Endothelium-Derived Relaxing Factor in Brain Blood Vessels Is Not Nitric Oxide

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Background: The endothelium-derived relaxing factor that mediates the actions of acetylcholine is now most frequently identified as nitric oxide. Nitric oxide is believed to have numerous important regulating actions in neurons, blood vessels, and several other biological systems.

Summary of Review: The literature concerning tissue other than cerebral blood vessels supports the conclusion that the endothelium-derived relaxing factor for acetylcholine is either nitric oxide or a compound formed from and containing nitric oxide (for example, a nitrosothiol). However, papers can be found indicating that this endothelium-derived mediator is not nitric oxide. In brain blood vessels the evidence is strongly against the conclusion that nitric oxide is the endothelium-derived mediator for acetylcholine. If this mediator is formed from nitric oxide, either in brain vessels or in other vessels, no data are available delineating how this synthesis is regulated or whether and where nitric oxide leaves the nitroso compound to initiate dilation. Indeed, cerebrovascular data now cast doubt on the commonly held belief that nitrosovasodilators regulate vascular tone by giving off nitric oxide to vascular smooth muscle.

Conclusions: In brain blood vessels the chemical identity of the endothelium-derived relaxing factor mediating the action of acetylcholine is unknown, but this relaxing factor does not appear to be nitric oxide. If the mediator contains nitric oxide, as is probably the case, the means by which it activates vascular guanylate cyclase and/or produces dilation is unknown. Since this relaxing factor inhibits platelet adhesion/aggregation in cerebral vessels as well as relaxing these vessels, the chemical identification of this relaxing factor and the elucidation of its mode of action are extremely important to our understanding and control of cerebrovascular phenomena in health and disease. (Stroke 1992;23:1527-1532)

KEY WORDS • acetylcholine • endothelium-derived relaxing factor • platelet aggregation • vasodilation

There has been a recent emphasis on the importance of endothelial cell products as mediators of vascular tone. The discovery of endothelium-derived constricting and dilating factors should have profound effects on our understanding of the regulation of cerebrovascular tone in health and disease. This is especially so since endothelial cell injury is a well-known phenomenon in atherosclerosis, ischemia, and hypertension and since subarachnoid hemorrhage has also been found to impair the synthesis/release of endothelium-derived mediators of cerebrovascular tone.1,2 The chemical identification of the various endothelium-derived mediators would be especially critical to our understanding of cerebrovascular control and to the development of appropriate interventions when deranged endothelial function contributes to morbidity. As has so often been the case, major differences appear to exist between extracerebral vessels and cerebral vessels, this time with respect to the identity of endothelial mediators that are responsible for the vasoreactivity of specific agonists. Recent widespread attention has been given to the role of nitric oxide (NO) as the key mediator of endothelium-dependent relaxation. This review of the available literature suggests that, at least in the cerebral circulation, this is not the case.

Acetylcholine (ACh) was the first dilator shown to have an endothelium-dependent action that was not a consequence of prostaglandin release by the endothelium. The endothelium-derived mediator of ACh action was called endothelium-derived relaxing factor (EDRF).3-6 EDRF is currently the focus of intense investigation. This mediator is an important regulator of vascular tone and intravascular platelet aggregation.4-8 There is a general assumption that EDRF is NO and that NO is also important as a messenger sent to effector tissues by a variety of cells, including neurons.4,6,9-12 However, evidence has been repeatedly presented by a variety of workers13-19 that NO is not EDRF. In the cerebral circulation, in particular, substantial evidence has accumulated indicating that NO is not EDRF. Tacit recognition of this situation may be reflected in the failure of extensive, recent reviews to contain any reference to the cerebral circulation.6,10,11 The following review is written with the purpose of describing the discordant data and encouraging a reex-
amination of present concepts concerning the role of NO in endothelial control of both cerebral and extracerebral vessels. At the very least, the reader will be reminded that studies of extracerebral vessels do not always provide accurate information about control of events in the vasculature of the brain. To set the stage, a recapitulation of the history of this field is in order.

