Endothelium-Dependent Regulation of the Ophthalmic Microcirculation in the Perfused Porcine Eye: Role of Nitric Oxide and Endothelins

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Purpose. The endothelium produces nitric oxide and endothelin (ET). This study was designed to investigate the endothelium-dependent regulation of the porcine ophthalmic microcirculation.

Methods. Isolated porcine eyes were perfused with a modified Langendorff setup (Hugo Sachs Elektronik KG, Freiburg, Germany) at a perfusion pressure of 80 cm H_2O with Krebs-Ringer bicarbonate solution (37°C; 95% O_2, 5% CO_2).

Results. The inhibitor of nitric oxide formation, L-nitroarginine methylester (L-NAME; 10^{-6} to 10^{-4} M), evoked decreases in flow (maximal decrease, 39% ± 6%; $P < 0.005$ versus control). The endothelium-dependent vasodilator bradykinin evoked increases in ophthalmic flow (maximal increase, 26% ± 2%; $P < 0.05$ versus control) prevented by L-NAME. The effect of endothelin-1 (ET-1; 10^{-12} to 10^{-11} M) on flow was biphasic, with early vasodilation (1 to 2 minutes) and late vasoconstriction. At 10^{-12} M, the increase in flow was most pronounced (24% ± 5%; $P < 0.05$ versus control), whereas 10^{-10} M caused only significant decreases in flow (59% ± 5%; $P < 0.001$ versus control). Endothelin-3 (ET-3) evoked similar vasodilator effects as ET-1 but less vasoconstriction. The vasodilator effects of ET-3 were prevented by pretreatment of the eye with indomethacin (10^{-5} M, to block the production of prostaglandins; $P < 0.05$ versus control). The endothelin$_A$ receptor antagonist FR-139317 significantly reduced vasoconstriction to ET-1 (10^{-10} M; $P < 0.001$ versus control). The thromboxane analogue (U-46619) reduced flow in a concentration-dependent manner ($P < 0.001$ versus control).

Conclusions. Endothelium-derived nitric oxide released under basal conditions or stimulated by bradykinin significantly regulated flow to the porcine ophthalmic microcirculation. This vasodilator system may play an important protective role against vasospasm. In contrast, ET-1 has vasodilator effects through the release of prostaglandins and potent vasoconstrictor properties mediated through ET_A receptors. Invest Ophthalmol Vis Sci. 1993;34:3614–3621.

The vascular endothelium modulates local vascular tone by the release of relaxing factors such as nitric oxide,[1–3] prostacyclin,[1] and endothelium-derived hyperpolarizing factor,[1,4] as well as by the formation of the potent vasoconstrictor peptide endothelin-1 (ET-1).[5–9] In extraocular porcine and human ophthalmic arteries, endothelium-derived vasoactive substances are potent regulators of vascular tone.[6–8,10–12] It is the ophthalmic microcirculation, however, that most significantly regulates the perfusion of the eye under normal and pathologic conditions. The regulatory role of the endothelium in ophthalmic microvessels has not been investigated. As judged from extraocular ophthalmic arteries, the importance of endothelial mediators increases with decreasing diameters of the blood vessel.[8] This would suggest a crucial role of these local mechanisms in the ophthalmic microcirculation. For technical reasons, however, it is difficult or impossible to investigate isolated arteries with a diameter of 100 μm or less in organ chambers.

To overcome these problems, an experimental setup has been developed for perfusion in isolated

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porcine eyes through the common ophthalmic artery, using a modified Langendorff system (Hugo Sachs Elektronik KG, Freiburg, Germany).[13] With the advent of specific inhibitors of endothelium-derived mediators, such as L-nitroarginine methylester (L-NAME),[14–16] and endothelin$_A$ (ET_A) receptor antagonists, this technique allows the characterization of local vascular regulatory mechanisms—in particular, the involvement of nitric oxide and ET—in the entire ophthalmic microcirculation in situ.

