Hydroxyl Radical–Dependent Inactivation of Guanylate Cyclase in Cerebral Arterioles by Methylene Blue and by LY83583

Hermes A. Kontos, MD, PhD, and Enoch P. Wei, PhD

Background and Purpose: Methylene blue and 6-anilino,5,8-quinolinedione (LY83583) are used extensively to block activation of guanylate cyclase. Both agents generate oxygen radicals. Therefore, it appeared profitable to investigate whether the generation of oxygen radicals by these agents is responsible for the blockade of responses to nitroldilators that act via activation of guanylate cyclase to relax vascular smooth muscle and cause vasodilation.

Methods: We tested in anesthetized cats equipped with cranial windows responses to topical application of nitroglycerin, nitroprusside, and adenosine before and during topical application of methylene blue (5 μM). Responses to the vasoactive agents were tested during application of methylene blue after permeabilization of the cell membrane with a detergent to allow methylene blue to enter vascular smooth muscle. Responses were also tested in the presence of superoxide dismutase, catalase, deferoxamine, or dimethyl sulfoxide to scavenge reactive products of oxygen metabolism or to eliminate catalytic iron. In additional experiments we tested the effects of topical application of nitroprusside or adenosine before and after application of LY83583. The responses to the vasoactive agents were also tested in the presence of superoxide dismutase, catalase, or dimethyl sulfoxide in addition to LY83583. We also tested responses to calcitonin gene-related peptide before and in the presence of LY83583 with or without superoxide dismutase.

Results: Methylene blue eliminated the arteriolar dilation in response to nitroprusside and nitroglycerin after permeabilization of the cell membrane with a detergent but not before. The responses to adenosine were unaffected. The blockade induced by methylene blue was reversed by superoxide dismutase, catalase, or dimethyl sulfoxide but not by deferoxamine. LY83583 blocked responses to nitroprusside but not to adenosine. The blockade was eliminated by superoxide dismutase, catalase, or dimethyl sulfoxide. LY83583 blocked the vasodilation induced by calcitonin gene-related peptide. This blockade was reversed by superoxide dismutase.

Conclusions: Methylene blue and LY83583 prevent the activation of soluble guanylate cyclase by nitroldilators or by calcitonin gene-related peptide by generating oxygen radicals. The mediator of this response is the hydroxyl radical. Methylene blue does not enter the vascular smooth muscle of cerebral arterioles unless the cell membrane is permeabilized. (Stroke 1993;24:427–434)

Key Words • microcirculation • superoxide dismutase • vasodilation • cats

The experiments reported below were undertaken to investigate the role of oxygen radicals in the mechanisms by which methylene blue and 6-anilino,5,8-quinolinedione (LY83583) prevent the activation of soluble guanylate cyclase and the associated vasodilation in cerebral arterioles. The rationale for these studies is as follows.

Methylene blue is used extensively to block guanylate cyclase–dependent vascular smooth muscle relaxation. Methylene blue blocks the relaxations induced by nitroldilators or by endothelium-derived relaxing factor (EDRF) released by acetylcholine from isolated large vessels.1,2 The inhibition of vascular relaxation by methylene blue is due to inhibition of activation of soluble guanylate cyclase. The mechanism for this effect of methylene blue has not been identified.

