A Retinal-Derived Relaxing Factor Mediates the Hypoxic Vasodilation of Retinal Arteries

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Purpose. To investigate the mechanisms involved in hypoxic vasodilation using an in vitro setup.

Methods. Retinal arteries with and without retinal tissue were mounted on a wire myograph. The segments were contracted with prostaglandin (PGF$_{2\alpha}$) (30 $\mu$M) or 120 mM K$^+$. Hypoxia was induced by replacement of O$_2$ by N$_2$ in the gas used to bubble the Krebs–Ringer bicarbonate organ bath solution.

Results. Hypoxia induced complete relaxation of preparations with adherent retinal tissue contracted with PGF$_{2\alpha}$. Preparations without retinal tissue were not affected by the change in oxygenation. When the retinal arteries were contracted with 120 mM K$^+$, hypoxia no longer induced relaxation of the preparation with adherent retinal tissue. The presence of an NO-synthase inhibitor (L-NA, 0.1 mM), a cyclooxygenase inhibitor (indomethacin, 50 $\mu$M), or an adenosine receptor antagonist (8-sulphophenyltheophylline, 1 mM) did not affect hypoxic vasodilation. Excitatory amino acids and lactate had no or only a limited effect on the PGF$_{2\alpha}$-induced contraction and are therefore unlikely mediators of hypoxic vasodilation. HCl (10 mM) reduced the pH to 6.1 ± 0.08 ($n = 4$) and induced a pronounced but transient relaxation of the retinal artery contracted with PGF$_{2\alpha}$ or 120 mM K$^+$, whereas hypoxia induced relaxation of the retinal artery contracted with PGF$_{2\alpha}$ only in the presence of adherent retinal tissue.

Conclusions. Adherent retinal tissue mediates the hypoxic vasodilatation of bovine retinal arteries in vitro. Neither NO, prostanoids, adenosine, excitatory amino acids lactate or changes in pH seem to be involved in this hypoxic response. (Invest Ophthalmol Vis Sci. 2000;41:3555–3560)

In most vascular beds, but not in the pulmonary circulation, hypoxia elicits a vasodilation, reflecting a physiological regulatory process that adapts blood flow to the metabolic needs of the tissues. Hypoxia-induced vasodilation may result from direct effects on the blood vessel wall as well as from indirect effects, such as the release of metabolic factors from the surrounding tissue. Blood flow in the retina is known to be strongly influenced by changes in arterial oxygen tension. Many in vivo studies have shown vasodilation of the retinal arteries during hypoxia and constriction during hyperoxia. However, the mechanisms involved in the adaptations of retinal vascular tone to changes in PaO$_2$ are not established. In vitro data are rare, and interpretation of in vivo data is difficult because they do not allow differentiation between direct and indirect responses of the retinal arteries. In addition, systemic changes and the presence of vasoactive substances in the circulating blood may confound the interpretation of in vivo observations.

The difficulties involved in identifying the mechanisms responsible for hypoxic vasodilation led us to investigate the influence of hypoxia on retinal arteries in the presence and absence of retinal tissue. A previous study, in which this innovative technique of incomplete removal of the perivascular tissue was used, has demonstrated that a diffusible factor released by retinal tissue influences retinal arterial tone in vitro. The present study now demonstrates that this or a similar retina-derived relaxing factor may be responsible for hypoxic vasodilation.

Materials and Methods

Bovine eyes, obtained from a local abattoir, were enucleated within a half hour after the animals died and were transported to the laboratory in ice-cold Krebs–Ringer bicarbonate (KRB) solution. The anterior segment and the vitreous were removed, and the eye cup was placed in cold and oxygenated KRB solution for further preparation. A segment located between the optic disc and the first branch of the most prominent artery was carefully excised with surrounding retinal tissue.

The arterial segments were transferred to an isometric myograph (model 500 A; JP Trading, Aarhus, Denmark) containing 10 ml KRB solution. Two stainless steel wires were guided through the lumen of the vessels. One wire was fixed to a force-displacement transducer, and the other was connected to a micrometer. After the first wire was fixed, the retinal tissue was either completely removed, or the excess tissue was trimmed away so that only a small strip of retinal tissue remained attached to the artery. The segments were equilibrated in oxygenated (5% CO$_2$ in O$_2$) KRB solution at 37°C (pH 7.4) for 30 minutes before the vessels were stretched to their optimal lumen diameter (212.5 ± 0.61 $\mu$m, $n = 116$). At the start of each experiment, the arteries were repeatedly activated...
with 120 mM K⁺. Maximal contractility was assessed by stimulating the arteries with a 120 mM K⁺ solution to which PGF₂α 30 μM and serotonin 10 μM was added.

