Responses of cerebral arterioles to ADP: eNOS-dependent and eNOS-independent mechanisms

Frank M. Faraci, Cynthia Lynch, and Kathryn G. Lamping

METHODS

Experimental animals. The animal protocol used in these experiments was reviewed and approved by the University of Iowa Animal Care and Use Committee. Most of the mice for this study were derived from the breeding of eNOS+/− mice with eNOS+/− mice to generate eNOS+/+, eNOS+/−, and eNOS−/− mice within the same litter. This approach allowed us to use eNOS+/+ mice as littermate controls. We included the study of eNOS+/− mice, because it has been observed previously that vascular responses to some stimuli are altered in eNOS+/− mice (14, 23). In addition, experts in the field of genetics encourage the study of heterozygous-deficient mice because, depending on the results, they may encourage the study of genetic polymorphisms in humans (38). Mice used for these experiments (both males and females) were derived from seven to eight generations of backcross breeding to C57BL/6 mice. Additional C57BL/6 mice were also used in some of the pharmacological experiments. Mice were fed regular chow, and water was available ad libitum. Genotyping of mice was performed by Southern blotting or PCR of DNA from tail biopsies as described previously (23, 24).

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RESULTS

Responses to acetylcholine. Control diameters of cerebral arterioles were similar in the various groups of mice and averaged 31.7 ± 0.5 μm. Acetylcholine produced concentration-dependent dilation of cerebral arterioles that was markedly inhibited by L-NNA (n = 7) (Fig. 1). Responses to acetylcholine were also examined in eNOS-deficient mice. Vasodilation in response to acetylcholine was not altered in eNOS+/− mice (n = 24) or eNOS−/− mice (n = 18) mice compared with wild-type controls (n = 25) (Fig. 2, left). The response to the high concentration of acetylcholine tended to be reduced in eNOS−/− mice, but this difference was not statistically different. Consistent with previous work (27, 28), the response to acetylcholine in eNOS−/− mice was markedly reduced by L-NNA (n = 5, data not shown).

Responses to ADP. In control mice, ADP produced concentration-dependent dilation of cerebral arterioles that was not affected by indomethacin (n = 4) (Fig. 3). This response was inhibited by ~50% by either L-NNA (n = 8) or ODQ (n = 9) (Fig. 3) or iberiotoxin (n = 7, data not shown). Vasodilation in response to ADP was markedly reduced by the combination of charybdotoxin plus apamin (n = 9) (Fig. 4, left) and completely eliminated by the combination of L-NNA plus charybdotoxin plus apamin (n = 4) (Fig. 5). All of the inhibitors have modest effects on baseline diameter of cerebral arterioles (L-NNA, −7 ± 3%; indomethacin, −9 ± 4%; ODQ, −1 ± 2%; iberiotoxin, −1 ± 3%; charybdotoxin + apamin, −4 ± 2%; and L-NNA + charybdotoxin + apamin, −5 ± 3%).

Responses to ADP were also examined in eNOS-deficient mice. Vasodilation in response to ADP (n = 19 control mice) was not altered in eNOS+/− mice (n = 13) but was reduced somewhat in eNOS−/− mice (n = 15) (Fig. 2, right). As was observed in eNOS+/− mice, dilation of cerebral arterioles in response to ADP was markedly reduced by charybdotoxin plus apamin in eNOS−/− mice (n = 8) (Fig. 4, right). The vasodilator response to the low concentration of ADP in eNOS−/− mice was not altered by L-NNA (10 ± 2 vs. 7 ± 2%), and responses to the high concentration of ADP were reduced by L-NNA by 50% (24 ± 3 vs. 12 ± 2%, P < 0.05) (n = 5).

Responses to nitroprusside and papaverine. Nitroprusside and papaverine produced concentration-dependent dilation of cerebral arterioles in control mice. The response to nitroprusside...
side was markedly reduced by charybdotoxin plus apamin ($n = 4$) (Fig. 6). This was a selective effect because responses to papaverine were not inhibited ($n = 4$) (Fig. 6).