With the discovery of prostaglandins came the realization that endothelium produced at least one messenger with the capacity to relax vessels and inhibit platelet aggregation. This messenger was, of course, prostacyclin. Then, about 10 years ago it was discovered that ACh-relaxed vessels, not by a direct action on vascular smooth muscle but by acting on endothelium which, when stimulated by ACh, released EDRF, which relaxed vascular smooth muscle, apparently by activating guanylate cyclase (g cyclase). EDRF is not only released by ACh and some other agonists, but it may also be basally released from many vascular beds including that of the brain. Subsequently a substantial body of work has led to the belief that EDRF is either NO or a nitroso compound, perhaps a nitrosothiol, that liberates NO. Moreover, NO has been implicated in other important biological reactions. For example, some neurons and macrophages are said to release NO that mediates the response of target cells. Some of these responses and the dilation produced by EDRF, ACh, or NO are all thought to be consequences of g cyclase activation. It became especially convenient to call the mediator of ACh action EDRF when it was found that several other dilators appeared to be mediated by the same compound, so that a single name would suffice for the common mediator. However, it is now known that even ACh may have two different EDRFs and that in the vessels of the brain the three classical stimuli of EDRF production (ACh, bradykinin, and calcium ionophore A23187) are each mediated by a different EDRF rather than by a single substance. Indeed, this was the first difference to emerge between studies of cerebral vascular endothelium and other preparations. We recommend use of terms like EDRF ACh, EDRF BK, and EDRF Ca, to indicate an EDRF for a specific agonist. This makes clear the possibility that each agonist has a different endothelial mediator and avoids the term NO for EDRF ACh. However, even this nomenclature fails to indicate the specific vascular bed in question, a point of significance since different vessels may employ different mechanisms of relaxation, even in the same species.

The evidence reviewed below clearly shows that, in the brain vessels, EDRF cannot be NO.

The hypothesis that NO is EDRF ACh originated with the recognition that NO synthase, the enzyme has been called NO synthase. It activates g cyclase. EDRF ACh and NO both activate g cyclase, both are extremely short-lived, and both are "stabilized" at acid pH. Therefore, it was suggested that EDRF ACh was NO. It was quickly established that L-arginine (L-ARG) can serve as substrate for an enzyme producing NO. In fact, two forms of this enzyme, with different cofactors, have been identified. The enzyme has been called NO synthase. It produces both NO and citrulline (CIT) in stoichiometric proportions from L-ARG. Because NO itself is difficult to measure, many workers have measured CIT or guanosine 3’-5’-cyclic monophosphate instead as markers of EDRF production and NO synthase activity. L-ARG analogues that competitively inhibit NO synthase have been found to inhibit dilators, like ACh, whose actions are dependent upon EDRF ACh. In addition, NO production has been measured in a variety of endothelial preparations, and these measurements have been presented as evidence that EDRF is NO and that endothelium-dependent dilators stimulate NO production and release. Finally, a variety of target tissues have been stimulated by NO and by authentic EDRF released in bioassays. The test organs have been said to respond identically to both agents. All of this evidence provides potent support for equating EDRF ACh with NO.

However, even outside the brain, a question has been raised concerning the identity of EDRF ACh as NO. Not all studies reveal agreement between bioassay profiles of NO and authentic EDRF. Moreover, the techniques for measuring NO are often nonspecific and generally indirect. They often depend on conversion of NO to another compound that is then measured and/or on treatment of the analyte in a reflux chamber with acetic acid, which would produce NO from that analyte. Therefore, in many studies (e.g., References 42 and 43), the NO being measured could reflect its precursor, which could then be the true EDRF. Indeed, techniques that would be more specific for NO have failed to detect it from preparations in which others, using less specific techniques, have detected it easily (e.g., References 42 and 43). In addition, when the amount of NO detected is compared with the amount required to mimic EDRF ACh in the same system, workers have sometimes failed to show sufficient NO to account for the dilation observed. Finally, several studies failed to detect NO even though they detected CIT from endothelial cells believed to be producing EDRF ACh. Some of these facts, notably that some NO-detecting systems may produce NO from a precursor, have led to the caveat that EDRF ACh is either NO or a nitroso precursor. A nitrosothiol is the most commonly suggested precursor. This alternative is especially attractive since nitrosothiols are potent vasodilators and in brain vessels might account for the biological activity of EDRF ACh. Moreover, a thiol intermediate has been strongly implicated in the action of nitrovasodilators like nitroglycerin. Nevertheless, the overwhelming impression given by article titles, texts, and the name used for the enzyme synthesizing a g cyclase-activating factor is that EDRF ACh is NO.