During the experiments, the KRB solution was bubbled with a mixture of oxygen (O₂), nitrogen (N₂), and carbon dioxide (CO₂). Throughout the experiments, the CO₂ content of the gas mixture remained fixed at 5%. The O₂ and N₂ content of the gas mixture varied between 95% and 0%. Hypoxia was induced by gradually reducing the O₂ content and increasing the N₂ content of the gas mixture. Oxygen tension (P O₂), carbon dioxide tension (P CO₂), and pH were analyzed on samples taken from the organ bath during the experiments. The samples were analyzed with a blood gas analysis system (model ABL-5; Radiometer Medical, Copenhagen, Denmark).

The experiments were performed using a KRB solution with the following composition (mM): 135 NaCl, 5 KCl, 20 NaHCO₃, 10 glucose, 2.5 CaCl₂, 1.3 MgSO₄, 1.2 KH₂PO₄, and 0.026 EDTA. Modified KRB solution containing 120 mM K⁺ was prepared by equimolar replacement of NaCl with KCl. Serotonin (5-hydroxytryptamine), indomethacin, N-ω-nitro-l-arginine (l-NA), taurine, l-lactic acid, isocitric acid, sodium cyanide, 8(p-sulphophenyl)-theophylline, and adenosine were all purchased from Sigma (St. Louis, MO). Prostaglandin (PG)F₂α, (dipropionyl trometamol, Dinolytic) was obtained from Upjohn (Puurs, Belgium). Stock solutions were made in water, except for indomethacin, which was dissolved in ethanol.

Statistical significance was evaluated using a Student’s t-test for paired and unpaired observations; n indicates the number of preparations tested.

RESULTS

Effect of Acute Hypoxia on Retinal Arteries with and without Adherent Retinal Tissue

The effect of acute hypoxia was studied on isolated bovine retinal arteries, both in the presence and absence of adherent retinal tissue. An original recording is shown in Figure 1. After PGF₂α (30 μM) induced a steady contraction, acute hypoxia was induced by an abrupt switch from an oxygenating gas (95% O₂-5% CO₂) to gas without oxygen (95% N₂-5% CO₂). This resulted in a complete loss of tone of the preparation containing retinal tissue. Reoxygenation of the solution rapidly restored the contraction. In contrast, the preparation without retinal tissue was little affected by the change in oxygenation. These initial observations were confirmed by additional experiments. Acute hypoxia consistently induced a pronounced relaxation (92.7% ± 7.53%, n = 9) of all the preparations with adherent retinal tissue. In contrast, none of the retinal arteries without retinal tissue (n = 6) relaxed during hypoxia.

Influence of l-NA and Indomethacin on the Hypoxic Response

The NO-synthase inhibitor l-NA and the cyclooxygenase inhibitor indomethacin were used to exclude the potential influence of NO or cyclooxygenase metabolites released from the hypoxic retinal tissue. The effect of hypoxia on the PGF₂α (30 μM)-induced contraction was studied on retinal arteries with adherent retinal tissue in the presence and absence of l-NA (0.1 mM) or indomethacin (10 μM and 50 μM).
tions with adherent retinal tissue, whereas arteries without retinal tissue relaxed only 12.3% ± 3.63% (n = 7).

In arteries with adherent tissue precontracted with 120 mM K+ changes in oxygen tension no longer induced a pronounced relaxation (Fig. 2B). The response was no longer significantly different from the response of preparations without retinal tissue; 23.2% ± 9.83% (n = 7) relaxation compared with 19.1% ± 8.61% (n = 7).

**Effect of Detached Retinal Tissue on Isolated Retinal Arteries during Hypoxia**

This series of experiments was performed to find out whether the hypoxia-induced relaxation was mediated by a diffusible factor released by the retina. A ring segment of a retinal artery, carefully cleaned of all retinal tissue, was mounted for isometric tension recording and contracted with PGF$_{2\alpha}$ in the presence of indomethacin and l-NA. A piece of retinal tissue was then placed in proximity with the retinal artery. The position and the size of the piece of retinal tissue was chosen so that application of the retinal tissue induced only weak relaxation (due to the continuous release of a relaxing factor). This was very difficult. Only 3 of 11 experiments were successful. Five attempts failed because application of the retinal tissue induced a full relaxation, and three attempts failed because the piece of retinal tissue was too small to induce any relaxation.