MATERIALS AND METHODS

Preparation of Eyeballs

Porcine eyes with surrounding tissue were obtained at a slaughterhouse 10 minutes after death and transported in cold (4°C) modified Krebs-Ringer bicarbonate solution containing: NaCl, 118.6 mM; KCl, 4.7 mM; $CaCl_2$, 2.5 mM; $MgSO_4$, 1.2 mM; KH_2PO_4, 1.2 mM; $NaHCO_3$, 25.7 mM; edetate calcium disodium, 0.026 mM; and glucose, 11.1 mM. All experiments adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Thereafter, the fatty tissue surrounding the extraocular muscles was removed carefully. During preparation, the ocular globes were kept in cold (4°C) modified Krebs-Ringer bicarbonate solution constantly. The globes then were cannulated under a microscope (Wild & Leitz, Zürich, Switzerland) with a short polyethylene tube (diameter, 600 μm) through the common ophthalmic artery[17] and secured with two sutures (8-0 Virgin Silk, Braun-SSC AG, Emmenbrücke, Switzerland).

Experimental Procedures

Experimental Set-Up. After preparation, the ocular globe with the tube inserted in the common ophthalmic artery was connected immediately with a needle (diameter, 500 μm) to a Langendorff perfusion system (Hugo Sachs Elektronik KG), and perfused within one hour postmortem with previously filtered (filter paper circles: middle fine, crystalline; Schleicher & Schuell, Dassel, Germany) and oxygenated (95% O_2, 5% CO_2) Krebs solution, containing 0.5% albumin (control solution), at a constant pressure (80 cm H_2O) and temperature (37°C). Pilot experiments (N = 6) showed that this concentration of albumin was sufficient to prevent a significant increase in ophthalmic weight (indicating no relevant edema formation). In additional pilot studies, sodium fluorescein was injected, and perfusion of the whole ophthalmic microcirculation was visualized (N = 6). Hence, catheterization of the common ophthalmic arteries assured complete perfusion of the choroidal and retinal vascular system, as well as the small extraocular muscular arteries.[17] The perfused ocular globe, hanging with the cornea downward, was immersed in a double-jacketed glass bath (volume, 200 ml) filled with control solution (37°C). The overflow from the bath in which the eye globes were immersed corresponded to ophthalmic flow (milliliters per minute) and was collected during 1- to 5-minute periods. Experiments were started after at least 20 minutes equilibration; all drugs were added after this period. If sudden changes in flow (<80%, >180% of initial flow) occurred after equilibration, the tissues were discarded.

Assessment of Perfusion Rate. The pressure–flow relationship of the isolated perfused porcine eye was determined by measuring flow (milliliters per minute) after equilibration (10 minutes) at increasing levels of perfusion pressure ranging from 60 to 90 cm H_2O and back to 60 cm H_2O, with excellent reproducibility of flow at each level of pressure. A perfusion pressure of 80 cm H_2O was chosen for all subsequent experiments for two reasons: this perfusion pressure and the corresponding flow are in good agreement with the pressure and flow reported in the literature for humans[18,19]; and this perfusion pressure corresponds well with estimated mean arterial pressure values in the porcine ophthalmic circulation.[20] In time-control experiments, ophthalmic flow (at 80 cm H_2O) remained constant for as long as 120 minutes (N = 6). Hence, all experiments were performed within 120 minutes after perfusion was started.

Protocol. The effects of bradykinin (10^{-8} to 10^{-6} M), thromboxane analogue U-46619 (10^{-9} to 10^{-6} M), and L-NAME (10^{-6} to 10^{-4} M) were tested by adding cumulative doses of the drug to the next respective perfusate. In some experiments, the effects of bradykinin (10^{-9} to 10^{-8} M) were tested in the presence of U-46619 (10^{-8} M) to preconstrict the ophthalmic circulation, or L-NAME (10^{-4} M) to inhibit nitric oxide production.[14–16]

To study the effects of ETs, the design was modified: In pilot experiments with increasing concentrations of continuously administered ET, no stable responses could be obtained. Hence, different doses of ETs were administered as a single bolus (one dose per experiment and eye; changes in flow were measured for 30 minutes). So that the effects of ET-1 or endothelin-3 (ET-3) (10^{-12} to 10^{-10} moles) could be studied, the peptides were added as a 1-ml bolus to the perfusion solution. ET-3 was studied under control conditions and after prior infusion of indomethacin (10^{-5} M, 30 minutes, to prevent the formation of prostaglandins[21]).

To study the effect of ET_A receptor antagonist FR-139317, ET-1 (10^{-10} M) was applied in different eyes in the presence or absence of an infusion of FR-139317 (10^{-6} M, started 30 minutes before the addition of ET-1).

