

Bradykinin Relaxation in Small Porcine Retinal Arterioles

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PURPOSE. To study changes in the spontaneous diameter of small retinal arterioles and bradykinin (BK)-induced vasodilation during inhibition of the synthesis of nitric oxide (NO), prostaglandins (PGs), and cytochrome P450 2C8/9-dependent endothelial-derived hyperpolarizing factor (EDHF).

METHODS. Forty-eight isolated porcine arterioles with a diameter of approximately 70 μm were mounted in a double-barreled pipette system placed in an organ bath, and diameter changes were studied under isobaric conditions. After an equilibration period, the arterioles were incubated with inhibitors of the synthesis of NO, PGs, or cytochrome P450 2C8/9-dependent EDHF, and spontaneous diameter changes were studied. Subsequently, the arterioles were precontracted, and the diameter was assessed after addition of BK in cumulative concentrations.

RESULTS. Inhibition of NOS elicited a significant decrease in the spontaneous diameter of the vessels ($P = 0.028$), whereas no change in the spontaneous diameter was induced by inhibition of PG or cytochrome P450 2C8/9 dependent EDHF synthesis ($P = 0.35$ and $P = 0.75$, respectively). The vasodilating effect of BK was decreased by inhibition of NO ($P = 0.002$) but not by inhibition of prostaglandin or cytochrome P450 2C8/9-dependent EDHF synthesis ($P = 0.82$ and $P = 0.94$, respectively).

CONCLUSIONS. The results suggest the presence of a spontaneous release of NO, which keeps the retinal microcirculation dilated under normal conditions. The finding of BK-induced relaxation being dependent on the NO synthase (NOS), but not on PGs or cytochrome P450 2C8/9-dependent EDHF may be of importance for understanding the microcirculatory effects of pharmacologic compounds affecting the BK metabolism, such as angiotensin-converting enzyme (ACE) inhibitors. (*Invest Ophthalmol Vis Sci.* 2002;43:1891-1896)

Retinal blood flow is disturbed in a variety of retinal diseases, such as retinal vein occlusion and diabetic retinopathy. These disturbances often involve vasodilation and hyperperfusion that may impose mechanical stress on the capillaries and disturb fluid homeostasis. The consequent damage to the retinal tissue may result in serious visual loss.

Previously, changes in retinal blood flow have been studied with different experimental techniques. The retinal blood flow has been assessed in vivo in the larger retinal vessels by examining the Doppler shift of light induced by moving blood cells

in the vessels¹ and by examining the passage of the dye front through the retinal circulation during fluorescein angiography.² The capillary circulation in the perifoveal area also has been studied by scanning laser ophthalmoscopic imaging of leukocyte shadows in this region³ and by blue-field entoptometry.⁴ However, none of these in vivo techniques has allowed the study of the precapillary arterioles that regulate the blood supply to single microcirculatory units. These arterioles have the largest capacity for regulating blood flow,⁵ and disturbances in the regulation of the tone of these vessels may be involved in the development of localized lesions in retinal disease. Previously, in vitro studies have been performed on large, isolated retinal vessels,⁶⁻⁸ whereas studies of smaller retinal vessels have been performed only on perfused whole bovine retina.⁹ However, the latter preparation did not allow the study of the regulation of vascular tone independent of counterregulatory mechanisms in the retinal tissue surrounding the vessels.

We therefore adapted an in vitro method developed for studying caliber changes of renal tubules and microvessels for the study of isolated retinal resistance vessels with a diameter of 30 to 90 μm .^{10,11} An experimental setup was established for studying the effect of vasodilating compounds on small resistance vessels from the pig retina. Vasodilating agents may either exert their effects directly on the smooth muscle cells in the vascular wall or indirectly by stimulating the release of vasodilating compounds from the vascular endothelium, such as nitric oxide (NO), prostaglandins (PGs), and endothelial derived hyperpolarizing factor (EDHF),^{12,13} but the relative contribution of these mechanisms varies among different vascular beds. Bradykinin (BK) is a vasodilating nonpeptide that is degraded by angiotensin-converting enzyme (ACE),¹⁴ and ACE inhibition is known to have beneficial effects on arterial hypertension and diabetic complications in the kidney and the retina.¹⁵ However, the relative contribution of endothelial cell-independent or endothelial cell-dependent mechanisms to the vasodilating effect of BK in the retina is unknown.