We recently reported a different mechanism by which methylene blue interferes with endothelium-dependent relaxations from acetylcholine in the cerebral microcirculation of the cat.3 In this preparation, methylene blue in low concentration (5 μM) inhibited the vasodilation from acetylcholine by generating oxygen radicals in the extracellular fluid, thereby destroying EDFR. Methylene blue generates superoxide at a low rate by autooxidation.3,4 Surprisingly, even much larger concentrations (1 mM) of methylene blue did not affect the vasodilation from topical sodium nitroprusside.3 We reasoned that the differences between large vessels in vitro and the cerebral microcirculation in vivo with
respect to the effects of methylene blue might be
dependent on differences in the cellular permeability of
vascular smooth muscle cells. If vascular smooth muscle
cells are less permeable in the in vivo preparation and
methylene blue was, therefore, unable to reach guany-
late cyclase in the vascular smooth muscle of cerebral
arterioles in vivo, although it did so in the in vitro
preparations, this might easily explain the results.
Accordingly, we tested whether differences in cellular
permeability might account for the differences in the
behavior of methylene blue in the cerebral microcircu-
lution of the cat versus what is seen in large vessels in
vitro. We also investigated the possibility that the
prevention of activation of guanylate cyclase by methyl-
en blue after increase in cell permeability might be due
to generation of oxygen radicals.
LY83583 also prevents activation of guanylate cyclase
by nitrodiolators, and it is used extensively for this
purpose. It is well known that quinones are reduced in
tissues via a univalent pathway to the semiquinones,
which then react with oxygen spontaneously to generate
superoxide. It appeared likely, therefore, that the
generation of oxygen radicals might be the mechanism
underlying the effect of LY83583 on guanylate cyclase.
Recent studies in isolated pulmonary vessels or in the
rabbit aorta are consistent with this interpretation.
Accordingly, we investigated the role of oxygen radicals
in the effect of LY83583 on activation of guanylate
cyclase.
Recent studies from our laboratories showed that the
vasodilator action of nitroprusside and nitroglycerin on
cerebral arterioles in the cat is to a large extent medi-
ated by the release of calcitonin gene-related peptide
(CGRP) from sensory nerve fibers. In the present
experiments we investigated the effect of LY83583 on
the response to CGRP and the role of oxygen radicals in
this response.

Materials and Methods
Experiments were performed in cats (2–3.5 kg) anes-
ethetized with sodium pentobarbital (30 mg/kg i.v.).
Additional doses of anesthetic were given as required to
maintain surgical anesthesia, based on testing of corneal
reflexes and responses to tail pinch. After tracheostomy,
each cat was ventilated with a positive pressure respira-
tor. The end-expiratory CO2 of the animals was
continuously monitored with a Hewlett-Packard CO2
analyzer and was maintained at a constant level of
approximately 30 mm Hg. Arterial blood pressure was
measured with a Statham pressure transducer con-
ected to a cannula introduced into the aorta via the
femoral artery. Arterial blood samples were collected
for determination of arterial blood oxygen, CO2 partial
pressures, and pH at appropriate intervals during the
experiment. Blood gas tensions and pH were measured
with Corning electrodes. The rectal temperature of each
animal was monitored continuously and was kept con-
stant with the aid of a heating blanket.
The cerebral microcirculation of the parietal cortex
was visualized through an acutely implanted cranial
window, as described in detail previously.11 The space
under the cranial window was filled with artificial cere-
brospinal fluid (CSF), identical in composition to that of
cats. One port of the window was connected to a
Statham low-pressure transducer for continuous moni-
toring of intracranial pressure. The intracranial pres-
sure was maintained at 5 mm Hg by connecting another
outlet of the window to a coiled plastic tube the free end
of which was placed at the appropriate height to give the
desired pressure. Two ports of the cranial window were
used as inlet and outlet, allowing topical application of
various solutions by superfusion. Pial arteriolar diam-
ter was measured with a Vickers image-splitting device
attached to a Leitz Universal microscope. In each ani-
mal, several arterioles were observed covering a wide
range of vessel caliber. The responses of small and large
arterioles (smaller or larger than 100 μm in diameter,
respectively) were analyzed separately to identify any
size-dependent differences in responses.
Acetylcholine chloride, methylene blue, superoxide
dismutase (SOD) (3,500 units/mg protein, from bovine
blood), catalase (40,000 units/mg protein, from bovine
liver), adenosine, sodium dodecyl sulfate (SDS), sodium
nitroprusside, and dimethyl sulfoxide (DMSO) were
obtained from Sigma. Deferoxamine was purchased
from CIBA. LY83583 was a gift from Eli Lilly and Co.
Nitroglycerin was prepared from tablets as described
previously. All solutions were prepared in CSF imme-
diately before use and were equilibrated at 37°C in a
water bath immediately before application under the
window. Solutions were prepared to achieve the follow-
ing final concentrations: acetylcholine 10–7 M, methyl-
en blue 5 μM, sodium nitroprusside 1 μM, nitroglyc-
erin 2 μM, SDS 20 μM, deferoxamine 1 mM, DMSO 10
μM, and adenosine 10–4 M.
The experimental design was as follows. We first
obtained control responses to topical application of
nitroprusside, nitroglycerin, and adenosine. Nitroprus-
side and nitroglycerin cause vasodilation via activation
of guanylate cyclase, and adenosine acts via adenylyl
cyclase. In each case, the space under the cranial
window was filled with the appropriate solution, which
was left to stand until vessel responses reached a steady
state. This was achieved within 2–4 minutes. Responses
are expressed as percent changes from the baseline
diameters and represent the steady-state values ob-
tained during this time. Subsequently, the responses to
these agents were retested in the presence of 5 μM
methylene blue. A third test of the responses to nitro-
prusside, nitroglycerin, and adenosine in the presence
of 5 μM topical methylene blue was carried out after a
15-minute application of 20 μM SDS. This detergent
increases cellular permeability. The intent was to in-
crease cellular permeability and thereby allow methyl-
en blue to enter vascular smooth muscle. The tech-
nique of permeabilization of cells to enhance entry of
various molecules into the intracellular compartment and
attain access to intracellular enzymes has been used
successfully in vascular and cardiac muscle as well as in
isolated hepatocytes and in ovarian cells in culture.12-15
Under these conditions, the cells remain viable and
retain normal histological features. In three experi-
ments we performed electron microscopic studies after
the application of SDS as described above. In these
experiments after completion of the physiological stud-
ies, the brain was fixed by perfusion. We first perfused
the brain transcardially with 0.9% sodium chloride
solution followed by a fixative consisting of 2% parafor-
maldehyde and 2.5% glutaraldehyde in 0.1 M phosphate
buffer. Subsequently, the arachnoid membrane in the
location underlying the cranial window was peeled off together with the pial vessels, and the same vessels that in the physiological studies showed blockade of the responses to nitrodilators with the application of SDS and methylene blue were selected for study. The tissues were then processed as described previously\(^{16}\) for examination by transmission electron microscopy. The endothelium and vascular smooth muscle appeared entirely normal. The only detectable change was an apparent increase in sarcolemmal pits. We found in earlier studies that after SDS application vasodilator responses to acetylcholine, nitroprusside, nitroglycerin, and adenosine were not altered.\(^ {17}\) In a final phase of the experiment, the responses to nitroprusside, nitroglycerin, and adenosine were tested for the fourth time in the presence of SOD 60 units/ml, catalase 40 units/ml, deferoxamine 1 mM, or DMSO 10 \(\mu\)M.

