<td>113 ± 3</td>
<td>107 ± 9†</td>
<td>114 ± 7†</td>
</tr>
<tr>
<td>8-Bromo-cGMP 10⁻³ M (n = 10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diameter</td>
<td>34 ± 2</td>
<td>33 ± 3</td>
<td>34 ± 2</td>
</tr>
<tr>
<td>Response (% diameter)</td>
<td>111 ± 3</td>
<td>111 ± 4</td>
<td>110 ± 3</td>
</tr>
</tbody>
</table>

All values in table are mean ± SD. MB, methylene blue.
data we have presented here does not represent proof of the validity of the cGMP hypothesis in pial arterioles, nor were our experiments meant to be a definitive test of that hypothesis. Nevertheless, our data have many points of similarity with in vitro biochemical and pharmacologic data that have been used to support the cGMP hypothesis. These similarities are discussed below.

**Possible Role of Guanylate Cyclase and cGMP**

With large vessels, studied in vitro, evidence has been gathered implicating increased guanylate cyclase activity and consequent production of cGMP as critical events leading to vasodilation by ACh, BK, and nitroprusside.14,16,19,20 These publications not only demonstrate that nitroprusside and the endothelium-dependent dilators ACh and BK increase guanylate cyclase activity with production of cGMP but also that the degree of relaxation parallels these events. In vitro experiments with large vessels used methylene blue to inhibit dilations produced by endothelium-dependent agents like ACh and BK as well as endothelium-independent dilators like nitro compounds.1,4-10 Several of these publications also showed that methylene blue inhibited the increase in cGMP that normally accompanied dilation.

Two possible explanations were suggested for the impairment by methylene blue of dilations and for the prevention of cGMP increases normally produced by ACh.20 Either methylene blue destroyed EDRF or it inhibited guanylate cyclase. Either explanation was compatible with the known facts that methylene blue inhibits guanylate cyclase15,26 and also produces radicals31 that can destroy EDRF.32 Our own data showing that methylene blue inhibits dilation by ACh is also compatible with either hypothesis.

However, in vitro studies of nitroprusside and our in vivo data with nitroprusside are more easily explained by assuming that methylene blue inhibited guanylate cyclase, which is activated by nitroprusside.14,16,19,20 The data cannot be explained by assuming inactivation of an EDRF because dilation by nitro compounds is independent of the endothelium. The latter fact is not only well known for large vessels,1,2 but Rosenblum et al11 have also demonstrated this endothelial independence of nitroprusside with the same in vivo preparation of pial arterioles used in the present study.

It is also easier to explain methylene blue's inhibitory effect on bradykinin by suggesting inhibition of guanylate cyclase, than by suggesting inactivation of the EDRF for BK by radicals produced by methylene blue. In the pial arterioles, Rosenblum did show that BK's action is endothelium dependent (Rosenblum,15 Rosenblum et al15). However, the EDRF involved appears to be different from that for ACh. The latter is preserved by superoxide dismutase or catalase, while the EDRF for BK appears to be synonymous with or dependent upon hydroxyl radical produced by superoxide so that dilation is blocked by scavengers of these radicals.8,11 Because the radicals produced by methylene blue are oxygen-centered radicals24 like those mediating the relaxation produced by BK, it seems unlikely that these identical radicals would inactivate each other.

Our results showed that the effect of methylene blue on ACh, BK, and nitroprusside was reversible. In each case, the vessels recovered from methylene blue, and the final responses to each dilator were significantly larger than the preceding responses obtained in the presence of methylene blue. This recovery indicates that the decline in the presence of methylene blue was not simply due to deterioration of the preparation. Recovery was least pronounced with ACh, a result paralleling the fact that the greatest inhibitory effect of methylene blue was observed on responses to ACh.

There are at least two possible reasons for the greater inhibitory effect of methylene blue on ACh. First is the fact that when endothelium-dependent relaxation to ACh is impaired, constriction occurs due to direct action of ACh on smooth muscle.1 The constriction represents a counterbalancing response to dilation and could make the inhibition of dilating mechanisms appear greater than it really is. A second possibility is the simultaneous action of the two different possible modes of attack by methylene blue on the response to ACh. These have already been discussed, namely, a direct attack by a methylene blue-generated radical on the EDRF for ACh and an inactivation of guanylate cyclase by methylene blue. Both inhibitory actions working together might account for the larger effect of methylene blue on ACh. The effect on BK was smaller, and this might be explained if only one inhibitory mechanism were acting, namely, inhibition of guanylate cyclase as discussed above. The fact that methylene blue had less influence on nitroprusside than on ACh would be in keeping with the inhibition of guanylate cyclase by methylene blue and with the suggestion that nitroprusside have two different mechanisms of action, only one of which is guanylate cyclase dependent.20

Our data with 8-bromo-cGMP appear to support the hypothesis that guanylate cyclase mediates dilation via production of cGMP. We obtained dilation with the analogue 8-bromo-cGMP in a concentration lower than the maximally effective dose reported in vitro against larger vessels.29 Our data appear to be the first in vivo data using cerebral microvessels. The 8-bromo-cGMP analogue is known to be more effective than exogenous cGMP itself, and this was confirmed by our study. The effectiveness of the analogue and ineffectiveness of cGMP itself is thought to be due to the greater capacity of the analogue to penetrate the cell35-37 and to the resistance of the 8-bromo derivative to phosphodiesterase.38 Because cGMP is beyond the step of guanylate cyclase activation, we would not expect an inhibitor of the enzyme to block the effect of the cGMP analogue. In keeping with this expectation, methylene blue did not alter the dilation produced by 8-bromo-cGMP. The data not only support a role for cGMP in mediating dilation of pial arterioles but also indicate that methylene blue was acting in a selective manner when it blocked ACh, BK, and nitroprusside.
References

17. Holzmann S: Endothelium-induced relaxation by acetylcholine associated with larger rises in cyclic GMP in coronary arterial strips. J Cyclic Nucleotide Protein Phosphor Res 1982;8:409-419

Key Words: acetylcholine • cerebral microcirculation • endothelium-dependent relaxing factor • guanylate cyclase • nitroprusside • bradykinin • methylene blue • cyclic guanosine monophosphate

Circulation Research Vol 62, No 1, January 1988

artery caused by nitrogen oxide-containing vasodilators and acetylcholine. J Pharm Exp Ther 1986;236:30-36
In vivo effect of methylene blue on endothelium-dependent and endothelium-independent dilations of brain microvessels in mice.
M Watanabe, W I Rosenblum and G H Nelson

doi: 10.1161/01.RES.62.1.86

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1988 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/62/1/86