In the present study, the spontaneous tone of small retinal arterioles was studied after inhibition of each of the endothelial cell-dependent mediators NO, PGs, and cytochrome P450 2C8/9-dependent EDHF. Subsequently, the effect of this inhibition on BK-induced vasodilation was studied.

METHODS

Equipment

Technique. A technique originally developed for perfusion of isolated renal tubules and further developed for the investigation of microvessels was modified for studying isolated small retinal arterioles.^{10,11} The vessels were mounted between two double-barreled pipette systems (Luigs and Neumann, Ratingen, Germany) in an organ bath (Danish Myotechnology, Århus, Denmark). Each pipette system consisted of an outer holding pipette and an inner perfusion pipette for cannulation of the arteriole. In the present experiments, only the right perfusion pipette was used. The left end of the arteriole was sealed by sucking it sideways into the holding pipette.

To ensure concentric tapering, the pipettes were created by pulling a glass tube (Drummond Scientific, Broomall, PA) vertically while it

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was rotating. The inner pipette was adapted so that it had a long, straight tip with a diameter of 15 to 20 μm . The holding pipette on the right side was pulled to have a tip diameter of 60 to 70 μm and an inner constriction of approximately 25 to 30 μm that sealed the arteriole around the perfusion pipette when the arteriole was sucked into the holding pipette. The tip of the holding pipette on the left side was adjusted to a diameter of approximately 45 μm to seal the arteriole when it was sucked in sideways.

The pipette system was mounted on an inverted microscope (Axiovert 25; Carl Zeiss, Oberkochen, Germany) and could be moved by micromanipulators (model M152; Narishige Scientific Laboratory, Tokyo, Japan). The inverted microscope was equipped with a camera (model CCD 72; Dage MTI, Michigan City, IN), which was connected to an S-VHS recorder (model HR-S7500EH; JVC, Yokohama, Japan) and a computer for diameter measurements.

Tissue. All experiments were conducted in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. Retinal arterioles from 48 domestic Danish pigs (both sexes, age \sim 4 months and weight 70 kg) were used. The eyes were obtained from a local abattoir and enucleated immediately after the pigs had been anesthetized with CO_2 and killed by stabbing. The eyes were immersed in physiologic saline solution (PSS) at 4°C containing (in millimolar) NaCl, 119; KCl, 4.7; MgSO_4 , 1.17; NaHCO_3 , 25; KH_2PO_4 , 1.18; EDTA, 0.026; glucose, 5.5; HEPES, 5.0; and CaCl_2 , 1.6; and were kept cool (4°C) during transport to the laboratory, which occurred within 1 hour.

The eyes were divided into two parts by a frontal section behind the ora serrata. The vitreous was removed, and subsequently the posterior part of the eye was divided into temporal and nasal parts by sagittal sectioning through the optic disc. In the temporal part, the retina was separated from the pigment epithelium by hydrodissection. The retina was placed under a stereomicroscope (Stemi SV11; Carl Zeiss) in PSS with bovine serum albumin (1%) at 4°C . The arterial tree was identified as the one with the smallest diameter, by the examination of paired venules and arterioles near the optic disc. In cases in which no evident difference in diameter was observed, the retina was discarded. The arterial tree was traced peripherally from the optic disc, and an arteriole with an outer diameter of approximately 70 μm was identified by using a scale in the ocular. A segment with a length up to 1 mm and no visible side branches was located. The arteriole was dissected from the surrounding retinal tissue using two pairs of forceps (Dumont Hand-Made Tools, Montignez, Switzerland), and the ends were cut using a beaver microblade. One arteriole was studied from each eye, and one eye was enucleated from each pig. All arterioles were mounted within 6 hours of enucleation.