The evidence from investigations of brain blood vessels provides the strongest data suggesting that, at least in that vascular bed, EDRF ACh is not NO. When evaluating the data from brain blood vessels one should keep in mind the well-known differences between species and even between vessels within a species with respect to the existence of, and mediators for, endothelium-dependent dilation. One should also note that studies of brain vessels often use the surface vasculature, or pial vessels, which are generally less than 300 μm in diameter. It is possible that these brain data reflect microvascular phenomena that might also be present in other organs and be distinct from the phenomena found in studies of large conductance arteries. It should also be noted that most studies examining the chemical identity
of EDRF in tissue other than brain blood vessels, and delineating the existence of NO synthase in those tissues, do not use intact blood vessels. Instead they use cultured endothelial cells harvested from large vessels or homogenates of these cells. In addition, homogenates of other organs such as adrenal gland have been used, as have brain slices. In these instances the data have been gathered to support a role for NO as an important messenger of cellular responses other than vascular responses. Perhaps caution is in order when using data gathered from other tissues to draw conclusions about blood vessels. Similarly, caution may be in order when using data from cultured endothelial cells or homogenates of these cells. Cultured cells do not have all the properties of normal endothelium. For example, they lose receptors for ACh so that other stimuli like bradykinin or calcium ionophore are used to initiate production of EDRF. It may not be appropriate to extrapolate from such studies to the cerebral circulation since in that circulation the effects of both bradykinin and calcium ionophore may be mediated by EDRFs that differ from EDRF ACh. The latter conclusion is based on in vivo studies of the cerebral circulation. Also based on in vivo studies is the conclusion that, in the brain, EDRF ACh is not NO.

In brain vessels methylene blue blocked ACh without affecting NO. In the same study nitro blue tetrazolium (NBT) blocked NO but did not interfere with ACh. In a subsequent study a concentration of hydrogen peroxide (H2O2) was found that prevented formation of EDRF ACh but did not inactivate bioassayable EDRF ACh once it had been formed. On the other hand, NO was blocked by the same concentration of H2O2. These two studies, of cat pial vessels in vivo, clearly show that NO and EDRF ACh are affected differently by identical treatments; one is blocked and the other not, and vice versa. Clearly, then, EDRF ACh cannot be NO.

These two studies taken together are also compatible with the suggestion that EDRF ACh in brain vessels is a nitrosothiol. In the study in which NBT was used the authors suggested that nitrosovasodilators work by interacting with a thiol in the vascular smooth muscle and that by oxidizing "SH groups on the thiol NBT prevented this interaction and prevented the nitrosovasodilators from working. If this explanation is correct and if EDRF ACh is a nitrosothiol, then we must ask why NBT failed to block the action of ACh. Perhaps the vascular smooth muscle of cat pial arterioles is permeable to NBT while the endothelium is not. Then nitrosovasodilators that directly affect the smooth muscle would be blocked but thiols in the endothelial cells would not be affected. If NO were EDRF ACh, then once it reached the smooth muscle its action there should have been blocked by NBT, just as the action of exogenous NO was blocked. Hence, these data indicate that NO is not EDRF ACh. On the other hand, if EDRF ACh were a nitrosothiol formed from NO and a thiol in endothelial cells, this would explain why NBT excluded from endothelial cells is unable to interfere with the action of ACh. Direct support for the hypothesis that a nitrosothiol is EDRF ACh in pial vessels was provided by the second paper from the same group. Selected doses of H2O2 were capable of preventing the formation of EDRF ACh but were not capable of inactivating EDRF ACh once it was formed. These concentrations of H2O2 inactivated NO but failed to interfere with the action of an S-nitrosothiol that was found to be a very potent dilator of the pial arteries and arterioles. It was suggested that H2O2 oxidized the "SH groups essential for formation of nitrosothiols and thus prevented both the formation of EDFR ACh and the action of nitrosovasodilators. However, once the nitrosothiol was formed in endothelial cells H2O2 could no longer affect it.