Drugs. Drugs were obtained from the following sources: bradykinin, indomethacin, and L-NAME from Sigma Chemical Company (St. Louis, MO); L-arginine from Fluka (Buchs, Switzerland); ET-1 and ET-3 from Novabiochem (Läufelfingen, Switzerland); FR 139317 from Fujisawa (Osaka, Japan); and U-46619 from Cayman Chemical Company (Ann Arbor, MI). All drugs were dissolved in distilled water—except U-46619 and indomethacin, which were dissolved in 10^{-5} M Na_2CO_3, and ET-1 and ET-3, which were dissolved in Krebs solution, containing 0.05% bovine serum albumin. All concentrations are expressed as final molar concentrations in the perfusate.

Statistical Analysis

Results are given as mean ± SEM. N equals the number of animals used for a given experimental protocol, in which only one eye per animal was used. The dose–response curves were fitted with the linear model (least-squares method) and compared with the analysis of variance (ANOVA) for repeated measurements. For bolus experiments, the area under the curve was compared with the unpaired Student's t-test or, when appropriate, with ANOVA followed by Sheffe's test. $P < 0.05$ was considered significant.

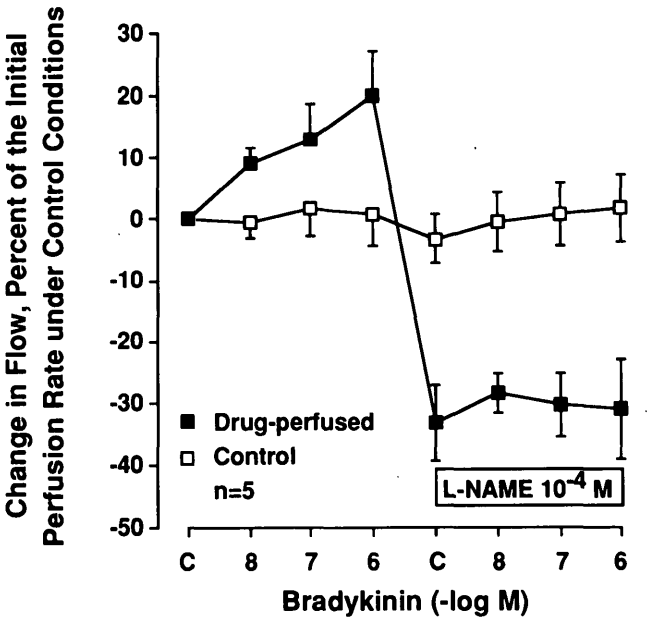

FIGURE 2. Effect of bradykinin on ophthalmic flow in the perfused porcine eye: Bradykinin caused concentration-dependent increases in ophthalmic flow ($P < 0.05$ versus time control). In the presence of L-NAME (10^{-4} M), flow was reduced significantly and addition of bradykinin (10^{-8} to 10^{-6} M) evoked no changes in flow. C: control flow after the effects of bradykinin (10^{-6} M) had faded.

RESULTS

Basal Release of Endothelium-Derived Nitric Oxide

The inhibitor of nitric oxide formation from L-NAME (10^{-6} to 10^{-4} M) evoked concentration-dependent decreases in ophthalmic flow (Fig. 1; $N = 5$; $P < 0.005$, ANOVA for repeated measurements). The time–control group remained stable. When L-arginine (10^{-4} M) was coinfused with L-NAME (10^{-4} M), the flow increased only slightly from 62% ± 6% to 66% ± 9% ($N = 5$, not significant [NS]).