Procedure. After dissection, the arteriole was moved to the organ bath with a pipette, sucked into the right holding pipette, and cannulated by the inner pipette. The other end of the arteriole was sucked sideways into the left holding pipette, forming a blind sack for the purpose of obviating flow.

A solution tube was placed in the inner pipette, and the tube was connected to a reservoir. The elevation of the reservoir was kept at 81 mm Hg (10.64 kPa) during the experiments. Preliminary experiments had shown that this pressure induces an optimal myogenic response. The fluid in the reservoir and inside the arteriole consisted of PSS with 0.5% bovine serum albumin, prefiltered through a 0.22- μm filter (Millipore, Molsheim, France), at pH 7.4. The PSS in the bath was continuously bubbled with a gas mixture of 95% O_2 and 5% CO_2 and was kept at 37.0°C by a thermostat.

A leak test was used to ensure that the arteriole was tight.¹¹ A stopcock between the reservoir and the perfusion pipette was turned off for 30 seconds. If a change in the vessel diameter occurred, it was considered to be leaking and was discarded (4/48 arterioles).

Experimental Procedures

Incubation. After an equilibration period of 30 minutes, the vessel was incubated with an inhibitor (test experiments) or no inhib-

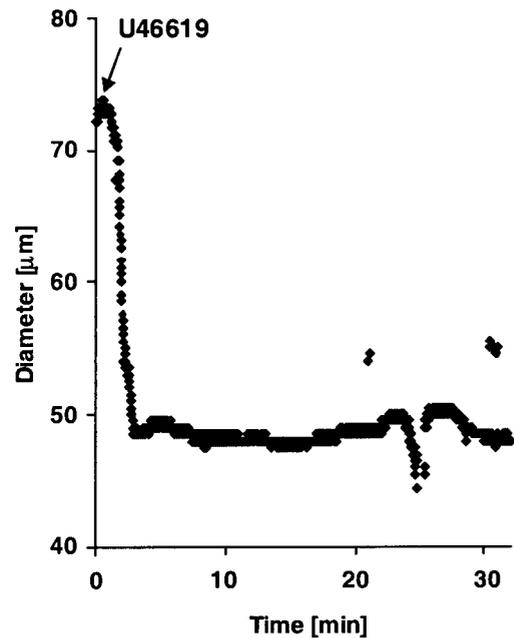


FIGURE 1. Trace showing the arteriole diameter as a function of time after contraction induced by U46619 (30 nM).

itor (control experiments). *N*-nitro-L-arginine methyl ester (L-NAME; 100 μM) was used to inhibit the nitric oxide synthase (NOS),¹⁶ ibuprofen (10 μM) or indomethacin (10 μM) was used to inhibit the cyclooxygenase,¹⁷ and sulfaphenazole (10 μM) was used to inhibit the cytochrome P450 2C8/9, which in some preparations has been shown to synthesize EDHF.¹⁸ The inhibitors were added to the chamber fluid. Sulfaphenazole was incubated for 30 minutes and the other compounds for 20 minutes each. In additional experiments, the vessels were incubated with L-NAME (100 μM) and L-arginine (1 mM) to exclude a nonspecific effect of L-NAME. No incubation period was included in the control experiments.

Precontraction. After the incubation period, the thromboxane analogue U46619 was added to the organ bath in a concentration of 30 nM. The arteriole was considered viable if U46619 induced a contraction. Otherwise, it was discarded (5/44 arterioles). Preliminary experiments had shown that the U46619 contraction persisted for more than 30 minutes (Fig. 1). Additional experiments with U46619 in a concentration of 100 nM tested the influence of the precontraction level on the BK-induced relaxation. Although the higher concentration of U46619 induced 8% more contraction, the pD_2 [$-\log(EC_{50})$] and the dilation after BK-induced relaxation were unaltered compared with the control curve ($P = 1.00$ and $P = 0.10$, respectively).

