REVIEW

Is the EDRF in the Cerebral Circulation NO?
Its Release by Shear and the Dangers in Interpreting
the Effects of NOS Inhibitors

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Abstract. Evidence from investigations of brain microcirculation (pial arterioles) reveals at least 3
different endothelium (EC) dependent mechanisms for dilation. Only one of the three can be triggered
by acetylcholine (ACh) and in this vascular bed it is only this path that is dependent upon endothelial
nitric oxide synthase (NOS) which produces nitric oxide (NO) from arginine. In this vascular bed the
ACh sensitive path cannot be triggered by bradykinin (BK). This state of affairs appears to differ from
that found in other beds or in endothelium cultured from conductance vessels. In the cerebral micro-
circulation there is considerable pharmacological evidence that the endothelium derived relaxing factor
(EDRF) for ACh is not NO itself but may contain NO. In many experimental vascular settings the
release of the NOS dependent EDRF is shear dependent. In the cerebral microcirculation there are
several studies suggesting, in vivo, that this is correct. Among these are the following: (1) vessels
narrow when shear is reduced after carotid ligation, and remain so along with unresponsiveness to ACh
for at least ten minutes following resumption of flow. This may be important in developing stroke. The
collapse is not passive due to low pressure. We know this because the narrowed vessels with their low
intraluminal shear and pressure are still capable of large dilation by the NO donor, sodium nitroprus-
side; (2) the antiplatelet effects of EC which are mediated, in part, by the EDRF for ACh are enhanced
for 10 to 20 minutes following the transient increase and return of shear within these vessels. If the
reverse is also true, reductions of shear may have important harmful proaggregant effects on platelets
(and leukocytes) in the microvascular bed of developing infarcts. However most of the cited work
depends upon pharmacological inhibitors of NOS to "prove" that NOS and an EDRF/NO are
involved. In the last three years evidence in cats and rats shows that many of the NOS inhibitors also
block K channels in cerebrovascular smooth muscle and that arginine, the "antidote" to the NOS
inhibitors keeps the channels open. This latter work must force a reexamination of the conclusions

Key words: acetylcholine, endothelium, vasodilatation, shear, nitric oxide

Introduction

With respect to the endothelial mechanisms controlling vascular diameter, the microcirculation of the
brain, at least in mice and cats, is clearly different from many other vascular beds, both microvascular and
conductance, in many other species.1,2 The situation in the human cerebral circulation and especially microcircula-
tion (e.g. small pial vessels) remains to be explored. In this review I will discuss the points of difference. In
doing this I will emphasize findings obtained with commonly used pharmacological probes. Then at the end I
will review some recent findings that open alternative possibilities for the interpretation of results with some of
these probes.

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The Diverse Nature of Endothelium Dependent Relaxing Mechanisms

In other vascular beds and in cultured endothelial cells, usually obtained from conductance vessels, it has been customary to note a similarity between the mechanisms responsible for the dilations produced by both acetylcholine (ACh) or bradykinin (BK). This mechanism has been shown to be dependent upon a normal endothelium and upon a mediator released by these agonists when they interact with receptors on the endothelial surface. The mediator has been identified by most, but not all workers, as nitric oxide (NO). Even when doubt has been cast upon its identification as NO by most workers, as nitric oxide synthase (NOS) on that substrate.1,2 I will call this endothelium derived relaxing factor EDRF/NO as have many workers. However I will use the term EDRF/NO only to refer to the mediator obtained from vessels or endothelial cells outside the brain.

In many tissue culture preparations of endothelial cells, the cells have lost the capacity to respond to ACh, presumably because the receptors to ACh have been lost (studies to investigate this point need to be performed). In these cultures, BK has been used as the agonist to release EDRF/NO and this EDRF/NO has the properties of the EDRF/NO released from extracerebral vessels in vitro by application of ACh. Consequently, the same EDRF/NO is thought to be released by ACh or by BK and sometimes also by a calcium ionophore in these preparations.1,2