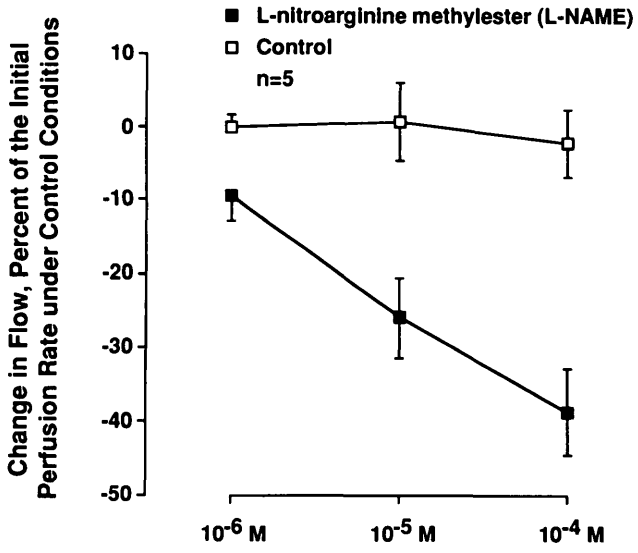

FIGURE 1. Effect of inhibition of nitric oxide production on ophthalmic flow in the perfused porcine eye: L-NAME (10^{-6} to 10^{-4} M) evoked concentration-dependent decreases in flow ($P < 0.005$ versus time control).

Stimulated Release of Endothelium-Derived Nitric Oxide

Bradykinin (10^{-8} to 10^{-6} M) caused concentration-dependent increases in flow (Fig. 2; $N = 5$; $P < 0.05$, ANOVA for repeated measurements).

In the presence of L-NAME (10^{-4} M), the flow was reduced by 32% ± 5% after 10 minutes as compared with control ($P < 0.05$ versus control). Also in the presence of L-NAME, bradykinin (10^{-8} to 10^{-6} M) evoked no changes in flow (Fig. 2; $N = 5$; NS versus L-NAME alone).

After infusion of the thromboxane analogue U-46619 (10^{-8} M), ophthalmic flow decreased from 3.5 ± 0.4 to 2.7 ± 0.3 ml/min ($N = 6$; $P < 0.01$). Coinfusion of bradykinin (10^{-8} to 10^{-6} M; with the continued presence of U-46619) increased the reduced flow in a concentration-dependent manner ($P < 0.005$, ANOVA for repeated measurements; Fig. 3).

Endothelins

Endothelin-1. The effect of ET-1 (10^{-12}, 10^{-11} M) on ophthalmic flow was biphasic, with early vasodila-

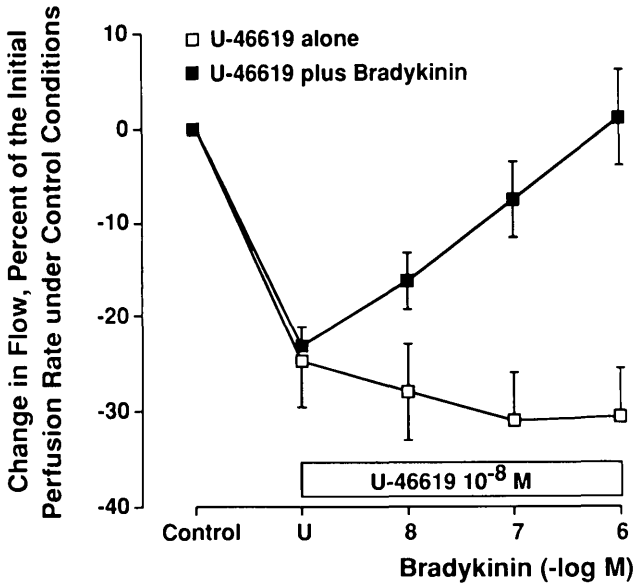

FIGURE 3. Effect of bradykinin on ophthalmic flow in the perfused porcine eye: After precontraction with a thromboxane analogue (U: U-46619), the addition of bradykinin (10^{-8} to 10^{-6} M) increased the reduced flow in a concentration-dependent manner ($P < 0.005$ versus time control).

tion after 1 to 2 minutes and late vasoconstriction after 5 to 7 minutes (Fig. 4, left panel, $N = 5$). At 10^{-12} M, ET-1 caused an increase in flow (24% ± 5%; $P < 0.05$ versus control), whereas 10^{-11} M ET-1 did not affect the response. At 10^{-10} M ET-1, only significant decreases in flow were observed. After prolonged exposure (15 to 20 minutes) to ET-1, significant vasoconstriction also occurred at 10^{-11} M ET-1, whereas 10^{-12} M caused no significant decrease in flow. The maximal reduction in flow averaged 4% ± 8% at 10^{-12} M (NS), 33% ± 7% at 10^{-11} M ($P < 0.01$ versus control), and 59% ± 5% at 10^{-10} M ($P < 0.001$ versus control; Fig. 4, left panel).