Concentration-Response Curves. The precontraction was followed by a BK concentration-response curve. Preliminary experiments had shown that the BK-induced dilation in precontracted retinal arterioles was blunted by repeated exposure to BK, whereas the precontraction level induced by U46619 was not changed. The arterioles contracted and dilate three times after repeated exposure to U46619 (30 nM) and histamine (100 μM), which confirms that the blunted response after repeated exposure to BK is not due to limited viability of the arteriole. As a consequence of the blunted response after repeated exposure to BK, only one concentration-response curve was obtained from each arteriole. A trace from one of the control experiments is shown in Figure 2. The effect of BK was measured as the largest diameter of the vessel in the 5-minute period between additions of BK. For each series six arterioles were studied. At the end of each experiment, the arteriole was relaxed maximally by papaverine (100 μM) and the diameter of the arteriole was measured. This diameter was used as a reference for the other diameters measured during

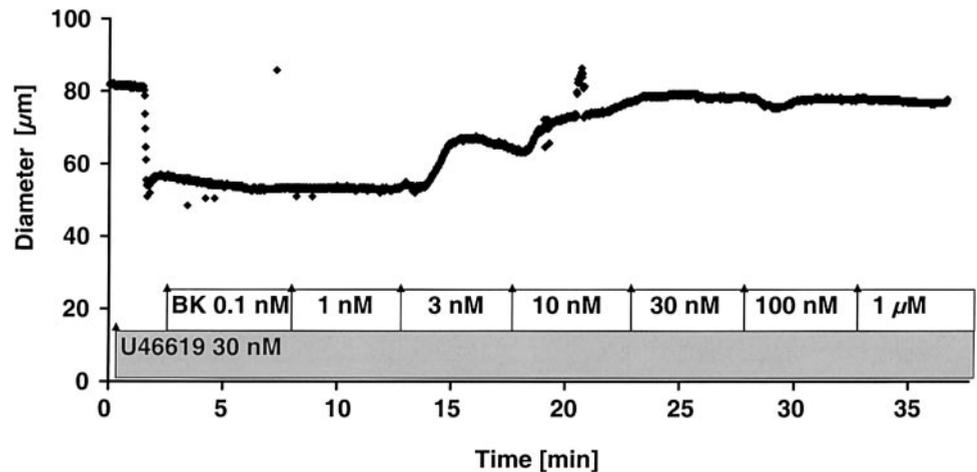


FIGURE 2. Trace from a control experiment. Addition of 30 nM U46619 led to a contraction of the arteriole. Subsequent addition of BK induced a concentration-dependent dilation of the vessel.

the experiment. The mean of the maximally relaxed outer diameter was $71 \pm 2 \mu\text{m}$ ($n = 36$), and there were no significant differences between the maximally relaxed diameters in the different series ($P = 0.13$). The basal diameter was identified as the diameter of the arteriole before the addition of the inhibitors in the test experiments and as the diameter before the addition of U46619 in the control experiments.

During the experiments, images of the vessel were captured every second and were analyzed by computer (Vesselview software; Danish Myotechnology, Århus, Denmark). The program calculates the outer diameter of the vessel on the basis of the contrasts between the two sides of the vessel wall and the surrounding PSS. Subsequently, diameter measurements are saved to hard disk.

Drugs

BK, U46619 (9,11-dideoxy-11 α , 9 α -epoxymethanoprostaglandin F 2 α), L-NAME, L-arginine, sulfaphenazole, and histamine were all dissolved in distilled water. Ibuprofen and indomethacin were dissolved in ethanol. The compounds were made as a stock solution and frozen for later use. The stock solutions were thawed and diluted on the day of the experiment, and the concentration of the solvent did not exceed 1% in the organ bath. The addition of 1% ethanol to the organ bath did not change the diameter of the arterioles. Bovine serum albumin was dissolved in PSS on the day of the experiment. All these compounds were purchased from Sigma Chemical Co. (St. Louis, MO). Papaverine (Dako, Glostrup, Denmark) was diluted in distilled water, and a fresh solution was made daily.

Data Analysis

All diameters in an experiment were expressed as the percentage of the maximally relaxed diameter after application of papaverine at the end of the experiments. The diameters of the arterioles among the six series were compared using the Kruskal-Wallis test. The changes in diameter during the incubation period were analyzed with the Wilcoxon signed rank test, comparing the arteriole diameter at the start and the end of the incubation period.