In the pial arterioles of the brain, at least in cats, mice and some other species, the situation is quite different.3 By directly injuring the pial arteriolar endothelium in mice it has been possible to show that there are many endothelium dependent dilators, including ACh, BK and calcium ionophore.4-6 However, the mediators for ACh, BK and calcium ionophore are each different. This has been shown in mice using pharmacological probes in vivo, and has been shown for ACh and BK in cats and other species also using pharmacological probes.7-11

Since these EDRFs are each different and since, as we will see, the EDRF for ACh is apparently not simple NO, I will denote each of them as follows: EDRF(ACh), EDRF(BK), and EDRF(ionomophore).3 Let us concentrate on the first two mediators. Like the EDRF/NO found elsewhere, the EDRF(ACh) is destroyed by reactive oxygen centered species like superoxide. However, EDRF(BK) is itself a radical, perhaps hydroxyl or perhaps some other species. In any case, unlike the EDRF(ACh), the EDRF(BK) cannot be eliminated by putative inhibitors of NOS. It can however be eliminated by radical scavengers. This is exactly the opposite of the situation with EDRF(ACh) whose activity (concentration) is reduced in any preparation with a significant level of superoxide present and whose activity is increased (rescued) by simultaneous application of superoxide dismutase.

Experiments in cats by Kontos and co-workers provided dramatic evidence that the EDRF(ACh) and the EDRF(BK) are different substances when they are released from pial arterioles. One set of experiments showed that simultaneous application of submaximal doses of ACh and BK to the pial surface resulted not in an additive dilatation but in elimination of dilation. In other words the EDRF(ACh) was destroyed by the EDRF(BK). That is exactly what one expects if EDRF(BK) is an oxygen centered reactive substance since such substances destroy EDRF(ACh). If the two EDRFs were identical, as they supposedly are in other vascular beds, the dilation produced by their combination should be greater than that produced by either one given alone.

A second set of experiments in cats used two cranial windows, one over each cerebral hemisphere. Fluid collected under one window could be transferred to the space under the other window. If ACh was placed under window #1 the arterioles dilated there. Fluid collected at that time could be transferred to window #2. If the transfer was very rapid the vessels in window #2 would be dilated by the donated fluid. The vessels in that window were pretreated by an inhibitor or ACh, so the dilation could not be due to residual ACh transferred from one window to the other. If the transfer was delayed there was no effect. A very short half-life is characteristic of EDRF/NO from extracerebral vessels and from cultured cells. Moreover the same short half-life is characteristic of NO. With respect to BK, unlike the EDRF(ACh), there was absolutely no transfer of dilating material from window #1 to window #2 when BK rather than ACh was placed under window #1.

A third set of studies in cats showed that nitroblue tetrazolium (NBT) prevented the action of BK. This may be because NBT can scavenge the radical that is the EDRF(BK). In contrast, the response to ACh was not impaired by NBT.

It should be noted that these studies of Kontos and most other investigators of cerebral blood vessels do not show that the response to any vasodilators are endothelium dependent. The belief that they are so, depends upon analogy with other vascular beds where responses have been tested in the presence and absence of endothelium1,2 and on the work of Rosenblum and co-workers4,6 who used two different light-dye techniques to damage pial arteriolar endothelium and showed that the responses to some dilators, including ACh and BK were blocked or impaired or in the case of ACh
even converted to constriction which represents the direct action of ACh on the vascular smooth muscle.

**What is EDRF(ACh) in the Brain's Microcirculation?**

With respect to the identity of EDRF(ACh), the work from the Kontos laboratory shows that in the cerebral circulation, at least in cats, EDRF(ACh) cannot possibly be simple NO. Several studies that show this are summarized as follows. All were conducted in vivo: (1) Nitroblue tetrazolium, which reacts with radicals such as NO, does indeed eliminate dilatation produced by the application of an NO donor such as nitroglycerin, but does not reduce the dilatation produced by ACh. (2) Superfusion of NO to the pial arterioles produced a dilation that is blocked by hydrogen peroxide in concentration of $10^{-6}$ M. However this concentration of H$_2$O$_2$ fails to inhibit a dilatation of equal size, produced by ACh.

