Effect of NO on EDHF response in rat middle cerebral arteries

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Schildmeyer, Lisa A. and Robert M. Bryan, Jr. Effect of NO on EDHF response in rat middle cerebral arteries. Am J Physiol Heart Circ Physiol 282: H734–H738, 2002; 10.1152/ajpheart.00583.2001.—Whereas the actual identity of endothelium-derived hyperpolarizing factor (EDHF) is still not certain, it involves a process requiring the endothelium and eliciting hyperpolarization and relaxation of smooth muscle. It is neither nitric oxide (NO) nor prostacyclin, and its presence has been demonstrated in a variety of vessels. Recent studies in peripheral vessels report that EDHF-mediated dilations were either attenuated or blocked by NO. Studies presented here demonstrate that NO does not block EDHF-mediated dilations in cerebral vessels. Rat middle cerebral arteries were cannulated, pressurized, and luminally perfused. EDHF-mediated dilations were elicited by the luminal application of ATP in the presence of Nω-nitro-l-arginine methyl ester (l-NAME) and indomethacin (inhibitors of NO synthase and cyclooxygenase, respectively). These dilations persisted when S-nitroso-N-acetylpenicillamine, an NO donor, was added exogenously in the presence of l-NAME, or when endogenous NO was present but its cGMP actions were blocked by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, an inhibitor of guanylate cyclase. These findings demonstrate that the EDHF response is not suppressed by NO in cerebral vessels and suggests a role for EDHF during normal physiological conditions.

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fundamental issue pertaining to a potential role for EDHF during normal physiological conditions. This is especially important in the brain where there is an abundance of neuronally derived NO. Therefore, we asked the question, does NO suppress the EDHF response in cerebral vessels?

We report that, unlike some peripheral vessels, NO does not inhibit the EDHF response in the rat cerebral circulation. Whereas it is still not clear whether EDHF has a role in the regulation of the cerebral circulation during normal physiological conditions, it is not inhibited by the presence of basal concentrations of NO.

MATERIALS AND METHODS

The Animal Protocol Review Committee at Baylor College of Medicine approved the experimental protocol. Male Long Evans rats (250–350 g) were anesthetized with 3% isoflurane and then decapitated. Brains were immediately removed and placed in physiological salt solution (PSS) (4°C). Middle cerebral arteries (MCAs) were visualized with the use of a dissecting microscope and carefully removed, beginning at the circle of Willis and extending distally 6–8 mm. A micropipette was inserted into each end of the MCA segment, and the segment was positioned between the micropipettes in such a way to avoid leakage of luminal perfusate through the side branches. The vessel was secured in place using nylon ties (4). Each MCA was bathed both luminally and abuminally in a 37°C PSS, which was equilibrated with a gas mixture of 20% O2-5% CO2 with a balance of N2 (4). The pH of the PSS was ~7.4, Pco2 was ~35 mmHg, and P02 was ~130 mmHg.

Each MCA was pressurized to 85 mmHg by raising PSS-containing reservoirs, connected to the micropipettes by tubing, above the vessel. Pressure transducers on either side of the MCA allowed measurement of the perfusion pressure across the system. Luminal flow was adjusted to 150 μl/min by setting inflow and outflow reservoirs at different heights. Each MCA was visualized using a video monitor at ×620. Experiments were recorded on videotape, and the diameter was continuously measured using Optimus image-analysis software (Bothell, WA).

After being warmed and pressurized to 85 mmHg, MCAs developed spontaneous tone over an hour by constricting to ~75% of their initial diameter. This diameter is defined as the resting tone diameter.

ATP (10⁻⁷–10⁻⁴ M) was added to the luminal perfusate to elicit dilations through NO and/or EDHF mechanisms (29–31). To avoid the risk of tachyphylaxis, each MCA was subjected to only one concentration-response curve.

Drugs and reagents. ATP, 2-methylthio-ATP (2-MeS-ATP), N(G)-nitro-L-arginine methyl ester (L-NAME), indomethacin (Indo), apamin (Apa), and charybdoxin (ChTX) were purchased from Sigma. S-nitroso-N-acetylpenicillamine (SNAP)