In the same preparation methylene blue inactivated EDRF ACh by generating an oxygen-centered free radical. Since it is well known that NO is inactivated by such radicals, we must ask why NO applied to cat pial vessels was not inactivated by methylene blue. It may take more radical to inactivate NO than it takes to inactivate EDRF ACh. Thus, the amount of radical generated, the rate of generation, and the peak concentration may be important. In vitro studies that demonstrate inhibition of EDRF ACh by oxygen-centered radicals generally use baths with 95% O2 rather than the physiological levels employed in the in vivo study. This might lead to a background of radical production upon which radical-generating systems generate still more radical in significant excess of the total amount produced by methylene blue during the in vivo studies. Indeed, in vitro studies frequently show relaxation of vessels by superoxide dismutase (SOD), implying basal production of a radical that inactivates EDRF ACh; in contrast, in vivo studies of pial vessels performed at much lower oxygen levels have not shown such an effect of SOD.

The cited studies of cerebral vessels do not invalidate the conclusion that L-ARG is required for synthesis of EDRF ACh. The studies do suggest that during the conversion of L-ARG to CIT by NO synthase an NO group is transferred to some other molecule, possibly a thiol, to form EDRF ACh. The suggestion that a thiol is involved is consonant with suggestions from work done with extracerebral vessels.

Nominating a nitrosothiol as EDRF ACh is also consonant with suggestions that nitrosovasodilators produce dilation by first releasing NO, which then forms a nitrosothiol that is the direct activator of g cyclase. However, direct evidence for this hypothesis is not available, and on occasion its proponents have suggested that NO directly activates g cyclase. Some workers have taken the position that it does not matter if a precursor of EDRF ACh (e.g., a nitrosothiol) is produced prior to production of EDRF ACh. This view is based on the belief that EDRF ACh is NO and that the precursor must give off NO prior to activation of g cyclase. Clearly, the evidence from in vivo investigations of brain vessels contradicts this belief since different treatments inactivated or blocked one of these dilators without inactivating or blocking the other. Thus, in the brain, if NO first combines with a thiol or other carrier, it would appear that the entire nitro complex activates g cyclase and that NO liberated from the carrier is not the effective EDRF ACh even though NO alone is, of course, an activator of g cyclase and dilates by that mechanism. Thus, in the cerebral vasculature the NO-carrying intermediate is not a precursor of EDRF ACh but is EDRF ACh itself. Moreover, whether the NO-carrying compound is a precursor or EDRF ACh itself, formation of the NO-carrying compound and its subsequent fate would clearly be controlled by reactions.
that may rival in importance the production of NO from L-ARG.

In cerebral vessels, evidence that the NO in EDRF AC1 comes from L-ARG rests largely on data showing inhibition of ACh-induced responses by L-ARG analogues known to block NO synthase.7,8,52 However, recent data suggest that, at least in the mouse, the analogues N⁴-monomethyl L-arginine (L-NMMA) or nitro-L-arginine may not work by inhibiting NO synthase. Rather, their actions appear to depend upon activation of cyclooxygenase and subsequent generation of superoxide. Thus, the actions of L-NMMA and nitro-L-arginine were totally blocked by inhibitors of cyclooxygenase and/or by SOD, a scavenger of superoxide, in doses having no other effect on the preparation or on responses to ACh.51

To explain the inhibitory effect of cyclooxygenase inhibitors or SOD on the actions of L-NMMA, it has been suggested that L-NMMA activates cyclooxygenase in some unknown manner, with subsequent generation of superoxide.51 However, it may be that nitroarginine analogues serve as substrates for some enzyme other than NO synthase and that interaction with some other enzyme generates superoxide. It has already been shown that, in addition to competitively inhibiting NO synthase, L-NMMA is substrate for at least one other enzyme.53 This enzyme converts L-NMMA to L-ARG. While it is unlikely that this reaction would lead to superoxide formation, it serves to alert us to the possibility that nitroarginine compounds may serve as substrates for multiple enzymes. If one such interaction is responsible for the generation of superoxide, then data from mouse pial vessels suggest that either the interaction is between L-ARG analogues and cyclooxygenase or that cyclooxygenase inhibitors that block the action of L-NMMA inhibit some enzyme other than cyclooxygenase. There is a precedent for the latter suggestion since indomethacin blocks phospholipase at concentrations that overlap with those on the concentration-inhibition curve for cyclooxygenase.54 The data showing inhibition of L-NMMA’s effects by either cyclooxygenase inhibitors or SOD were not replicated in a study of cat vessels,52 but in the latter study there was no attempt made to prevent the action of L-NMMA. Instead, SOD was used in an attempt to reverse the effect of L-NMMA after it had been present for over an hour. On the other hand, a recent in vitro study has confirmed blockage of L-NMMA’s action by SOD.55

If the effects of some nitroarginine analogues are due to destruction of EDRF AC1 by radicals, this does not negate the use of analogues to establish a role for EDRF AC1 as mediator of the response being investigated. However, in such vascular beds the inhibitory effect of L-NMMA analogues is clearly not due to a specific interaction with NO synthase.54 It also could not serve as proof of a role for NO synthase or L-ARG in production of EDRF AC1. But, since L-ARG dilates pial arterioles, at least in some species,7 and since this is prevented by endothelial damage, there is, in fact, evidence favoring L-ARG as one precursor of EDRF AC1.