In cats, the Kontos laboratory showed that commonly used inhibitors of NOS, inhibit the dilatation produced by ACh. These inhibitors include arginine analogs such as N Nitro L arginine (NOLA) or N nitro L monomethyl arginine (LNMMMA). This implies that the responses to ACh are, in fact, generated by release of EDRF(ACh), synthesized by the action of NOS on the substrate L-arginine. Why, then, does the EDRF(ACh) behave pharmacologically like something other than NO?

Other workers have also questioned the dogma that EDRF(ACh) is NO. They have suggested that the substance released from endothelium is a compound that contains NO which was synthesized by the action of NOS on arginine. Prime candidates for this compound are nitrosothioles. In cats, the Kontos laboratory showed that S-nitroso cysteine is a very potent dilator of pial arterioles and, like the EDRF released by ACh, the effect of this nitrosothiol is not inhibited by H$_2$O$_2$, $10^{-6}$ M.

If EDRF(ACh) is a molecule that contains NO and the latter is first synthesized by NOS, then the highly reactant and short-lived NO must be efficiently incorporated into the carrier compound. To my knowledge no investigations have been published showing how this occurs.

Such work may be held back by the attitude of those dominant investigators who insist that EDRF(ACh) is NO itself, a view based largely upon work in preparations other than the cerebral circulation. Moreover these dominant investigators, even when conceding the possibility that EDRF(ACh) may be a more complex compound, have often stated that “it doesn't matter” because the NO must be released from the carrier compound at the site of the critical enzyme activated by NO. This enzyme is guanylate cyclase. The consequent elevation of cyclic guanosine monophosphate (GMP) is the apparent cause or at least usually correlated with the resultant relaxation of the vascular smooth muscle. In other words, even when the possibility of a carrier compound is acknowledged the attitude has been that the final result is still a consequence of NO released by the carrier. Such an attitude misses completely the point that if this is the case, then the diffusible substance released by the endothelium is not NO but the carrier containing NO. If the latter situation is the true one, then consideration must be given to the steps required for the synthesis of the carrier, for example S-cysteine and to the place and manner in which the NO in the carrier is finally released (if it is released) to activate g cyclase. There has been relatively little work of this kind.

The possibility of a NO carrier is related to the possibility that there is a storage form for NO in the endothelial cells. The question would then arise as to whether or not it is this storage form that is the diffusible EDRF(ACh) and whether or not application of ACh or some other adequate stimulus causes release of this storage molecule or, instead, releases only newly synthesized NO either attached to or free of a carrier. Almost no one has looked at these possibilities although some workers have certainly considered the possibility of a storage form of NO perhaps consisting of an iron based molecule. In unpublished work, I found that successive applications of ACh ultimately resulted in the failure of the response to ACh. The response could be restored by arginine but not immediately. This suggested that the synthesis of EDRF(ACh) was not simply stopped because of depletion of the substrate, arginine, but rather that this substrate had not only to be converted to NO but the NO had to be deposited into a storage form which must accumulate in adequate amounts in order to produce vasodilatation when released by ACh.

**Release of EDRF(ACh) by Shear**

It is known that EDRF(ACh) is released continuously from endothelial cells in vitro and from many blood vessel preparations provided that there is flow and hence shear over the endothelium. Again the question should be raised as to whether this EDRF(ACh) [or the NO if one prefers that term] is released from a storage form or simply as free NO during shear stress of the endothelium. Regardless of the answer to this question it is of interest to seek evidence demonstrating the release of NO from the pial arteriolar endothelium during blood flow in vivo.

One evidence for this would be the constriction of arterioles following application of inhibitors of NOS. This occurs in the mouse. The effect is not present if the...
endothelium is injured first, hence the constriction must be due to elimination of endothelial NOS and hence elimination of a basally released EDRF(ACh). It is of interest that the same light/dye injury does not constrict the arteriole as one might expect if the source of the basally released EDRF(ACh) was impaired by the injury. However the injury is only at a point less than 100 µm in diameter and the EDRF(ACh) arriving from the nearby upstream source is available to maintain the diameter. On the other hand the NOS inhibitors are applied to all the surface vessels at once in the suffusion of mock CSF. Consequently, there is no opportunity for upstream EDRF(ACh) to enter the picture. In addition, the injury may also eliminate endothelial derived constrictors at the same focus so that the net effect is no change in diameter.