Fig. 1. A: effect of luminal application of ATP on diameter in individual middle cerebral arteries (MCAs). Successive increases in ATP concentration from 10⁻⁷ to 10⁻⁴ M (in log increments) are indicated. Top, diameter changes in a control MCA (ATP only); middle, response in presence of N(G)-nitro-l-arginine methyl ester (l-NAME, 3×10⁻⁶ M) and indomethacin (Indo, 10⁻⁵ M) to block nitric oxide (NO) and prostacyclin production, respectively; bottom, response in presence of l-NAME, Indo, charybdotoxin (ChTX, 10⁻⁷ M), and apamin (Apa, 3×10⁻⁷ M). Combination of ChTX and Apa blocked the three classes of Ca-activated potassium channels (KCa). Dashed line represents maximal diameter at 85 mmHg determined by removal of Ca²⁺ from the buffers. Summary data are shown in B. B: concentration-response curves to luminally applied ATP in control MCAs (circles, n = 6), in the presence of l-NAME (3×10⁻⁵ M) and Indo (10⁻⁵ M) (squares, n = 10), in the presence of l-NAME, Indo, and ChTX (10⁻⁷ M) (triangles, n = 8), and in the presence of l-NAME, Indo, ChTX, and Apa (3×10⁻⁷ M) (inverted triangles, n = 5). *P < 0.05 compared with corresponding control. All other groups were significantly different from other groups with the exception of the groups treated with ChTX and ChTX-Apa (2-way repeated measures ANOVA with post hoc Tukey test).

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was purchased from RBI. 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) was purchased from Tocris. Br-A23187 was purchased from Molecular Probes. Indo was prepared by dissolving in a 15 mM Na2CO3 solution; ChTX was dissolved in 150 mM NaCl; ODQ and Br-A23187 were dissolved in dimethyl sulfoxide. All other reagents were dissolved in distilled water. L-NAME and Indo were administered 30 min before adding the dilating agent (ATP, 2-Mes-ATP, or Br-A23187).

Statistical analysis. All data are reported as means ± SE. For concentration-response curves to ATP or 2-MeS ATP, the results are presented as the percentage of the maximum diameter and calculated by the following equation

\[ \% \text{ maximum diameter} = \left( \frac{D_{\text{ATP}} - D_{\text{base}}}{D_{\text{max}} - D_{\text{base}}} \right) \times 100 \]

where \( D_{\text{ATP}} \) is the diameter after luminal administration of ATP, \( D_{\text{base}} \) is the baseline diameter before addition of ATP, and \( D_{\text{max}} \) is the maximum diameter at 85 mmHg (measured using Ca-free PSS).

For statistical analysis, two-way repeated measures ANOVA was used followed by the Tukey test for individual comparisons when appropriate. \( P < 0.05 \) defined the acceptable level of significance.

RESULTS AND DISCUSSION

Experiments were conducted on 90 rat MCAs. After being mounted, warmed to 37°C, and pressurized to 85 mmHg, the MCAs constricted 24% from a maximum diameter of 271 ± 3 μm (in Ca²⁺-free PSS) to a resting tone diameter of 205 ± 3 μm. In an individual MCA, the luminal application of ATP produced a dose-dependent dilation with near-maximal dilation occurring at 10⁻⁶ M ATP (Fig. 1A, top). We have previously demonstrated that this dilation elicited by ATP does not occur as a result of ectonucleotidase degradation of ATP to ADP or adenosine (30). When NO and prosta-cyclin production had been inhibited with L-NAME (3 × 10⁻⁵ M) and indomethacin (10⁻⁵ M), respectively, ATP still produced a maximal dilation; however, dilation did not occur at 10⁻⁶ M ATP (Fig. 1A, middle). This residual dilation in the presence of L-NAME and Indo has been previously shown to be due to EDHF (29, 31). The dilation to ATP was almost completely blocked in an L-NAME-Indo-treated MCA after inhibition of K_{CaS} (Fig. 1A, bottom). ChTX (10⁻⁷ M) was used to inhibit the large and intermediate conductance K_{CaS}, and Apa (3 × 10⁻⁷ M) was used to inhibit the small conductance K_{CaS}.

Summary data of this study are shown in Fig. 1B. Note that the presence of L-NAME and Indo nearly

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Fig. 3. A: effects of 10⁻⁶ M 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) on the dilations elicited by the luminal application of 2-methylthio-ATP (2-MeSATP). 2-MeSATP dilates exclusively through the generation of NO (30). Circles, control MCAs (n = 11). Squares, MCAs in the presence of ODQ (10⁻⁶ M) (n = 4). *P < 0.05 compared with control values (2-way repeated measures ANOVA with post hoc Tukey). B: effects of 10⁻⁶ M ODQ, a selective inhibitor of guanylate cyclase, alone or in combination with L-NAME on the ATP-mediated dilations in rat MCAs. Circles, control MCAs where no inhibitors are present (n = 4). Squares, MCAs (n = 6) in the presence of L-NAME and Indo. Triangles, MCAs (n = 7) in the presence of Indo and ODQ. Inverted triangles, MCAs in the presence of L-NAME, Indo, and ODQ (n = 5). *P < 0.05 compared with control values (2-way repeated measures ANOVA with post hoc Tukey).
abolished the dilations at $10^{-6}$ M ATP but did not significantly alter the maximum dilations at $10^{-5}$ and $10^{-4}$ M ATP. The additional presence of ChTX alone or in combination with Apa further diminished the dilation to ATP. The presence of ChTX and Apa inhibited the dilations more than ChTX alone; however, this difference was not statistically significant. In earlier studies, ChTX alone completely abolished all dilations to EDHF (18, 29, 31). More recently in our laboratory, residual EDHF dilations have occurred in the presence of ChTX (14, 15). Consequently, we employed the combination of K channel blockers, ChTX, and Apa as previously described (32). We cannot offer an explanation for the recent incomplete blocking of the EDHF response by ChTX alone or ChTX in combination with Apa. Nevertheless, the combination K channel blockers still blocked the major portion of the dilation in the presence of L-NAME and indo.