Endothelin-3. ET-3 (10^{-12} to 10^{-11} M) evoked an early vasodilator effect similar to that of ET-1 (Fig. 4, right panel; NS versus ET-1). The vasoconstrictor effect of ET-3 was much less pronounced than that of ET-1 at 10^{-11} M (19% ± 8%; $P < 0.05$ versus ET-1) and 10^{-10} M (34% ± 3%; $P < 0.001$ versus ET-1).

The vasodilator effects of ET-3 were prevented by pretreatment of the eye with indomethacin (10^{-5} M, to block the production of prostaglandins[21]; $P < 0.05$ versus control; Fig. 5). The vasoconstrictor effects of ET-3 were increased under these conditions ($P < 0.05$ for ET-3 at 10^{-12} M and 10^{-10} M; $P < 0.32$ for 10^{-11} M versus control).

ET_A Antagonist (FR-139317). The ET_A receptor antagonist FR-139317 (10^{-6} M) was infused for 30 minutes. No changes in flow occurred during infusion of the antagonist (NS versus control). The vasoconstrictor effect of ET-1 (10^{-10} M) was reduced significantly in the presence of FR-139317 (Fig. 6; area under the curve, $P < 0.001$ versus control). In the presence of the antagonist, ET-1 (10^{-10} M) caused only a small maximal decrease in flow (maximal decrease, 16% ± 4% after 4 minutes versus 60% ± 5% under control conditions; $P < 0.001$). The maximal effects of ET-1 occurred 4 to 6 minutes after injection of the peptide in the presence and absence of the ET_A antagonist.

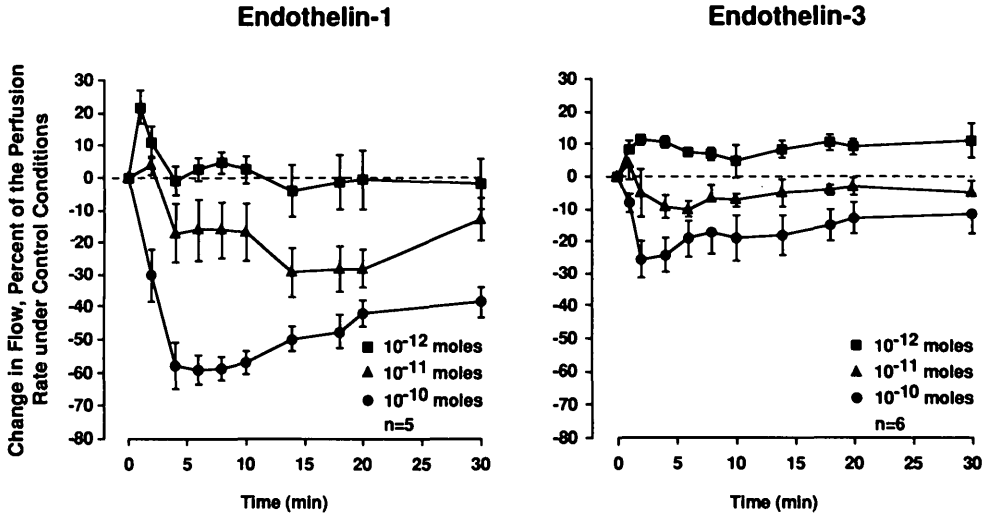

FIGURE 4. Effect of ET-1 (*left*) and ET-3 (*right*) on ophthalmic flow in the perfused porcine eye: ET-1 showed a biphasic component with early vasodilation after 1 to 2 minutes at 10^{-12} M and 10^{-11} M and late vasoconstriction. ET-1 (10^{-10} M) caused only significant decreases in flow. ET-3 evoked similar vasodilator effects as ET-1, but the vasoconstrictor effect was less pronounced ($P < 0.01$ versus ET-1). The different concentrations of ET have been studied in different eyes.