A second group of results also imply the release of basal EDRF(ACh) under shear conditions. In these studies, a more severe laser/dye injury (longer duration of laser) was used which resulted in sufficient endothelial damage to initiate platelet adhesion/aggregation at the injured spot. This adhesion/aggregation in itself may imply basal release of EDRF(ACh) since the latter is a potent antiplatelet substance. In other words diminution in the local concentration of EDRF(ACh) may be one of the factors promoting local adhesion/aggregation. But since the lesion is so focal, as pointed out above, one might ask why the EDRF(ACh) delivered from intact upstream sites was not sufficient to maintain the antiplatelet properties of the downstream injured site. There are two possible reasons. One is the fact that the local injury is more severe than that used to test endothelium dependence of vasooactive substances. Therefore the lack of local constriction after the latter injury may reflect a milder loss of local EDRF(ACh) than in studies where platelet adhesion/aggregation is induced. A second factor in the latter studies may be the induction of proaggregant factors by the local injury which can overcome the antiplatelet action of EDRF(ACh) arriving from upstream sites. Upregulation or exposure of endothelium Platelet Endothelial Cell Adhesion Molecule may be one such additional factor.

A third type of study implying the basal release of EDRF(ACh) under shear conditions also involves platelet adhesion/aggregation. Here we found that the adhesion/aggregation at an injured site could be enhanced by suffusion with inhibitors of NOS. This means that active NOS must be present in the untreated situation. Similarly, parenteral treatment with arginine, an enzyme that destroys arginine [the substrate for NOS and hence the source of the NO in EDRF(ACh)], also enhances the local adhesion/aggregation, again indicating that there must normally be local release of endothelial EDRF(ACh).

Finally, in yet another group of experiments employing platelet adhesion/aggregation as an endpoint, my laboratory studied the effect of increasing shear on the time it took for a standard light/dye injury to initiate local adhesion/aggregation. Such studies would be nonsensical if we had attempted to initiate adhesion/aggregation during the period of increased shear because increased shear exerts mechanical effects that sweep away platelets from a site of potential adhesion/aggregation. However, we waited at least 10 and sometimes 20 minutes after shear returned to normal following a sharp but very short increase in blood pressure. Two studies were performed, each with a different type of light/dye injury and each with a different pressor — angiotensin in one study and norepinephrine in the other. The results of the two studies were similar — the preceding episode of increased shear had a residual effect. That was the inhibition of platelet adhesion/aggregation after the injury, so that the interval between injury and onset of adhesion/aggregation was significantly prolonged when compared with the interval in a parallel control group infused only with saline and showing no increase in BP prior to endothelial damage. These data support the view that there is shear dependent basal release of EDRF(ACh) in these vessels and that the impaired adhesion/aggregation was a result of increased release of the EDRF(ACh) because of the increased shear. However for this interpretation to be valid it is necessary that the effect of increased shear be prolonged for at least 10 to 20 minutes following the return of the shear to normal. To my knowledge these are the first in vivo studies in any microvascular bed to suggest that this can occur. In vitro studies of cultured endothelial cells by others suggest the same phenomenon. In addition other studies in my laboratory failed to support an alternative theory which was prolonged release of antiplatelet prostacyclin following the injection of angiotensin which is known to release that antiplatelet compound. We failed to find prolonged release of prostacyclin in the plasma following the injection of angiotensin and the return of shear to normal.

It should also be noted that in the preceding paragraphs I have used BP as a surrogate marker for shear. Of course shear is the product of both pressure and diameter. There were no changes in diameter in these studies which could nullify our conclusion that when BP was dramatically elevated the shear was elevated. Nor were there changes in diameter that could nullify the conclusion that shear had returned to normal at the time that platelet adhesion/aggregation was induced. Moreover in the first of the two studies, both pial arteriolar pressure and systemic BP were measured to show that intraluminal pressure paralleled systemic pressure. In that study, we monitored arteriolar diameter and...
red cell velocity within the arterioles so we could directly calculate shear and demonstrated that it did indeed rise during the abrupt increase of systemic pressure and that it did return to normal later.