In the concentration-response experiments, the maximum response of BK for the different inhibitors was compared with the control curve by Mann-Whitney test. For each experiment, the EC_{50} was calculated using the Hill equation: $E = (E_{\text{max}} - C^n)/(C^n + EC_{50}^n)$, where E is the measured response; E_{max} the maximum response achievable, C the concentration of BK, EC_{50} the concentration that produces a 50% maximal response, and n the slope parameter. EC_{50} was transformed to pD_2 [$-\log(EC_{50})$] and used for comparison among the different series by an independent-sample t -test.

RESULTS

Incubation Period

The addition of L-NAME to the organ bath induced a significant contraction of the arterioles ($P = 0.028$), whereas the combination of L-NAME and L-arginine did not change the diameter significantly ($P = 0.25$). The addition of indomethacin, ibuprofen, or sulfaphenazole did not change the diameter significantly ($P = 0.35$, $P = 0.35$, and $P = 0.75$, respectively).

BK Concentration-Response Curve

L-NAME. Inhibition with L-NAME (Fig. 3A and Table 1) significantly reduced the vasodilating effect of BK ($P = 0.002$) and right shifted the curve (increase in pD_2) for the BK relaxation significantly ($P = 0.009$). When L-NAME was combined with L-arginine the BK-induced maximum relaxation did not differ significantly from that in the control experiments ($P = 0.94$), but the combination significantly right shifted the curve (increase in pD_2) during the BK relaxation ($P = 0.023$).

Cyclooxygenase Inhibitors. Inhibition with ibuprofen and indomethacin (Fig. 3B, 3C; Table 1) did not alter the maximum relaxation significantly ($P = 0.13$ and $P = 0.82$, respectively), whereas these compounds induced a significant right shift of the curve (increase in pD_2) during BK relaxation ($P = 0.027$ and $P = 0.045$, respectively).

Sulfaphenazole. Inhibition with sulfaphenazole (Fig. 3D, Table 1) did not alter the maximum significantly ($P = 0.94$), and no shift of the concentration-response curve was induced ($P = 0.131$).

DISCUSSION

BK and other vasodilating substances may exert their effects through endothelial cell-independent mechanisms or by stimulating the release of vasodilators from these cells, such as EDHF, PGs, and NO. However, the relative contribution of these factors to vasodilation varies among different vascular beds. In humans, BK has been found to mediate relaxation through an EDHF-dependent mechanism in coronary arteries,¹⁹ BK increases the concentration of metabolites from PG degradation in plasma,^{20,21} and through an NO-dependent mechanism in the forearm arterial bed.²² BK-induced endothelium-dependent vasodilation is mainly mediated by G protein-coupled cell surface receptors, designated B_2 receptors.^{13,23} The signal transduction from the B_2 receptor to NOS activation involves Akt protein kinase activation and calcineurin-mediated dephosphorylation of eNOS.²⁴

