Hydrogen peroxide acts as an EDHF in the piglet pial vasculature in response to bradykinin

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THERE IS CONSIDERABLE EVIDENCE showing that bradykinin (BK) is a mediator of pathophysiological reactions such as inflammation and edema formation (22, 46). All parts of the kininogen system have been described in the cerebral circulation (54), and kinins have been extensively studied for implications in the transmission of nociceptive information (34) and the control of blood pressure (9, 35). In the central nervous system, BK is found to be the main mediator of edema formation (46, 47, 55), and it is also a potent dilator of arteries (36, 41, 54, 58). However, the mechanisms that contribute to BK-induced cerebral vasodilation are not well understood (54).

The actions of BK are mediated via specific G protein-coupled receptors of which two different types have been identified so far. The BK1 receptor is located mainly on neurons (44) and can also be found on the surface of adventitial fibroblasts of cerebral arteries (51). BK2 receptors are represented in all layers of the cerebral vessel wall: their presence has been documented in cerebral endothelial as well as smooth muscle and adventitial cells (16, 44, 51, 53). In a variety of preparations, the vasodilator effect of BK is abolished by removal of the endothelium and is mediated by the BK2 receptor subtype (54).

Vasorelaxation to BK is mediated in part by nitric oxide (NO) formation (6, 21, 27, 30, 41) and dilator prostanoids (8, 28, 41). Furthermore, BK is a widely used agent for evoking the release of the hypothetical endothelium-derived hyperpolarizing factor (EDHF) in different regions (12, 14, 24). Hyperpolarization induced by opening of the Ca2+-dependent K+ (KCa) channels is characteristic of EDHF (7, 12, 50, 56); however, EDHF-like actions can also be mediated via ATP-dependent K+ (KATP) channels (see Refs. 13, 40, 56). Arachidonic acid metabolites through the cytochrome P-450 (19) or the lipooxygenase pathways acting via KCa channels are widely accepted candidates for EDHF (40, 56). However, recent studies indicated that endothelial-derived H2O2 can also dilate arteries in an EDHF-like fashion.

The involvement of EDHF in agonist-induced cerebral vasodilation is not well understood. In isolated rat middle cerebral arteries, intraluminal application of uridine-5′-triphosphate induces vasodilation, which is mediated in part by EDHF (37, 62). In the same artery, BK is a potent vasodilator and its effect is still pronounced even after the blockade of the cyclooxygenase (COX) and guanylyl cyclase enzymes (Z. Benyó, Z. Lacza, and M. Wahl, unpublished observations). However, evaluation of the contribution of EDHF to BK-
induced cerebral vasodilation has not yet been undertaken.

The aim of the present study was to characterize the vasodilation induced by topical application of BK in the porcine cerebral circulation in vivo. To exclude the participation of the NO and prostanoid pathways, all experiments were carried out in the presence of N\textsuperscript{-} nitro-L-arginine methyl ester (L\textsuperscript{-}NAME) and indomethacin. Using topical application of specific blocking agents, the involvement of K\textsuperscript{+} channels and putative EDHFs were studied in the BK-induced pial arterial dilation.

MATERIALS AND METHODS

**Measurement of pial arterial diameter.** All procedures were approved by the Animal Care and Use Committee of Wake Forest University. Newborn (1–7 days old) piglets of either sex that weighed 2.39 ± 0.06 kg were anesthetized with thiopental sodium (50 mg/kg iv), and anesthesia was maintained with α-chloralose (75 mg/kg iv). The right femoral artery and vein were cannulated for blood pressure recordings, blood gas sampling, and drug administration. The piglets were intubated via a tracheotomy and artificially ventilated with room air. The respiratory parameters were set to maintain arterial pH, P\textsubscript{CO\textsubscript{2}}, and P\textsubscript{O\textsubscript{2}} values in the physiological range (pH = 7.51 ± 0.01; P\textsubscript{CO\textsubscript{2}}, 31 ± 1 mmHg; P\textsubscript{O\textsubscript{2}}, 95 ± 2 mmHg). Body temperature was maintained at 37°C with a heating pad.

The head of the animal was fixed in a stereotaxic frame. The scalp was incised and the skin was removed along the incision. A circular (19-mm diameter) craniotomy was made in the left parietal bone, and the dura was cut and reflected over the skull. A stainless steel cranial window with three needle ports was placed into the craniotomy, sealed with bone wax, and cemented with cyanoacrylate glue and dental acrylic.