**Isoforms of NOS: Is There NOS in the Endothelium?**

There are three isoforms of NOS, one known as endothelial NOS (eNOS), one as neuronal NOS (nNOS) and one as inducible NOS. The latter can be induced by diverse stimuli, including inflammatory stimuli, in diverse cell types. The first two take their names from the principal cells in which they are basally expressed.

However nNOS may be found in cells other than neurons and eNOS in cells other than endothelial. I have shown that both nNOS and eNOS are constitutively expressed in the pial arteriolar endothelium of mice. I showed this by combining antisense technology with standard studies employing pharmacological probes or focal endothelial injury. Other workers demonstrated by immunohistochemical means at both the light and electronmicroscopic level, that an nNOS as well as eNOS is present in the endothelium of vessels in several species. Under the experimental conditions in my laboratory and with the Swiss derived strains of mice used there, antisense studies showed that about half of the dilation produced by ACh or by tetrahydrobiopterin, a cofactor for NOS, can be accounted for by nNOS and half by eNOS. Again, since these responses are completely endothelium dependent the nNOS and eNOS that account for the responses must reside in the endothelial cells. More recently, others have demonstrated the mRNA for nNOS as well as the mRNA for eNOS in the endothelium of rat pial arterioles and have reported preliminary data showing the nNOS protein in cultured endothelial cells. Evidence for nNOS in endothelium is important because studies by others of knockout mice, have indicated that in the absence of eNOS, an nNOS may take over in the regulation of some cerebral vasomotor responses. Investigators reporting such studies have interpreted them to mean that the nNOS which takes over has its origin in neurons. However, the latter conclusion depends upon the use of pharmacological probes such as tetrodotoxin that are thought to be specific for the action of neurons or 7-nitro indazole (7-NI) thought to selectively inhibit nNOS. However the basis of the action of tetrodotoxin is a blockade of sodium channels. The role of such channels in cerebrovascular endothelium has not been explored. Moreover it may be that tetrodotoxin will be found to work on other channels as well or to have additional modes of action. Thus the effect of tetrodotoxin on the response to mediators of cerebral blood flow may not necessarily prove that neurons are the target of tetrodotoxin in every experiment. As for the effect of 7-NI, that might be explained, if nNOS can be located, as I and others have shown, in the endothelium. Then the action of this supposedly selective inhibitor of nNOS could reflect, not an increased importance of neurons in cerebral vasomotor control, but simply an increased importance of nNOS in the endothelial cells of eNOS knockouts. There may be other problems with the use of 7-NI. Some workers using rats, found that 7-NI inhibited eNOS. This, of course, would not be a factor in eNOS knockouts since the latter do not have any eNOS. However 7-NI may also inhibit a K channel. This effect, described below, may confound interpretations of the 7-NI effect.

**Inhibitors of NOS Also Block Potassium Channels**

Many of the studies reviewed here and innumerable unmentioned studies by a large number of workers, have employed inhibitors of NOS to "prove" that NOS is the critical enzyme producing EDRF/NO or EDRF(ACh). They also employ arginine, the substrate for NOS, to reverse the effects or to prevent the effects of the NOS inhibitors and they use this pharmacologic evidence to reinforce the interpretation that NOS and arginine are the key factors in the experiment. However recent work from Kontos' laboratory shows that the common inhibitors of NOS can have another very important action, namely the inhibition of the K<sub>ATP</sub> channel, presumably in the vascular smooth muscle and not the endothelium. Moreover L-arginine is essential to keep the channel open. N nitro arginine (NNA) and LNMMA, the common inhibitors of NOS, in doses used to inhibit NOS, were found to interfere with the dilating action of drugs known to work by opening the K<sub>ATP</sub> channels. This effect is reversed by L-arginine which also reversed the effects of established blockers or closers of those potassium channels. This data might explain the findings of some workers that the dilation produced by carbon dioxide (reduced local pH) is inhibited by NOS inhibitors (NOSI).