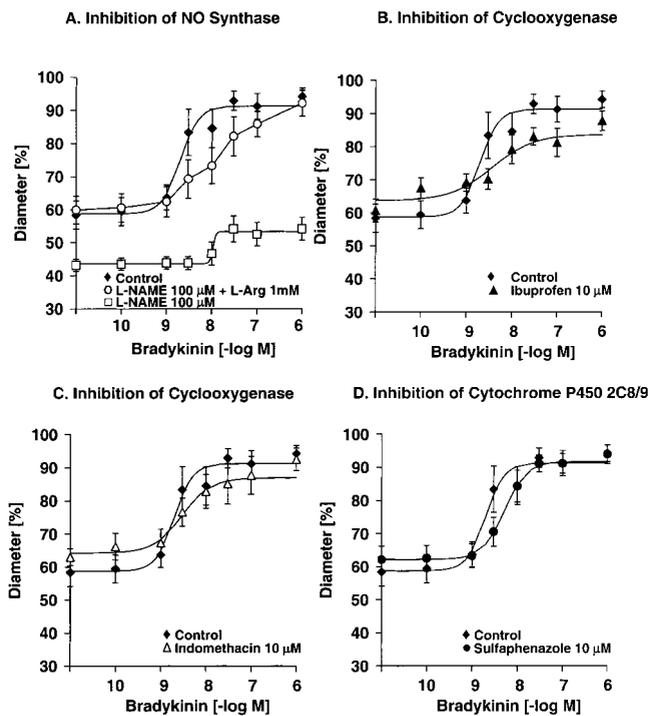


FIGURE 3. Effect of inhibition of NOS (A), cyclooxygenase (B, C), and cytochrome P450 2C8/9 (D) on the BK concentration-response curve in porcine retinal arterioles. L-NAME significantly decreased the potency and maximum effect of BK. The combination of L-NAME and L-arginine (L-Arg) significantly right shifted the concentration-response curve, but did not alter the maximum dilation induced by BK. Inhibition with ibuprofen and indomethacin significantly right shifted the concentration-response curve for bradykinin, but did not alter the maximum dilation induced by BK. No significant effect of inhibition with sulfaphenazole was observed. Diameter expressed as a percentage of the maximally relaxed diameter obtained at the end of each experiment. Data are the mean \pm SEM ($n = 6$ for each series).

In the ocular circulation, BK's effects on the larger ciliary and ophthalmic arteries have been studied,²⁵ whereas the effects on the small retinal arterioles regulating blood supply to single microcirculatory units are unknown. The lack of knowledge of BK's effect on small retinal arterioles is partly due to difficulties in setting up a suitable experimental model in vivo for studying small-caliber retinal vessels. We therefore modified a method originally developed to study renal tubules for the purpose of studying caliber changes in vitro of small isolated retinal arterioles with an outer diameter between 30 and 90 μm .¹⁰ This is the caliber of arterioles supplying individual microcirculatory units.⁵

The effect of inhibition of the three endothelium-dependent vasodilators, NO, PGs, and cytochrome P450 2C8/9-dependent

EDHF, was studied in porcine retinal arterioles. Incubation of the arteriole with inhibitor was performed at the beginning of the experiments, which enabled us to study its effect on the spontaneous tone of the arterioles. After the incubation period, the arterioles were precontracted, and a concentration-response curve for BK was obtained. Thereby, BK's influence on the arterioles during inhibition of the vasodilators NO, PGs, and cytochrome P450 2C8/9-dependent EDHF could be studied. In preliminary experiments, we found a blunted vasodilatory response after repeated exposures to BK, which is in contrast to previous studies of ophthalmic and ciliary arteries.²⁵ However, the ability to contract was unaltered, which indicates a persistent viability of the arterioles, but desensitization to BK. This was further confirmed by the preservation of a vasodilatory response when BK was replaced with histamine. The blunted vasodilation after repeated exposures to BK may be because BK's effect on these vessels is predominantly mediated through the B₂ receptor, which is desensitized and internalized after exposure to BK.^{26,27}

Inhibition of cytochrome P450 2C8/9-dependent EDHF or PGs did not alter the diameter of the retinal arterioles significantly during the incubation period, suggesting no spontaneous release of these compounds. The BK concentration response was not significantly altered in experiments in which cytochrome P450 2C8/9-dependent EDHF production was inhibited by sulfaphenazole, which is an inhibitor of EDHF in some but not all blood vessels.²⁸ Inhibition of cyclooxygenase showed a significant right shift of the BK concentration response, but did not diminish the maximum dilation induced by BK. This suggests that BK can fully dilate the arterioles using one or more vasodilating pathways different from the cyclooxygenase pathway, but it also indicates a minor role of the cyclooxygenase pathway in BK-induced vasodilation, because of the right shift of the BK concentration response.