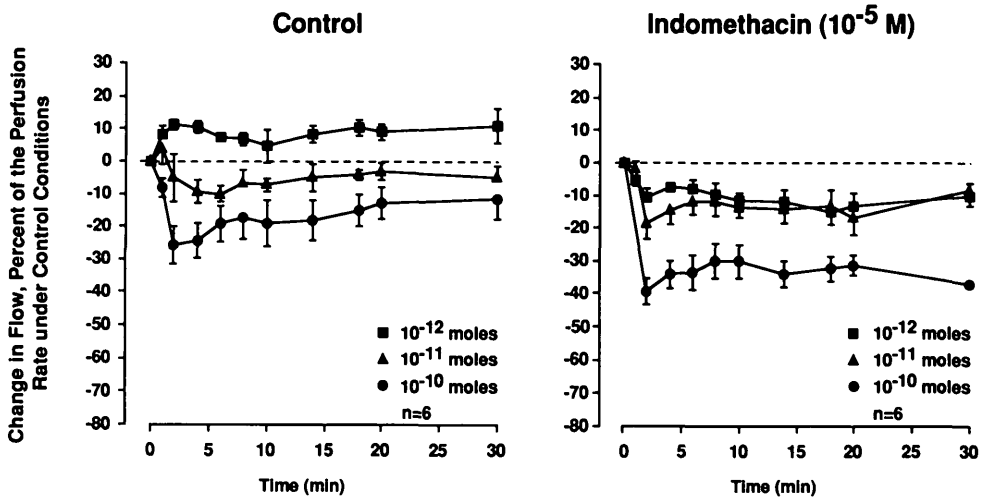

FIGURE 5. Effect of ET-3 on ophthalmic flow in the perfused porcine eye before (*left*) and after preinfusion of indomethacin for 30 minutes (*right*). Notice that inhibition of cyclooxygenase with indomethacin prevented vasodilation and increased the vasoconstrictor effects of ET-3 (area under the curve: $P < 0.05$ at 10^{-12} M and 10^{-10} M versus control, NS at $P < 10^{-11}$ M).

Contractile Responses to the Thromboxane Analogue (U-46619)

Addition of U-46619 (10^{-9} to 10^{-6} M) to the perfusion solution reduced flow in a concentration-dependent manner (Fig. 7, $N = 6$; $P < 0.001$, ANOVA for repeated measurements).

DISCUSSION

In the current study, a modified Langendorff perfusion system for the perfusion of intact porcine eyes[22,23] was used to delineate the role of endothelium-derived local mediators in the regulation of flow to the entire ophthalmic microcirculation. The results show the profound influence of nitric oxide on ophthalmic flow when it was released under basal conditions and after stimulation by bradykinin. Furthermore, ETs can cause vasodilation through the production of prostaglandins and profound vasoconstriction through the ET_A receptors.

Previous experiments have shown that endothelial mediators significantly regulate the vascular tone of

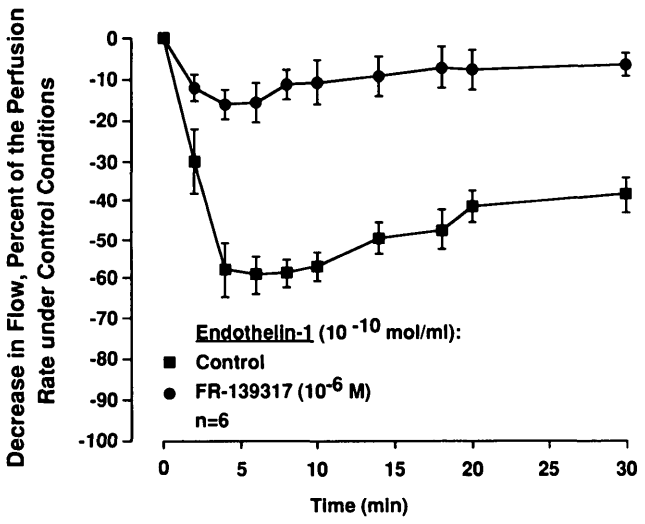

FIGURE 6. Effects of the ET_A receptor antagonist FR-139317 (10^{-6} M) on the response to ET-1 (10^{-10} M) in the perfused porcine eye: The decrease in ophthalmic flow with ET-1 (■) was reduced significantly in the presence of FR-139317 (●) (area under the curve: $P < 0.001$ versus control).