Those who successfully inhibit, with NOSI, the hyperemic response to CO<sub>2</sub>, interpret such data to mean that there is a role for neuronal NOS in the response to carbon dioxide. They exclude a role for eNOS from consideration, not only because of the supposedly selective effect of 7-NI on nNOS, but also because of work showing the persistence of the response to CO<sub>2</sub> after endothelial damage. With respect to the action of 7-NI the work from the Kontos group presents an alternative explanation for its ability to block the response to carbon dioxide. In unpublished data kindly provided to me by Dr Kontos, he has found that the 7-NI, like the other NOS inhibitors he has tested, can close or prevent the opening of the K<sub>ATP</sub> channel. Thus we must ask again...
whether the inhibitory effect of a NOS inhibitor on a vascular response (or on any other response) is necessarily accounted for by inhibition of NOS. Instead the effect might be due to the closure or the preventing of opening of a K\textsubscript{ATP} channel.

Before discussing the response to CO\textsubscript{2} further it should be noted that not all workers have succeeded in inhibiting this response with NOSI.\textsuperscript{32} In the case of humans and non human primates, there have been reports of significant,\textsuperscript{33} slight\textsuperscript{34} and no\textsuperscript{35} inhibition by NOSI.

From Kontos' work it is clear that when the NOS inhibitors block or impair the response to CO\textsubscript{2} they may do so because they were blocking a K channel.\textsuperscript{28} This alternative explanation is strongly supported by Kontos, finding that the response to carbon dioxide can be blocked by other well established blockers of K channels.\textsuperscript{28} The explanation is also supported by data from other laboratories that shows that the response to carbon dioxide can occur without an increase in cyclic GMP.\textsuperscript{36-38} The effect of nitric oxide or EDRF(ACh) is thought to depend upon activation of guanylate cyclase and elevation of cyclic GMP.

If nNOS is not, in fact, a mediator or modulator of the response to carbon dioxide then we would expect that response to be normal in nNOS knockout mice. In fact, in such mice the response to carbon dioxide is normal.\textsuperscript{38} Moreover in such mice, NOSI do not inhibit the response to carbon dioxide. The NOSI only inhibit that response in wild type mice or in eNOS knockouts.\textsuperscript{39} Why should there be this difference among the three types of mice? First of all it may be that, as said earlier, the NOSI, when they succeed in inhibiting the response to carbon dioxide, they do so because of an effect on K channels and not because of an effect on NOS. Secondly, the importance of K channels may be different in nNOS knockouts than in other animals. There is certainly a precedent for such shifts in the relative importance of K channels if one looks at pathophysiological states.\textsuperscript{40,41}

The latter work from the Kontos laboratory concerning K\textsubscript{ATP} channels was performed \textit{in vivo}. However, in unpublished \textit{in vitro} electrophysiological studies performed with the collaboration of Dr Harders, (personal communication from Dr Kontos) the NOS inhibitors were also shown to block calcium activated K channels. This is significant because some of the literature places more importance on the K\textsubscript{ca} channel than on the K\textsubscript{ATP} channel.\textsuperscript{40}

If the findings of the Kontos laboratory are replicated, the implications reach far beyond concerns about the actions of NOS and of EDRF/NO or EDRF(ACh). In fact K\textsubscript{ATP} channels have been cloned and found to be dimers, one half of which is the glyburide sensitive site.\textsuperscript{42} This site, then, may be the site that is sensitive to arginine and to NOSI. It is also known that a part of the N-methyl-D-aspartate (NMDA) receptor or the calcium channel connected to that receptor, has a site sensitive to glycine. If Kontos is correct about the sensitivity of the K\textsubscript{ATP} channel to arginine, we may be seeing here the beginning of a pattern in which different amino acids are critical to the maintenance of diverse open ion channels.