Figure 2 shows the effects of SNAP on EDHF-mediated dilations. In the presence of L-NAME and Indo (control group in Fig. 2), MCAs dilated to the luminal administration of ATP (indicative of an EDHF-mediated dilation when SNAP was administered luminally to ATP). Figure 2 also shows that ChTX-Apa attenuated the dilations at 10% ($n = 15$) compared with their resting tone diameters due to inhibition of NO synthase. Sufficient concentrations of SNAP, an NO donor, were added either luminally ($6 \times 10^{-7}$ M) or abuminally ($3 \times 10^{-7}$ M) to restore the MCAs to 105 ± 3% ($n = 15$) and 108 ± 2% ($n = 6$), respectively, of their diameters before the addition of L-NAME-Indo (resting tone). Neither abuminonal nor luminal SNAP administration significantly altered the EDHF-mediated dilation to ATP (Fig. 2). When the group receiving abuminonal SNAP was subdivided into those MCAs that ranged from 79 to 104% (mean = 96 ± 3%, $n = 8$) of diameter before addition of L-NAME-Indo (resting tone) and those that ranged between 108 and 124% (mean = 114 ± 2%, $n = 7$), there were still no significant differences among the groups when comparing the dilation to ATP. Figure 2 also shows that ChTX-Apa attenuated the dilation when SNAP was administrated luminally ($n = 4$). Similar results with SNAP were obtained with Br-A23187, a calcium ionophore that has been demonstrated to elicit an EDHF response in rat MCAs (16, 17). In the control group, the addition of L-NAME-Indo constricted the vessels by 16 ± 4% ($P = 0.016$, $n = 5$). In the experimental group receiving SNAP, in addition to L-NAME-Indo, the vessels were not significantly different from the diameter before L-NAME-Indo ($n = 5$). The EDHF responses elicited by the luminal application of Br-A23187 ($10^{-6}–10^{-4}$ M) were similar in the two groups ($P = 0.91$, data not shown). The addition of SNAP did not affect the EHDF response when elicited by either ATP or Br-A23187.

In another study, dilations to ATP were measured in the presence of ODQ, a selective inhibitor of guanylate cyclase (25). In the presence of ODQ, the endothelium would be capable of NO generation, but the NO would not produce dilation through stimulation of guanylate cyclase. If NO were to directly inhibit EDHF synthesis as suggested in peripheral arteries (1), then the EDHF dilation should be inhibited in the presence of ODQ.

Figure 3A demonstrates that NO-mediated dilations in the rat MCA occur solely through stimulation of guanylate cyclase and the generation of cGMP. 2-MeS-ATP, an agonist that dilates exclusively through the production of NO (30), dilated the vessel. This dilation was completely blocked by ODQ ($10^{-6}$ M). ODQ constricted the resting MCAs (19 ± 4%) to a similar degree as did L-NAME.

Figure 3B shows dilations to ATP: 1) alone, 2) in the presence of L-NAME + Indo, 3) in the presence of ODQ + Indo, and 4) in the presence of ODQ + L-NAME + Indo. Dilation of the vessel was not blocked in the presence of ODQ + Indo alone. Because dilations in the presence of ODQ and in the presence of L-NAME were similar, and because the combination of L-NAME and ODQ had no greater effect than either alone, NO is not inhibiting EDHF-mediated dilations by directly inhibiting an EHDF synthase. The studies using ODQ support the studies shown in Fig. 2 where NO was added exogenously (SNAP).

We have demonstrated that neither basal concentrations of NO nor concentrations above basal levels inhibit the EDHF response in the rat MCA. The dual approach (exogenously added NO and endogenously generated NO) provides convincing evidence for this conclusion. Unlike peripheral vessels, NO did not abolish or attenuate the EDHF-mediated dilations (1, 21). This finding is consistent with the idea that the EDHF response does occur during normal physiological conditions and may be important in the regulation of the cerebral circulation (29).

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