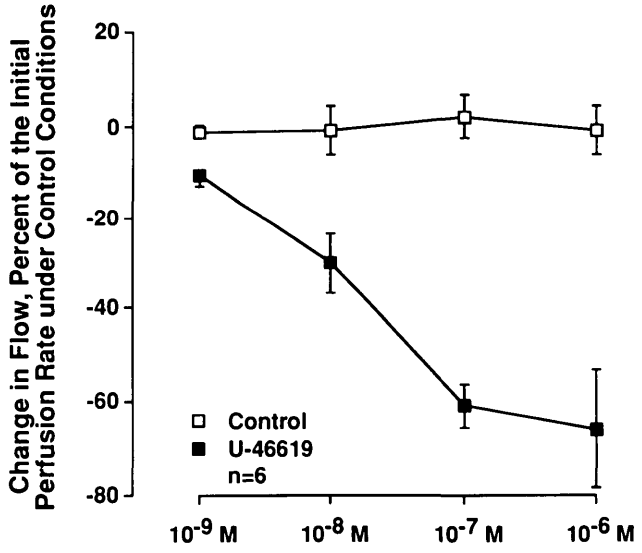

FIGURE 7. Effect of the thromboxane analogue U-46619 on ophthalmic flow in the perfused porcine eye: U-46619 (10^{-9} to 10^{-6} M) evoked concentration-dependent and significant decreases in ophthalmic flow ($P < 0.0001$ versus time control).

extraocular arteries.[7,8,10,11] The newly developed perfusion system for the intact porcine eye allowed testing of the hypothesis regarding whether these mediators can affect flow to the ophthalmic microcirculation. With a modified Langendorff perfusion system,[13] porcine eyes could be perfused with modified Krebs-Ringer Henseleit solution, provided albumin was present to prevent edema formation. Under these conditions, ophthalmic flow exhibited a direct relation to perfusion pressure (indicating the absence of autoregulation in the entire ophthalmic microcirculation).[24–26] For at least 2 hours, flow remained stable at a given perfusion pressure under these conditions, allowing appropriate experiments for studying the regulatory mechanisms of flow to the ophthalmic microcirculation during that period. The perfusion pressure selected in these experiments corresponds well with the mean arterial pressure in that part of the circulation.[19] Because small arteries with a diameter of 100 μm or less contribute the most to peripheral vascular resistance, the responses observed under these experimental conditions in the intact porcine eye must occur in intraocular small arteries and arterioles.

Nitric oxide is produced from the amino acid L-arginine through the activity of a recently cloned constitutive form of the brain isomer of nitric oxide synthase.[27] Experiments in isolated porcine ophthalmic arteries of the extraocular circulation suggested a basal formation of nitric oxide because the inhibitor of nitric oxide formation, L-N^G-monomethyl arginine, caused endothelium-dependent contractions.[10] The experiments using the perfused porcine eye showed that L-NAME, a potent inhibitor of nitric oxide production, caused significant decreases in ophthalmic flow, averaging almost 40% of the flow rate observed under control conditions. This indicates that the ophthalmic circulation is in a state of continuous vasodilation through the basal production of nitric oxide. Hence, the endogenous nitrovasodilator nitric oxide may contribute significantly to the maintenance of ophthalmic flow.

Bradykinin is an agonist for the release of endothelial mediators such as nitric oxide,[1,3] prostacyclin,[28] and the putative endothelium-derived hyperpolarizing factor.[1,4,29] In the perfused porcine eye, bradykinin caused concentration-dependent increases in ophthalmic flow under control conditions and after preconstriction of the ophthalmic circulation by a thromboxane analog. After inhibition of nitric oxide production by L-NAME, the vasodilator effects of bradykinin were prevented fully, indicating that in the ophthalmic microcirculation, nitric oxide is the primary mediator of the vasodilator effects of the kinin. This contrasts with extraocular arteries, in which inhibitors of nitric oxide production only shifted the concentration–response curve to bradykinin to the right but did not prevent the maximal effects of the endothelium-dependent vasodilator.[8,10] Because the effects of bradykinin were observed under control conditions and after precontraction of the ophthalmic circulation by the thromboxane analog U46619, the inhibitory effects of L-NAME cannot be attributed to its effect on ophthalmic flow (ie, contraction of the ophthalmic microvessels) but must reflect specific inhibition of the L-arginine/nitric oxide pathway.