**DISCUSSION**

There are several major findings in this study. First, responses to ADP were reduced in eNOS−/− mice and were inhibited partially by l-NNA or ODQ in control mice. All these findings support the conclusion that dilation of cerebral microvessels to ADP is partially NO mediated. Second, vasodilation to ADP was partially reduced by iberiotoxin but was markedly inhibited by the combination of charybdotoxin plus apamin in both control and eNOS−/− mice. Importantly, this prominent inhibitory effect was seen in the absence of NOS inhibition. The combination of charybdotoxin plus apamin plus l-NNA totally abolished responses to ADP in control mice. Third, there was a significant component of the ADP response that was insensitive to l-NNA in eNOS−/− mice. Fourth, responses to nitroprusside, but not papaverine, were substantially reduced by charybdotoxin plus apamin, suggesting that NO produces dilation of cerebral arterioles in large part via activation of calcium-activated potassium channels. Together these results provide strong evidence that an eNOS-independent mechanism, most likely an EDHF, mediates part of the response to ADP in cerebral microvessels in vivo.

Microvascular responses to ADP are partly mediated by NO. ADP is known to produce endothelium-dependent relaxation in cerebral blood vessels (11, 17, 34, 41). Dilation of cerebral arterioles in response to ADP was partially inhibited by l-NNA or ODQ in the present experiments. These results are consistent with previous studies (1, 27, 40–42) and suggest that a portion of microvascular dilation in response to ADP is NO mediated. In the present study and in previous studies in mice (37), l-NNA essentially completely abolished responses to acetylcholine. This observation is important because it suggests that the efficacy of l-NNA as an inhibitor of eNOS is very high in this model. Despite this demonstrated efficacy of l-NNA, approximately half of the response to ADP was l-NNA (and ODQ) insensitive in cerebral arterioles.

In addition to these pharmacologically based findings, vasodilation in response to ADP was reduced in eNOS−/− mice. These findings represent the first genetic evidence that a portion of the response of cerebral microvessels to ADP is eNOS dependent.

We found that responses to acetylcholine were preserved in eNOS+/+ and eNOS−/− mice. These findings are consistent with previous work, which suggested that this response is maintained by a compensatory increase in expression of neuronal NOS in eNOS−/− mice (28, 29). Consistent with this concept, we found that l-NNA markedly reduced vasodilation to acetylcholine in eNOS−/− mice. In contrast, l-NNA had no effect on the vasodilator response to the low concentration and only partially inhibited responses to the higher concentration of ADP in eNOS−/− mice. Thus it appears that in relation to ADP responses, there may be partial compensation in eNOS−/− mice. Importantly, a significant component of the ADP response was not affected by l-NNA in eNOS−/− mice.

Role of eNOS-independent mechanisms in cerebral arterioles. Previous work has suggested that ROS are important mediators of eNOS-independent responses in some blood vessels. We (3) have shown previously that dilation of cerebral arterioles is partially NO mediated. All these findings support the conclusion that dilation of cerebral microvessels to ADP is partially NO mediated.
arterioles to ADP is not affected by superoxide dismutase plus catalase, scavengers of superoxide, and hydrogen peroxide, respectively. Thus ROS do not appear to contribute to responses of cerebral arterioles to ADP. We also found that indomethacin had no effect on responses to ADP in the present experiments, suggesting that neither prostacyclin nor ROS generated by cyclooxygenase activity were involved.

In previous studies of non-NO mediated, endothelium-dependent relaxation, the greatest effort has been to define the functional importance of EDHF. For many blood vessels, the use of charybdotoxin plus apamin is most effective in inhibiting EDHF-mediated responses (4, 8, 18). Because of this pharmacological profile, a defining feature of an EDHF-mediated response for many blood vessels is one that is inhibited by charybdotoxin plus apamin (4, 8, 18).

In the cerebral circulation, several studies have attempted to define the functional importance of EDHF in vitro using large cerebral arteries (in the presence of inhibitors of NOS and cyclooxygenase). In these experiments, charybdotoxin plus apamin markedly inhibited responses to endothelium-dependent agonists (26, 32, 35, 39). The effectiveness of charybdotoxin alone in inhibiting these responses has varied from moderate to high (7, 26, 35, 42, 43). Iberiotoxin has been reported to have no effect (26, 32) or to be less effective than charybdotoxin or the combination of charybdotoxin plus apamin (7, 43). Overall, these studies have suggested that in some species and in response to select stimuli, an EDHF contributes to endothelium-dependent relaxation in cerebral arterioles.