The closed window was filled with artificial cerebrospinal fluid (aCSF) warmed to 37°C and equilibrated with 6% O\textsubscript{2}, 6.5% CO\textsubscript{2}, 87.5% N\textsubscript{2} to yield a pH of 7.33, a P\textsubscript{CO\textsubscript{2}} of 46 mmHg, and a P\textsubscript{O\textsubscript{2}} of 43 mmHg. The aCSF consisted of the following (in mmol/l): 132 NaCl, 2.9 KCl, 1.2 CaCl\textsubscript{2}, 1.4 MgCl\textsubscript{2}, 24.6 NaHCO\textsubscript{3}, 6.7 urea, and 3.7 glucose. Diameters of pial arterioles were measured with a microscope (Wild M36) equipped with a digital videocamera and a video microscaler (model IV-550, For-A-Co). After a 45-min postsurgical equilibration period, a pial arterial segment with an initial diameter of ~100 μm was selected for the measurements. Only one segment was used from each animal. At the end of the experiments, the anesthetized animals were killed with an intravenous bolus of KCl.

After the determination of baseline diameter, a dose-response curve for topicaly applied bradykinin (0.03, 0.3, and 50 μmol/l) was measured. BK was dissolved in aCSF and was given in 3-ml infusions under the window. Pial arterial diameter was monitored continuously and the peak dilation was used for further evaluation.

In another group of animals, 20 min after intravenous administration of 15 mg/kg L\textsuperscript{-}NAME and 5 mg/kg indomethacin (a generous gift from Merck; Rahway, NJ), a dose-response curve for topically applied bradykinin (0.03, 0.3, and 30 μmol/l) was measured. We have previously shown that these doses effectively block the respective enzymes in the experimental preparation (3, 4, 11). Repeated application of BK after the first dose-response curve had an inconsistently lower effect; therefore, BK was applied only for one response (3 μM) in each L\textsuperscript{-}NAME plus indomethacin-treated animal.

In all other experimental groups, specific inhibitors were applied topically 10 min before and in coapplication with BK. The role of the bradykinin receptor subtypes involved was assessed by application of the specific BK\textsubscript{2} receptor blocker [d-arg\textsuperscript{9}-Hyp\textsuperscript{5},Thio\textsuperscript{2}-d-Tic\textsuperscript{7}-Oic\textsuperscript{8},BK (HOE-140, 0.3 μmol/l). This dose was sufficient to completely suppress the vasodilation that was induced by 10 μmol/l BK in rat cerebral arteries (21). To assess the role of cytochrome P-450 metabolites, arteries were pretreated with miconazole (20 μmol/l). The dose of miconazole applied was two times higher than what was enough to completely suppress EDHF-like responses in rat and piglet pial arteries (32, 42). The involvement of lipooxygenase products was tested by the application of baicalein (10 μmol/l) or cinnamyl-3,4-dihydroxy-α-cyanoacnamate (CDC, 1 μmol/l, BioMol; Plymouth, PA). The applied dose of baicalein was sufficient to block the vasodilation to arachidonic acid in pial arteries in vivo (17). The chemically different lipooxygenase inhibitor CDC was applied in a dose that completely blocked dilatory responses in rat mesenteric and porcine coronary arteries (39, 63). H\textsubscript{2}O\textsubscript{2} accumulation was prevented by the application of catalase (400 U/ml), which was previously shown to inhibit the vascular effects of endogenous H\textsubscript{2}O\textsubscript{2} but not hypotension in the piglet (33). KC\textsubscript{a} channels were blocked by coapplication of charybdotoxin (0.1 μmol/l) and apamin (0.5 μmol/l), which are doses sufficient to block EDHF (26). K\textsubscript{ATP} channels were inhibited by glibenclamide (10 μmol/l). This dose of glibenclamide was able to block the vasodilation induced by activators of the K\textsubscript{ATP} channel but not by PGE\textsubscript{2} in piglet pial arteries (5). Baicalein and miconazole were prepared as stock solutions in DMSO and further diluted with aCSF; final DMSO content was 0.1 and 0.2%, respectively. All other drugs were diluted with aCSF. After the determination of BK-induced dilation, animals received 20 μmol/l sodium nitroprusside (SNP) topically to test smooth muscle responsiveness. To determine whether endothelial impairment alters the BK response, phorbol-12,13-dibutyrate (PDB) was infused into the left common carotid artery. PDB was dissolved in DMSO and diluted to the final concentration of 10 μM at a volume of 5 ml. This procedure of PDB injection has been shown to produce transient endothelial dysfunction for at least 15 min, whereas dilator responses to non-endothelium-dependent agents are intact (1, 3, 10). We tested bradykinin reactivity of arterioles 8–10 min after PDB infusion. Only one BK response was performed in each animal. All drugs were obtained from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

**Western blot analysis.** Tissue samples were isolated from piglet brain cortex or pial vasculature and were frozen immediately at ~60°C. Protein was extracted by the addition of boiling lysis buffer (which contained 1% of 1 mol/l Tris and 1% sodium dodecyl sulphate). The samples were sonicated, boiled at 95°C for 5 min, and centrifuged for 20 min at 12,000 rpm and 4°C. The supernatant was used for immunoblotting. Protein concentration was measured by a Bio-Rad (New York, NY) assay kit.