The newly discovered 21-amino acid peptide ET has complex cardiovascular actions.[1,5,30] Although in most isolated blood vessels the peptide only causes vasoconstriction, ET induces a transient decrease in blood pressure in vivo in experimental animals.[5,31] Similar observations have been made with intraarterial infusion of ET-1 in the human forearm circulation of normal subjects.[9] The current study shows that, although ET only causes vasoconstriction in isolated extraocular ophthalmic arteries, in the perfused ophthalmic microcirculation (in which ET is infused intraluminally and thereby first reacts with endothelial cells), the peptide evokes vasodilation at lower concentrations, followed by a pronounced contraction at higher concentrations. The fact that the vasoconstrictor effect only reached its maximum after 5 to 7 minutes indicates that the peptide had to diffuse through the intima toward vascular smooth muscle. The ET concentrations causing significant decreases in flow (ie, 10^{-11} to 10^{-10} moles) were remarkably lower than those previously found to exert vasoconstriction in extraocular arteries of pigs and humans.[7,10] This indicates that intraocular vessels are more sensitive to the effects of the peptide; even in extraocular arteries, the sensitivity to ET increases as the size of the blood vessel decreases.[8] The definition of the exact anatomic site of action of endothelial mediators within the ophthalmic circulation will await additional experiments visualizing arteries of a different size and studies of receptor distribution within the eye. A predominant action of ET in the venous circulation can be excluded, however, because this (with fixed perfusion pressure) would have been associated with an increased capillary pressure and edema formation, a phenomenon that could not be observed under our experimental conditions.

Two main ET receptors have been cloned recently: ET_A[32] and ET_B receptors.[33] The two receptors are expressed differently on vascular smooth muscle and the endothelium, and they have different affinities to the isoforms of ET.[34] ET-1 has a particular affinity for the ET_A receptor, whereas the ET_B receptor binds ET-1 and ET-3 with similar affinity. It appears that endothelial cells primarily express ET_B receptors, whereas the ET_A receptor is the predominant form in vascular smooth muscle cells.[30,32–34] The current experiments in the perfused porcine ophthalmic circula-

tion are in line with that concept. ET-1 and ET-3 caused similar vasodilation, indicating that vasodilation is mediated by the endothelial ET_B receptor. The fact that indomethacin, an inhibitor of cyclooxygenase, prevented vasodilation and augmented the vasoconstrictor effects of ET-3 strongly suggests that activation of the ET_B receptors on endothelial cells is linked to the formation of vasodilator prostaglandins, most likely prostacyclin, which mediate the vasodilator effects of ET and reduce its vasoconstrictor action on vascular smooth muscle.[35] Similar observations have been made in perfused rat mesenteric resistance arteries[36] and in the forearm circulation of normal humans.[9,37] On the other hand, the vasoconstrictor effects of ET-1 on vascular smooth muscle cells must be mediated by the ET_A receptor. The specific ET_A receptor antagonist FR-139317 significantly reduced the vasoconstrictor effects of ET-1. The fact that, even in the presence of high concentrations of the ET_A antagonist, ET-1 still caused a small but significant decrease in ophthalmic flow indicates that, in addition to the predominant ET_A receptor, an ET_B receptor on vascular smooth muscle[34] also may contribute to the vasoconstrictor effects of the peptide in the ophthalmic microcirculation.

Therefore, the current study strongly suggests that endothelial mediators are involved significantly in the local regulation of ophthalmic flow under physiologic conditions. On the other hand, alterations in the synthesis, release, or action of these mediators may alter flow to the ophthalmic microcirculation profoundly and contribute significantly to disease states associated with alterations in local blood flow in the eye. In particular, hypertension[30,38,39] and diabetes[40,41] are associated with significant alterations in endothelial function, decreased formation of endothelium-derived relaxing factors, and enhanced production of endothelium-derived contracting factors.[38] Furthermore, disease states with increased vasoconstrictor responses of the ophthalmic microcirculation, such as low-tension glaucoma,[42–45] may be related at least in part to alterations in the endothelium-dependent regulation of ophthalmic flow. Finally, drugs designed to improve ophthalmic flow partially may affect local vascular tone through their effects on the production or action of endothelial mediators. ET_A receptor antagonists could be useful tools to inhibit the profound vasoconstrictor effects of ET in the ophthalmic circulation, particularly in disease states associated with increased production of the peptide.

Key Words

bradykinin, endothelin-1, FR-139317, Langendorff perfusion system, nitric oxide, U-46619

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