We have already discussed the alternative views concerning either the direct activation of g cyclase by a compound carrying NO, such as a nitrosothiol, or the activation of g cyclase by NO that must first leave its carrying compound. No matter which of these theories is embraced, they presuppose that dilation by com-

pounds like the nitrovasodilators work directly on vascular smooth muscle.20,49 The most recent data suggesting a discrepancy between the literature in general and brain vessels in particular bear directly on this issue. In the cat the dilating action of sodium nitroprusside (SNP) and nitroglycerin is markedly inhibited by eliminating the sensory peptidergic innervation to the cerebral vessels.56 Several peptides with ability to dilate arterioles are released from these nerves, notably substance P and calcitonin gene–related peptide (CGRP). However, substance P has generally been reported to be an endothelium-dependent dilator while CGRP is not.57,58 Since dilation of brain vessels by nitrovasodilators or NO is not dependent upon the endothelium,7,59 CGRP became the candidate for mediator of the response. A CGRP antagonist, eCGRP(8-37), was then tested and found to block the response to the nitrovasodilators and NO.56,60 All other literature suggests that SNP works by releasing NO, which activates g cyclase in vascular smooth muscle. However, in the cat, because SNP-induced dilation of pial arteries or arterioles is blocked or greatly inhibited by sensory peptidergic denervation or by a CGRP antagonist, it appears that the effect of SNP on diameter is mediated by release of CGRP from sensory peptidergic perivascular nerves. If g cyclase is activated, this activation must be by CGRP, not by NO. How CGRP activates g cyclase is unknown.

More important, sensory peptidergic denervation and the CGRP antagonist do not block the response to ACh or S-nitrosocysteine.56,60 Thus we see again that in cerebral vessels the response to EDRF AC1 is not blocked by maneuvers that block NO and that a nitrosothiol, rather than NO, appears to best mimic EDRF AC1. Moreover, NO, at least in low concentrations, does not even seem to directly activate g cyclase in cerebrovascular smooth muscle. If it did, it would have relaxed the vessels after the CGRP receptor was blocked. Hence it is unlikely that NO must leave the nitrosothiol to activate g cyclase in cerebrovascular smooth muscle.

In our opinion the literature permits the hypothesis that a nitrosothiol that need not give off NO could be EDRF AC1 in all vascular beds. The nitrogen in the NO moiety could come from L-ARG in a reaction catalyzed by NO synthase. But another compound, a thiol, must also be synthesized and available to form the critical nitrosothiol that is EDRF AC1. This could travel across cell membranes and would directly activate g cyclase. If this unifying hypothesis is not correct, then we are left with three choices: 1) critical experiments already reported have faulty data, 2) the data are correct but have been misinterpreted in ways not yet clear, or 3) there is a real difference between EDRF AC1 in brain blood vessels—especially surface arterioles—and EDRF AC1 in vessels from other organs. Since it is not only a regulator of vessel diameter but also a potent inhibitor of platelet adhesion/aggregation,111 EDRF AC1 clearly has implications for disease. These implications are beyond the scope of this review. Some have been mentioned in another review cited here.11 Broadly stated, stroke and many conditions predisposing to stroke produce endothelial damage. Because this damage will impair the synthesis/release of EDRF AC1, the ability of the cerebral vasculature to adjust to atherosclerosis, hypertension, etc., or to stroke itself will be altered. It is clear that efforts that un-equiv-
ocally define the chemical composition of EDRF\textsubscript{AD} in brain vascular endothelium will have at least three effects: 1) they will greatly assist in the elucidation of all components needed for the synthesis of EDRF\textsubscript{AD}, 2) they will increase our understanding of how the cerebral vasculature responds to a variety of insults, and 3) they may lead to therapies based upon interventions that will increase EDRF synthesis or modify its degradation.

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