In another series of experiments, we investigated the mechanism of action of LY83583. We first tested responses to topical application of nitroprusside, nitroglycerin, and adenosine as described above for methylene blue. We then applied 10 \(\mu\)M LY83583 for 15 minutes. Subsequently, we retested responses to the vasoactive agents. In a final phase of the experiment, we tested responses to nitroglycerin, nitroprusside, and adenosine in the presence of either SOD 60 units/ml, catalase 40 units/ml, or DMSO 10 \(\mu\)M.

In the final series of experiments we investigated the effect of LY83583 on the vasodilator action of CGRP. We first tested responses to topical application of CGRP. We then applied 10 \(\mu\)M LY83583 for 15 minutes. Subsequently, we retested responses to CGRP. In a third phase of the experiment, we tested responses to CGRP in the presence of SOD 60 units/ml.

Statistical analysis of the results was done with analysis of variance followed by \(t\) tests modified for multiple comparisons.

**Results**

Methylene blue in a dose of 5 \(\mu\)M did not affect the responses to nitroprusside, nitroglycerin, or adenosine (Figures 1–4). The results with respect to nitroglycerin and nitroprusside confirm our findings in earlier experiments.\(^ {3}\) After application of SDS to increase cellular permeability, methylene blue blocked the responses to nitroprusside and nitroglycerin but did not affect the responses to adenosine. The blockade of the responses to nitroprusside and nitroglycerin by methylene blue was reversed in the presence of catalase, SOD, or DMSO (Figures 1–3) but not in the presence of deferoxamine (Figure 4). Baseline arteriolar caliber was unaffected by methylene blue (before and after SDS), SOD, catalase, DMSO, or deferoxamine.