Because of the discoveries of the Kontos laboratory it is important that all investigators who have or will use these NOS inhibitors, check their test preparations to be sure that they are unresponsive to K\textsubscript{ATP} openers or blockers. If they are responsive to such blockers then the effects of the NOS inhibitors might actually be due to an effect of the inhibitors on the K channel rather than on NOS. When the data suggest that this is a possibility, further tests must be conducted, for example examining the effect of other known inhibitors of the K channel on the response being investigated (for example the response to ACh). In this regard, it is of interest to report here that in my laboratory the responses of mouse pial arterioles, under the experimental conditions that existed in my studies, were quite resistant to manipulation by openers or inhibitors of K channels. In addition, in Kontos' preparation the K\textsubscript{ATP} channel is kept open by either L-arginine or L-lysine\textsuperscript{29} while the mouse pial arterioles in my laboratory were only dilated by L-arginine and not by L-lysine. Even in Kontos' laboratory the dilatation produced by ACh was not altered by application of established blockers of K channels. Hence the inhibition of the response to ACh by NOS inhibitors was not caused by the effect of the same NOS inhibitors on the K channel in either cats in Kontos' laboratory or in mice in my laboratory.

Summary

(1) Endothelium dependent dilation exists in the brain microcirculation as proved by studies in which diverse endothelial injuries impaired the response to some but not all vasoactive agents. There are at least three different endothelium dependent dilating pathways in the pial arterioles of mice, each with its own endothelium derived mediator.

(2) The path from ACh to dilation is mediated by an EDRF that appears to be formed, at least in part by the action of NOS on arginine. Contrary to findings in other vascular beds or in some preparations of cultured endothelium, this mediator of dilation is not the same as the agent that mediates the endothelium dependent dilation produced by bradykinin. The mediator for ACh I have called EDRF(ACh) rather than NO because compelling evidence from the studies of cat pial arterioles shows that it cannot be NO by itself. It may be a compound containing an NO derived from the
action of NOS on arginine. If so, nitrosothioles are one possible group of candidates. The identity of the diffusible substance released from the endothelium of cerebrovascular resistance vessels in the brain is important and it is unfortunate that acceptance of its identity as simple NO has held back research in this area. The identity of the other portion of the actual EDRF(ACh) molecule is important as is the path to its synthesis and the means by which it joins NO. Also important is the question of whether it must give off its NO to activate guanylate cyclase and if so where and how this occurs.

(3) The EDRF(ACh) is released from the endothelium of pial arterioles as a consequence of shear – hence there is basal release in the living animal at least in mice. The EDRF(ACh) has antiplatelet properties in vivo, just as others have shown in vitro. Thus the basal release of EDRF(ACh) can function as one factor in the maintenance of a platelet free endothelial surface. This is probably one reason why the release of EDRF(ACh) has been conserved in large conductance vessels where it is unlikely to play an important role in the maintenance of diameter. When the shear rate is transiently elevated to high levels and then returns to normal there is a prolonged increase in the antiplatelet properties of pial arteriolar endothelium in vivo. This suggests that the increased release of EDRF(ACh) produced by the increase in shear can persist for at least 10 to 20 minutes after the shear itself has returned to baseline levels.

(4) The neuronal isoform of NOS can be found in endothelial cells where it may be functionally active. Studies of endothelial NOS knockout mice, in which an inhibitor of the nNOS becomes an effective inhibitor of dilation might be due to the effect of the inhibitor on nNOS in endothelial cells.

(5) Moreover there are pitfalls in the interpretation of studies that use inhibitors of NOS. Recently these inhibitors have been found to also inhibit potassium channels and particularly KATP channels in pial arterioles, at least in cats and rats. Moreover, arginine, the NOS substrate, has another function, namely that of opening the K channels or of maintaining their open state. Therefore one must be sure when using these inhibitors of NOS that the results are correctly interpreted and that they are really due to NOS inhibition rather than to an effect on potassium channels. Each preparation must be checked to see whether potassium channels can play a role under the experimental conditions employed. This might be done, for example, by seeing whether the preparation is sensitive either to established openers or closers of the channels. If so then one must see if the NOS inhibitors employed will also block the effect of the K channel opener in the preparation being studied. When diseased animals are studied, for example diabetic or hypertensive, it is not enough to show a lack of K channel activity in the control animals without disease, but one must test channel activity in the diseased animals because the importance of the channels may be disease dependent. It is quite likely that studies showing an effect of NOS inhibitors on the dilating response to hypercapnia may have been misinterpreted and that the pharmacological probes were working because they were blocking K channels which have, in fact, been shown to play a role in the dilation to carbon dioxide.

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