The inhibition of the synthesis of NO induced a significant decrease in the diameter of the porcine retinal arterioles during the incubation period, and this decrease did not occur when L-NAME was combined with L-arginine, which makes it unlikely that the inhibition of the BK-induced response during NO inhibition was due to an unspecific effect of L-NAME. This concurs with findings in human and porcine ophthalmic arteries,^{29,30} the porcine ciliary artery,²⁵ and the bovine retinal artery,^{31,32} where inhibition of NO synthesis has been shown to induce vascular contraction. In a study by Su et al.,³³ no significant change in diameter was observed after intraluminal addition of L-NAME in porcine retinal arterioles. However, this may have been a consequence of the experimental conditions in which a high potassium concentration in the perfusion media led to a strong contraction of the vessels under study and masked the L-NAME-induced contraction. The findings of the present and other studies imply the presence of a basal release of NO that keeps the retinal vascular bed in a constantly dilated condition.^{31,32} The observed reduction in arteriolar

TABLE 1. Inhibitors Effects on BK Relaxation

Inhibitor	MD	Basal Diameter (% of MD)	Precontraction Level (% of MD)	E_{max} (% of MD)	pD_2
None (control)	76 \pm 3 μm	87 \pm 7	58 \pm 4	94.2 \pm 2.5	8.7 \pm 0.2
Indomethacin	63 \pm 5 μm	88 \pm 4	63 \pm 2	92.5 \pm 3.4	7.9 \pm 0.3*
Ibuprofen	69 \pm 4 μm	80 \pm 7	61 \pm 4	87.7 \pm 3.0	8.1 \pm 0.2*
Sulfaphenazole	62 \pm 5 μm	83 \pm 7	62 \pm 4	93.8 \pm 2.9	8.3 \pm 0.2
L-NAME	79 \pm 6 μm	88 \pm 3	43 \pm 2*	54.2 \pm 3.5*	7.9 \pm 0.2*
L-NAME and L-Arginine	75 \pm 6 μm	87 \pm 6	60 \pm 4	92.2 \pm 3.9	8.0 \pm 0.2*

Maximally relaxed outer diameter (MD), basal diameter, precontraction level, and maximum relaxation by BK (E_{max}) expressed as percentages of the MD. pD_2 is calculated as $-\log EC_{50}$. Data are expressed as the mean \pm SEM.

* $P < 0.05$ versus control.

diameter of 38% after inhibition of NO synthesis in the present study corresponds to an approximate sevenfold reduction in the flow. Thus, NO is a potent regulator of blood flow in both small and large arterioles, and disturbances in the NO-mediated vasodilation could lead to changes in retinal blood flow of the magnitude seen in retinal diseases such as early diabetic retinopathy.³⁴⁻³⁶

Previous studies on ophthalmic and ciliary arteries have shown that NO-dependent BK-induced dilation is most pronounced in small-caliber vessels and that the dilation is dependent on the presence of the endothelium.²⁵ Our study showed a similar effect of NO inhibition on BK-induced dilation in the smaller retinal arterioles, and it is therefore likely that the BK response in these vessels is also endothelium dependent. However, a definite test of this has not been conducted.

Because of the marked effect of NO inhibition on the arterioles, it may be surmised that NO is a key mediator for regulating retinal microcirculation, and it is possible that microcirculatory disturbances in retinal disease involve changes in the activity of the BK system.

BK is degraded in vascular endothelial cells by ACE, and treatment with ACE-inhibiting drugs accordingly increases the plasma level of BK.³⁷ ACE inhibitors lower the blood pressure, but evidence also suggests a reduced risk of progression of retinopathy in diabetic patients,^{15,38} with an effect that is additional to the effect induced by the lowering of the blood pressure.³⁹ This may be due in part to a direct effect of ACE inhibition on retinal flow regulation mediated through a change in the level of circulating BK.⁴⁰

In conclusion, our in vitro studies of isolated small porcine retinal arterioles showed vascular contraction after inhibition of NOS. This finding suggests that a spontaneous release of NO keeps small retinal arterioles dilated under physiological circumstances. Furthermore, the maximum BK-induced vasodilation was inhibited by the NOS inhibitor, indicating that this dilation is mediated by NO presumably released from the vascular endothelium. The findings contribute to an understanding of the regulation of retinal microcirculation. The experimental setup may be further used to test the effect on retinal vessels of new and existing drugs believed to influence blood flow in retinal disease—for example, ACE inhibitors.

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