LY83583 completely inhibited the vasodilation from nitroprusside but did not affect the response to adenosine (Figures 5–7). The blockade induced by LY83583 was completely reversed by SOD, catalase, or DMSO (Figures 5–7). LY83583 did not alter baseline arteriolar caliber.

LY83583 completely inhibited the vasodilation from CGRP. The blockade induced by LY83583 was reversed by SOD (Figure 8).

**Discussion**

There are two important findings from the present experiments. First, LY83583 and methylene blue, when the latter is applied after an increase in cellular permeability, prevent activation of guanylate cyclase by generating oxygen radicals. The mediator for this response is the hydroxyl radical. Second, the inhibition of the vasodilator action of the nitrodilators nitroprusside and nitroglycerin by methylene blue in the cerebral microcirculation occurs only after cellular permeability is increased with a detergent but not under normal baseline circumstances. The dependence of the effect of methylene blue on the response to nitroprusside and nitroglycerin on cellular permeability explains important differences in the effect of methylene blue on the responses to the nitrodilators in different preparations. In the cerebral microcirculation, where permeability to this agent is low under normal conditions, even very high concentrations of methylene blue do not affect the vasodilation from nitroprusside and nitroglycerin. In in vitro preparations, on the other hand, where permeabil-
ity is high and access by methylene blue to vascular smooth muscle is easier, inhibition of these responses is the rule. This difference is not a peculiarity of the cerebral microcirculation because other investigators have found that it is more difficult to block responses to the nitrodlators than responses to acetylcholine.\textsuperscript{3,18}

The present study and earlier studies\textsuperscript{3} show that methylene blue can interfere with endothelium-dependent responses in the cerebral microcirculation of the cat by two mechanisms. The first mechanism operates only when cellular permeability is abnormally high and methylene blue has access to the interior of vascular smooth muscle. This effect depends on the blockade of guanylate cyclase. A second mechanism of action was identified earlier\textsuperscript{3} by showing that methylene blue inhibited the vasodilatation from acetylcholine but did not affect responses to nitrodlators. This is the only effect of methylene blue when permeability is normal and methylene blue is restricted to the extracellular space. This action is due to direct oxidation of EDRF by oxygen radicals produced by methylene blue.\textsuperscript{3} Thus, both mechanisms of action depend on the generation of oxygen radicals but in two different locations. The inactivation of EDRF occurs in the extracellular space, whereas the inactivation of guanylate cyclase requires access by methylene blue to the interior of vascular smooth muscle cells. Methylene blue is a photosensitive dye that, when exposed to light, is capable of generating superoxide.\textsuperscript{3,4} This ability of methylene blue is enhanced in the presence of electron donors.\textsuperscript{3,4}

Our findings show that the inhibition of the responses to nitroprusside and nitroglycerin brought about by methylene blue in the presence of high cellular permeability after SDS application is due to generation of oxygen radicals. The findings suggest that the most probable immediate cause for the inactivation of guanylate cyclase is the hydroxyl radical. This is based on the fact that SOD as well as catalase and DMSO

![Figure 2](http://stroke.ahajournals.org/content/24/3/430/f2)

**Figure 2.** Bar graphs show vasodilator responses to topical nitroprusside, nitroglycerin, and adenosine in the control period, in the presence of methylene blue (MB), in the presence of MB after permeabilization with sodium dodecyl sulfate (SDS), and in the presence of MB plus catalase after permeabilization with SDS. Values are mean±SEM from 18 small and 15 large arterioles in five cats. Note that responses to nitroprusside and nitroglycerin were significantly lower than control in both small and large arterioles in the presence of MB after permeabilization with SDS. All other responses were comparable to control values.

![Figure 3](http://stroke.ahajournals.org/content/24/3/430/f3)