A recording of a successful experiment is shown in Fig. 3. After careful positioning, application of a piece of retinal tissue induced a slight relaxation of the retinal artery (4.5% ± 2.39%, n = 3). Ten to 15 minutes later, hypoxia was induced (95% N$_2$-5% CO$_2$), which led to nearly complete relaxation (78.1% ± 21.93% relaxation, n = 3). Reoxygenating the KRB solution restored the contraction to 93.9% ± 3.07% (n = 3) of its initial tone. A small additional contraction (to 116% ± 2.25% of the preexisting tone, n = 3) was seen when the piece of retinal tissue was finally removed. In the absence of retinal tissue, hypoxia had no effect on the tone of these preparations.

**Effect of Inhibition of ATP Formation on Retinal Arterial Tone**

In this series of experiments the effect of adenosine triphosphate (ATP) exhaustion on retinal arterial tone was investigated. During these experiments, the organ bath was continuously bubbled with 95% O$_2$-5% CO$_2$. Retinal arterial segments with and without adherent retinal tissue were contracted with PGF$_{2\alpha}$ in the presence of indomethacin (50 μM) and l-NA (0.1 mM). ATP formation was inhibited by either blocking the glycolysis with iodoacetate (1 mM to 0.1 mM) or by blocking the oxidative phosphorylation with sodium cyanide (1 mM to 1 mM). Iodoacetate (0.1 mM) induced complete relaxation (100%, n = 4, Fig. 4A) of the preparations with adherent retinal tissue, whereas it only moderately relaxed the arteries without retinal tissue (22.5% ± 15.62%, n = 6). Cumulative addition of sodium-cyanide to the organ bath induced a concentration-dependent relaxation of arteries with adherent tissue and induced 100% relaxation at the highest concentration (n = 5, Fig. 4B). However, sodium cyanide also had a pronounced effect (68.3% ± 7.52%, n = 6) on preparations without adherent tissue relaxation at the highest concentration.

**Influence of Hypoxic Metabolites on Retinal Arterial Tone**

The results of the previous experiments suggest that hypoxia promotes the release of vasodilator molecules from the retina. Potassium ions, hydrogen ions, lactate, excitatory amino acids, and adenosine have all been suggested as possible mediators of the hypoxic vasodilatation. This series of experiments investigated the effect of these molecules on retinal arteries cleaned from all surrounding retinal tissue and contracted with 30 μM PGF$_{2\alpha}$.

Cumulative addition of small amounts of potassium (KCl, 2.5–10 mM, n = 8) failed to induce a substantial relaxation of
the retinal artery. Taurine (10 nM to 1 mM, n = 4) was also without any effect and lactate (0.1 nM to 1 mM) induced only a slight and transient relaxation (20.2% ± 7.67%, n = 9) at the highest concentration. Important changes of pH were needed to induce relaxation similar to the hypoxic relaxation. HCl (10 mM) reduced the pH from 7.4 ± 0.00 (n = 4) to 6.1 ± 0.08 (n = 4) and induced 81.0% ± 2.89% (n = 7) relaxation of vessels contracted with PGF$_{2\alpha}$ and 84.1% ± 15.05% (n = 7) relaxation of arteries contracted with 120 mM K$^+$. These relaxations were transient, and the restoration of contractile tone was accompanied by partial recovery of the pH (6.95 ± 0.015; n = 4).

**DISCUSSION**

The main finding of the present study is the clear demonstration of an indirect vasodilatory influence by a diffusible retinal factor in hypoxia on bovine retinal arteries in vitro. In vitro studies on isolated arterial rings usually reveal only the direct effects of hypoxia on the arterial wall. This can be either a contraction, a relaxation, or a biphasic response. In the present study, no direct effects of hypoxia could be demonstrated on the tone of bovine retinal arteries. The tone of a particular artery can, however, also be influenced by the surrounding tissue. A previous study in which we found that surrounding tissue has a pronounced influence on the tone of isolated retinal arteries led to the idea to study the potential role of adherent retinal tissue in response to hypoxia. We were surprised to find that hypoxia induced a pronounced relaxation of preparations with retinal tissue but not of preparations without the tissue.

These retina-mediated responses do not require direct contact between the vascular and retinal cells but are mediated by a diffusible factor from the retina. This is most clearly demonstrated in the experiments in which detached retinal tissue is brought in close apposition with a cleaned retinal artery. Although the cleaned retinal artery does not relax in response to hypoxia, the vertical scale shows the active force in millinewtons; horizontal scale shows time.
response to hypoxia, it relaxes when retinal tissue is in proximity.

The release or the effect of this retinal factor is possibly triggered by reduced ATP levels rather than by hypoxia itself. This is suggested by the fact that under hyperoxic conditions, inhibition of ATP formation with iodoacetate or sodium cyanide induces complete relaxation of the retinal arteries with adherent retinal tissue in contrast to the partial relaxation seen in preparations without retinal tissue.