An equal volume of sample buffer (120 mmol/l Tris, 20% glycerol, 0.02% bromophenol blue, 4% sodium dodecyl sulphate, and 200 mmol/l dithiothreitol at pH 6.8) was added to each sample. Equal amounts of protein (50 μg) were separated on a 4–20% gradient mini gel (Bio-Rad) and transferred to nitrocellulose membrane. After samples were blocked with 5% milk, primary antibody against the BK\textsubscript{2} receptor (BD Transduction Laboratory; San Diego, CA) was applied in a 1:1,000 dilution and horseradish peroxidase-
conjugated secondary antibody was subsequently applied. Chemiluminescence was used to visualize the bands. Standard for the protein (rat pituitary lysate, Transduction Laboratory) and molecular weight markers (Bio-Rad) were included on each blot. The visualized blots were scanned and evaluated by measuring pixel numbers in the respective bands with a computer protocol (NIH Image software).

Statistical analysis. Data are presented as means ± SE. Statistical analysis was carried out by one-way ANOVA with subsequent Duncan’s post hoc test for comparing control and treated values; power of the calculations was 0.9961. For data presented in Fig. 1, ANOVA for repeated measures was performed with subsequent Fisher’s least-significant-difference test for post hoc comparisons. Number of animals in the text and figures is indicated as n. *P < 0.05 was considered significant.

RESULTS

Topically applied BK induced dose-dependent vasodilation. The dilation to each dose was transient, reached its peak within 1 min, and returned to baseline within 3 min. Administration of aCSF alone did not change vascular diameter. Systemic application of L-NAME and indomethacin increased blood pressure (from 68 ± 2 to 93 ± 3 mmHg; *P < 0.01) and significantly constricted pial arteries (from 107 ± 2 to 87 ± 3 μm; **P < 0.01). The dilator response of BK was significantly shifted to lower diameter values due to the constricted initial diameter in the presence of L-NAME and indomethacin (Fig. 1).

There was no significant difference in baseline vascular diameter values among the experimental groups. None of the applied additional treatments (HOE-140, baicalein, CDC, catalase, miconazole) affected baseline arterial diameter or smooth muscle responsiveness to SNP (data not shown).

![Fig. 1. Dose-response curves of bradykinin (BK)-induced vasodilation in naïve animals (n = 7) and after the blockade of the nitric oxide synthase and cyclooxygenase enzymes (n = 10) by systemic application of 15 mg/kg N-nitro-L-arginine methyl ester (L-NAME) and 5 mg/kg indomethacin (Indo). Initial vessel diameter was identical in the two groups (naive, 105 ± 3 μm; L-NAME + Indo, 107 ± 4 μm). Data are presented as means ± SE arterial diameter. **P < 0.01 vs. control.](http://ajpheart.physiology.org/)

The remaining substantial dilation to BK (62 ± 12%) after L-NAME and indomethacin was completely inhibited by the application of the selective BK2 receptor antagonist HOE-140 (54 ± 11 vs. 3 ± 2 μm increase in vessel diameter; *P < 0.01; Fig. 2). Furthermore, endothelial impairment with intracarotid application of PDB markedly decreased the BK response (54 ± 11 vs. 16 ± 6 μm increase in vessel diameter; **P < 0.01; Fig. 2).

Western blotting experiments using a monoclonal antibody against the BK2 receptor showed a double immunoreactive band at 42 kDa in both brain cortex and pial vascular tissue samples (Fig. 3). The bands were doublets in the case of the samples as well as the company-provided standards, probably representing two slightly different splice variants of the BK2 receptor (48).

The cytochrome P-450 antagonist miconazole as well as the lipoxygenase inhibitors baicalein and CDC had no effect on BK-induced dilation. In contrast, the H2O2 scavenger catalase abolished the BK response (54 ± 11 vs. 0 ± 2 μm increase in vessel diameter; *P < 0.01; Fig. 4).

The KATP channel inhibitor glibenclamide significantly reduced the BK response (54 ± 11 vs. 16 ± 5 μm increase in vessel diameter; **P < 0.01). Conversely, the KCa channel inhibitors charybdoxin (large- and intermediate-conductance KCa channels) and apamin (small-conductance KCa channels) in coapplication failed to reduce the BK-induced vasodilation (Fig. 5).