**Figure 3.** Bar graphs show vasodilator responses to topical nitroprusside, nitroglycerin, and adenosine under control conditions, in the presence of methylene blue (MB), in the presence of MB after permeabilization with sodium dodecyl sulfate (SDS), and in the presence of MB plus dimethyl sulfoxide (DMSO) after permeabilization with SDS. Values are mean±SEM from 16 small and 12 large arterioles in five cats. Note that responses to nitroglycerin and nitroprusside in the presence of MB after permeabilization with SDS were significantly lower than control in both small and large arterioles. All other responses were comparable to control values.
reverses the inactivation caused by methylene blue. The absence of an effect of deferoxamine in the present studies may be due to a high concentration of iron in the cells so that the amount of deferoxamine used in our experiments was inadequate to eliminate all the iron. In contrast, the concentration of catalytic iron for the Haber-Weiss reaction in the CSF is in the micromolar range. Hence, deferoxamine easily eliminates the catalytic iron in that location and thus removes the blockade induced by methylene blue on the vasodilation induced by EDRF generated by acetylcholine. An alternative explanation for the absence of an effect of deferoxamine in the present experiments may be that the agent is unable to reach the effective site of the catalytic iron responsible for the generation of the hydroxyl radical, which inactivates guanylate cyclase. We prefer the first explanation because cellular permeability is high under the conditions of the experiment, and SOD and catalase, despite their higher molecular weight, are effective. Methylene blue after SDS inhibited the responses to the nitrodlators but did not influence the response to adenosine. This shows that the effect of the dye is specific for guanylate cyclase-mediated responses and suggests that adenylyl cyclase is not sensitive to oxygen radicals.

Our findings show that the blockade of guanylate cyclase by LY83583 is also mediated by the generation of oxygen radicals and that, as is the case with methylene blue, the immediate cause of the blockade is the generation of the hydroxyl radical. It is well known that quinones are reduced in cells by a univalent pathway to generate semiquinones, which, in turn, interact with oxygen to generate superoxide. The generation of superoxide by LY83583 has been demonstrated in pulmonary arteries by chemiluminescence techniques by Cherry et al. Similarly, Gidari and colleagues showed that LY83583 generated superoxide and inactivated EDRF in the isolated rabbit aorta. Our present findings confirm our earlier observations that the action of CGRP on cerebral arterioles of the cat is mediated by

**FIGURE 4.** Bar graphs show responses to topical nitroprusside, nitroglycerin, and adenosine in the control period, in the presence of methylene blue (MB), in the presence of MB after permeabilization with sodium dodecyl sulfate (SDS), and in the presence of MB plus deferoxamine after permeabilization with SDS. Values are mean ± SEM from 12 small and 11 large arterioles in five cats. Note that responses to nitroglycerin and nitroprusside were significantly lower than control in both small and large arterioles in the presence of MB after permeabilization with SDS with or without deferoxamine. All other responses were comparable to control values.

**FIGURE 5.** Bar graphs show vasodilator responses to topical nitroprusside and adenosine in the control period, in the presence of 6-anilino, 5,8-quinolinedione (LY83583), and in the presence of LY83583 plus superoxide dismutase (SOD). Values are mean ± SEM from 16 small and 14 large arterioles in five cats. Note that responses to nitroprusside were significantly lower than control in both small and large arterioles in the presence of LY83583. All other responses were comparable to control values.
activation of guanylate cyclase because it is blocked by LY83583. The present findings also show that the effect of LY83583 on the response to CGRP is also due to generation of oxygen radicals because it is reversible by SOD, and these findings exclude the possibility that the radicals might be acting on the sensory fibers to prevent the release of CGRP by the nitrodilators.

Our findings with both methylene blue and LY83583 confirm in part the results of Cherry et al.⁹ who found that in pulmonary arteries the generation of oxygen radicals inactivated guanylate cyclase. In their experiments the blockade appeared to be dependent on superoxide, whereas in ours it was due to hydroxyl radical generation.

The mechanism by which hydroxyl radical prevents the activation of guanylate cyclase by nitrodilators and CGRP is not known. We presume that it is due to oxidation of sensitive components of the enzyme or a change in the redox state of the enzyme. In either case, the effect is readily reversible by antioxidants.