Despite the fact that the combination of charybdotoxin and apamin has commonly been used to examine EDHF-mediated responses in the peripheral circulation and has produced positive results in studies of large cerebral arteries (26, 32, 35, 39), it is surprising that this experimental approach has rarely been used to examine responses of cerebral arterioles in vitro or in vivo. To our knowledge, only a single study tested the effects of charybdotoxin plus apamin on responses to an endothelium-dependent agonist (25). In that study, the toxins had no effect on responses to bradykinin in cerebral arterioles of newborn pigs. In contrast, we found that charybdotoxin plus apamin markedly inhibited responses of cerebral arterioles to ADP in control and eNOS−/− mice.

The effect of iberoiotoxin on endothelial microvascular responses has been tested previously. Iberiotoxin produced partial inhibition of responses to ADP in rat cerebral arterioles in a previous study (40) and in mouse cerebral arterioles in the present study. We found that the combination of charybdotoxin plus apamin produced greater inhibition of ADP-induced vasodilation than did iberoiotoxin.

Some studies in the peripheral circulation have suggested that EDHF-mediated responses are more prominent in the microcirculation than in larger arteries, although all data do not support this view. This generalization may be too broad for the cerebral circulation. For example, NO affects basal tone and mediates the vast majority of the response to acetylcholine in large cerebral arteries, pial arterioles, and parenchymal arterioles (9, 12, 13, and present study). With respect to bradykinin, another commonly studied endothelium-dependent agonist, most studies indicate that NO mediates responses in large cerebral arteries (12) and ROS mediate dilatation in cerebral microvessels (12, 13, 25). In contrast, previous work (40, 42, 43) and the present study suggest that responses to some purine and pyrimidine nucleotides are mediated by an EDHF in large cerebral arteries and microvessels. Thus an EDHF appears to be functionally important in cerebral microvessels for some stimuli.

Role of potassium channels in responses to NO. Previous work has suggested that activation of potassium channels (calcium-activated potassium channels in particular) can mediate relaxation of cerebral blood vessels to NO (14, 31). An important observation in the present experiment is the finding that responses to nitroprusside were greatly reduced by charybdotoxin plus apamin. This result is consistent with previous studies, which suggested that calcium-activated potassium channels are major mediators of vasodilation in response to NO (and cGMP) in cerebral blood vessels (20, 31, 40). Because inhibitory effects of charybdotoxin plus apamin are generally considered to be a hallmark of EDHF-mediated responses, the present study suggest that in at least some blood vessels, NO-mediated vasodilation can also be very sensitive to these inhibitors. Thus charybdotoxin plus apamin was extremely effective in inhibiting responses to ADP because it blocks both NO- and EDHF-mediated responses. The effects of charybdotoxin plus apamin on responses to ADP and NO are selective, however, because vasodilation to papaverine was not inhibited by these toxins.

Ideally, one would like to obtain a direct patch-clamp recording of channel activity and/or membrane potential to confirm activation of potassium channels and membrane hyperpolarization in response to ADP. A limitation of our study is that we did not measure membrane potential in vascular muscle. In cerebral arterioles of the size that we studied (30 μm), we are not aware of any laboratory that makes such measurements in vivo. Membrane potential could potentially be studied in very small arterioles in vitro under pressurized conditions. However, that method is most typically done without the presence of blood flow (blood flow is an additional determinant of membrane potential), and we are not aware of in vitro studies of arterioles that include the use of pulsatile pressure and blood flow as occurs in vivo.

Implications. Most studies that examined the functional importance of EDHF tested effects of charybdotoxin plus apamin (or other potassium channel blockers) in the presence of inhibitors of NOS. With this approach, one can implicat
role for EDHF and its potential functional importance but only under conditions of NOS inhibition. This distinction is important because EDHF can functionally compensate for reductions in expression or activity of eNOS and may function in large part as a backup vasodilator mechanism (15, 16, 19, 21). As noted by others (19), there has been very little insight into the functional importance of EDHF under normal conditions (i.e., in the absence of NOS inhibition). Thus it is noteworthy in the present study that charrybotoxin and apamin produced marked inhibition of microvascular responses to ADP in the absence of NOS inhibition.

Studies on vascular effects and mechanisms of action of ADP in cerebral circulation are potentially important. ADP is the primary mediator of endothelium-dependent relaxation in response to platelets (22). In addition, ATP may be used as an extracellular signaling molecule between astrocytes and endothelium in the brain (2). Once released by cells, ATP can be converted to ADP by ectonucleotidases, which are abundantly expressed in the brain (44). Thus there are potentially local sources of ADP in the cerebral microcirculation. This study suggests responses to ADP in cerebral microvessels is mediated by NO and an EDHF.

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REFERENCES