DISCUSSION

In the present study, we have shown that BK is a remarkably potent vasodilator in the piglet pial vasculature even after the blockade of NO synthase (NOS) and COX. This dilation is dependent on endothelium and requires the action of BK2 receptors. The effect is mediated via H2O2 formation and the opening of KATP.
channels. To the best of our knowledge, this is the first indication of the role of an EDHF in the newborn cerebral circulation.

In this study, the specific BK$_2$ receptor antagonist HOE-140 abolished the vasodilation induced by BK, which indicates the prominent role of this receptor subtype in the mediation of vasorelaxation to BK. A similar exclusive participation has already been described in other species including the rat (21) and cat (58). The BK$_1$ receptor appears to have no contribution to vasorelaxation, at least in the preparations studied so far (54, 58). BK$_2$ receptors were found in both neural and vascular tissue in the present study. Receptors at each location may have a role in the mediation of vasodilation; however, the BK$_2$-induced response is primarily endothelium dependent in various preparations (21, 43, 54). In the present study, endothelium impairment with PDB markedly reduced the BK response. This in vivo approach of endothelial impairment has been shown to reduce endothelium-mediated vasodilation and constriction, whereas the smooth muscle responsiveness remained intact. Accordingly, in the present study, the endothelium-independent vasodilation to SNP remained intact after PDB infusion. Furthermore, in a recent study by Willis and Lefler (59), endothelial impairment with the light dye technique also abolished the BK response, making it less likely that neural or direct smooth muscle action can account for the effect. Although endothelial denudation cannot be adequately performed in vivo, two independent approaches provided the same results. Therefore, this substantial, non-NO, non-prostanoid-mediated vasodilation appears to be the specific action of BK$_2$ receptors of the endothelial cell layer. Because BK induces EDHF release in different vascular beds, our next aim was to test which of the various EDHF candidates could be responsible for the observed vasodilation.

There is considerable evidence that cytochrome P-450 products dilate cerebral arteries and thus act like EDHF (2, 15, 20, 32). In the present study, blocking arachidonic acid metabolism at the lipoxygenase or the cytochrome P-450 pathways failed to reduce the BK response, making it unlikely that vasodilator arachidonic acid derivates contribute to the response.

Previous in vivo observations showed that BK relaxes the pial arteries via H$_2$O$_2$ formation in mice, rats, and cats (28, 29, 45, 60), as well as in porcine coronaries (43). Furthermore, H$_2$O$_2$ has recently been shown to fulfill the criteria to be an EDHF in mouse isolated vessels (38, 52). Because H$_2$O$_2$ is also a potent vasodilator in the piglet cerebral circulation (31), we hypothesized that it may contribute to the BK-induced vasodilation. Catalase, the endogenous H$_2$O$_2$ scavenger, specifically breaks down H$_2$O$_2$ to less vasoactive products. In the present study, the BK-induced vasodilation.
was abolished by catalase, which indicates the primary role of H$_2$O$_2$ in the mediation of this vasodilator response. Considering factors such as the dose of BK used and the initial baseline diameter, we estimate that H$_2$O$_2$-driven mechanisms provide at least one-half of the dilator response to BK.

The dilator action of H$_2$O$_2$ is reported to be mediated by hyperpolarization through K$^{+}$ channels (23, 38, 49, 57, 61). The main contributing channel type is dependent on the species; for example, in the cat pial vasculature, K$_{ATP}$ channels mediate the response, whereas in rats or dogs, K$_{Ca}$ channels are responsible (25, 49, 57). Patch-clamp studies provided direct evidence that H$_2$O$_2$ induces hyperpolarization of the cell membrane, which was inhibited by the K$_{ATP}$ channel blocker glibenclamide (18). In the rat cerebral circulation, the dilator effect of BK was inhibited to a similar extent by either catalase or the K$_{Ca}$ channel blocker ibetioxin (49). In this study, both catalase and glibenclamide markedly reduced the response to BK in the piglet pial vasculature, which indicates that the H$_2$O$_2$-induced vasodilation is mediated by K$_{ATP}$ channel-derived hyperpolarization. Because glibenclamide did not totally abolish the BK-induced dilation, it seems likely that other mechanisms, including cyclic nucleotides, also contribute to the dilator response.

In conclusion, H$_2$O$_2$ has been found to be an EDHF-like mediator of the BK-induced vasodilation in the piglet cerebral circulation. Together with NOS and COX metabolites, EDHF appears to be an important mediator of vasodilation in the neonatal cerebral circulation.

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