Mittal and Murad²⁰ reported that hydroxyl radical activated partially purified liver-soluble guanylate cyclase. This preparation produced superoxide spontaneously. They found that the addition of SOD activated guanylate cyclase, whereas catalase and several agents that directly scavenge hydroxyl radical inhibited this activation. They concluded that guanylate cyclase was activated by hydroxyl radical generated via the Haber-Weiss reaction from superoxide and hydrogen peroxide. Some of their observations, however, are not consistent with this conclusion. For example, SOD would be expected to inhibit the Haber-Weiss reaction by eliminating one of the precursors of hydroxyl radical and should have had an inhibitory effect on guanylate cyclase activation, but the reverse occurred. Also, not all scavengers of hydroxyl radical used were consistently effective. The findings of Mittal and Murad²⁰ would be more consistent with inhibition of guanylate cyclase by superoxide and activation by hydrogen peroxide. Burke and Wolin²¹ described activation of guanylate cyclase in pulmonary arteries in vitro by hydrogen peroxide. The mechanism of activation involved the generation of compound I of catalase. If this is the correct explanation for the findings of Mittal and Murad,²⁰ the effect of some of the hydroxyl radical scavengers used may be explained by the fact that they inactivate catalase rather than by direct scavenging of hydroxyl radical.

It is worthy of note that in our experiments, the generation of oxygen radicals by methylene blue or LY83583 was not accompanied by any changes in baseline caliber of cerebral arterioles. Superoxide, hydrogen peroxide, and hydroxyl radical generated in the extra-
cellular space all dilate cerebral arterioles in cats. The absence of an effect of methylene blue or LY83583 in the present experiments may be due to the fact that the effects of oxygen radicals generated intracellularly may be different or because the rates of production are less than those required to produce significant vasomotor effects. The first explanation is unlikely because methylene blue when confined to the extracellular space also did not induce significant alterations in baseline arteriolar caliber. The rates of generation of superoxide observed in vitro from the concentration of methylene blue used in the present experiments of 0.02 nmol/min in the absence of reducing agents and 1.44 nmol/min in the presence of a reducing agent are sufficiently low to be consistent with this interpretation. Also, the absence of changes in baseline arteriolar caliber despite effective inhibition of guanylate cyclase by methylene blue after SDS treatment or by LY83583 suggests that guanylate cyclase is not active under resting conditions.

References

Figure 8. Graphs show dose–response curve to calcitonin gene-related peptide (CGRP) in the control period, in the presence of 6-anilino,5,8-quinolinedione (LY83583), and in the presence of LY83583 plus superoxide dismutase (SOD). Values are mean±SEM from 21 small and 19 large arterioles in six cats. Note that LY83583 eliminated the vasodilator response to CGRP and that the addition of SOD restored the response to control values. Baseline diameters from which the percent changes were derived are given in the insert in micrometers.
Relaxation of vascular smooth muscle involves the activation of soluble guanylate cyclase and subsequent dephosphorylation of myosin light chain kinase.\textsuperscript{1–3} Methylene blue and 6-anilino,5,8-quinolinedione (LY83583) have been used extensively to inhibit relaxation of vascular smooth muscle, and it has been proposed that inhibition of relaxation by methylene blue and LY83583 is related to inhibition of activation of soluble guanylate cyclase.\textsuperscript{4,5} The precise mechanism for the effects of methylene blue and LY83583 on guanylate cyclase, however, is not clear.

The present studies by Kontos and Wei examine the mechanism by which methylene blue inhibits diatation of guanylate cyclase by generation of oxygen radicals. Finally, the authors examined the possible role of oxygen radicals in inhibition of cerebral vasodilatation by LY83583. The authors found that methylene blue prevented diatation of cerebral arteries in response to nitrovasodilators only after increasing the permeability of vascular smooth muscle with sodium dodecyl sulfate (SDS). In addition, diatation of cerebral arteries in response to nitrovasodilators could be restored using enzymatic inhibitors of oxygen radicals. Finally, LY83583 prevented diatation of cerebral arteries in response to nitroprusside, and this inhibition could be reversed by application of enzymatic scavengers of oxygen radicals. The present studies by Kontos and Wei demonstrate that methylene blue prevents dilatation of cerebral arteries in vivo only after increasing cellular permeability to methylene blue. In addition, methylene blue and LY83583 inhibit diatation of cerebral arteries in vivo by production of oxygen radicals, presumably production of hydroxyl radical. Thus, these studies provide important new information concerning the mechanism by which methylene blue inhibits dilatation of cerebral arteries in vivo.

\textbf{Editorial Comment}

William G. Mayhan, PhD, Guest Editor
Department of Physiology and Biophysics
University of Nebraska Medical Center
Omaha, Neb.

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H A Kontos and E P Wei

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