Several mediators have been proposed in relation to the effects of hypoxia. Hydrogen ions, perhaps in the form of lactate, and potassium ions were among the first molecules suggested to mediate hypoxic vasodilation in the cerebral circulation. Measurements of perivascular pH and potassium concentration however demonstrated that the changes during hypoxia were too small and too slow to fully explain the hypoxic cerebral vasodilation. Also in the present study, changes in pH and potassium concentration did not seem to mediate hypoxic vasodilation. Small increases in the potassium concentration had no effect on the retinal arterial tone. Measurements of pH did not reveal any change during hypoxia. To obtain a pH-induced relaxation similar to the hypoxic relaxation, the pH of the organ bath solution had to be reduced far below physiological levels (6.1 ± 0.08; n = 4). Moreover, changes in pH also induced relaxation of the retinal artery contracted with 120 mM K⁺, whereas hypoxia-induced relaxation were suppressed in the presence of 120 mM K⁺.

Changes in pH are sometimes attributed to the release of lactate from hypoxic tissue. The relaxations induced by lactate, however, are not necessarily due to reductions in extracellular pH. Brazitikos et al. demonstrated that microinjections of both acid and neutral solutions of L-lactate in the preretinal tissue induced segmental retinal vasodilation and that microinjection of L-lactate caused acidification but no vasodilation. Although these observations apparently support the involvement of lactate in the retinal hypoxic vasodilation, the concentration needed to induce strong segmental relaxation was much higher than that measured in the normal retina, a fact that was acknowledged by the authors. In our experiments, high concentrations of lactate induced only weak transient relaxation. Moreover, iodoacetate, which inhibits glycolysis and thus pyruvate and lactate formation, induced complete relaxation of the preparation with adherent retinal tissue. This excludes the involvement of lactate at least in the relaxation induced by metabolic inhibitors.

The possibility that hypoxia may act on the retina to promote the release of vasodilator prostaglandins was explored by Pournaras et al. on miniature pigs. Although these authors demonstrated that indomethacin completely blocked the hypercapnic vasodilation, inhibition of hypoxic vasodilation was less convincing. During our experiments, indomethacin failed to inhibit hypoxic vasodilation, excluding the involvement of cyclooxygenase metabolites released from the retinal tissue.

Similarly, addition of L-NA, an NO synthase inhibitor, enhanced the prostaglandin-induced contraction but failed to inhibit the hypoxic relaxation. This confirms the results of an in vivo study in which inhibition of NO-synthase induced a prominent arterial constriction but had no influence on the autoregulatory responses to hypoxia. These results suggest that despite a sustained release of NO, autoregulatory dilatory responses to hypoxia occur independent of NO synthesis.

Recently, much attention has been given to the release of excitatory amino acids (e.g., γ-aminobutyric acid, glutamate, and glycine) during hypoxia and ischemia. Excitatory amino acids, however, appear to have little direct effect on retinovas-
cular tone. Glutamate, glycine, and γ-aminobutyric acid were found to have no effect on the prostaglandin-induced contraction. In the present study, taurine also failed to relax the retinal arteries. Moreover, the release of these compounds is probably more important during the first minutes of reperfusion or reoxygenation than during the actual onset of hypoxia. Adenosine is often considered as the most likely mediator of hypoxic vasodilation. Hypoxia promotes the release of adenosine and inhibition of the adenosine receptor attenuates hypoxic vasodilation in the retina of the miniature pig. Our results, however, do not support the involvement of adenosine in hypoxic vasodilation. The adenosine receptor antagonist 8-sulphonylthephyllyline significantly blocked the adenosine-induced relaxation but was without any effect on the relaxation induced by hypoxia. Moreover, adenosine (in contrast to hypoxia) was equally effective in relaxing the prostaglandin-induced contractions as the 120-mM K⁺-induced contractions. On the basis of these results, adenosine can be excluded as mediator of the retinal hypoxic vasodilation.

As yet, no study has been able to fully explain the response of the retinal circulation to hypoxia. The present study reinforces the view that an unknown factor probably participates in the hypoxic response. Evidence for the existence of an unknown retina-derived relaxing factor has recently been reported. It is conceivable that the hypoxic relaxation is mediated by this retinal relaxing factor. However, the possibilities of testing this hypothesis are limited, because the identity of this factor is not known. It is known, however, that a contraction induced by 120 mM K⁺ remains almost unaffected by the retinal relaxing factor. Similarly, hypoxia does not induce a marked relaxation of the retinal artery contracted with 120 mM K⁺.

From this study, it can be concluded that retinal arterial tone adapts to metabolic needs by an indirect effect mediated by an as yet unknown mediator released from retinal tissue.

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References