Oxygen Modulation of Guanylate Cyclase-Mediated Retinal Pericyte Relaxations With 3-Morpholino-Sydnonimine and Atrial Natriuretic Peptide

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Purpose. This study explores at which level of the guanylate cyclase pathway oxygen modulates retinal pericyte relaxation induced by nitric oxide (NO).

Methods. Bovine retinal microvascular pericytes were grown on silicone. On silicone, pericyte contractile tone induces wrinkles. Drug-induced relaxation was quantified as a reduced number of wrinkles after exposure to 3-morpholino-sydnonimine (SIN-1) or atrial natriuretic peptide (ANP) in the absence or in the presence of either 0.3 μM methylene blue (MB), a guanylate cyclase inhibitor, or 10 μM hemoglobin, a NO scavenger; and under 100% oxygen (hyperoxia), ambient air (normoxia), or 100% nitrogen (hypoxia).

Results. Pericytes were relaxed with SIN-1 and ANP in a concentration-dependent manner (EC50: 0.1 μM and 0.01 μM, respectively). Relaxations induced by SIN-1 or ANP were inhibited (P < 0.001) by MB, whereas hemoglobin inhibited only SIN-1 relaxations (P < 0.001). Relaxations induced by SIN-1, but not by ANP were increased (P < 0.001) under hypoxia and decreased (P = 0.002) under hyperoxia.

Conclusions. SIN-1 and ANP relax pericytes through the activation of guanylate cyclase (inhibited by MB), but only SIN-1 through an extracellular release of NO (inhibited by hemoglobin). That oxygen only modulates pericyte relaxations induced by SIN-1 (NO-mediated) but not those induced by ANP suggests that an interaction between oxygen and NO might participate in the capillary network’s blood-flow modulation according to local tissue oxygen tension.

Pericytes are the main contractile cells of capillaries. Just as vascular smooth muscle cells modify the diameter of larger vessels, pericytes may modify the diameter of retinal capillaries and thus potentially modulate blood flow within the retinal capillary network.
oxygen affects pericyte relaxation when guanylate cyclase is stimulated by NO, but not when it is stimulated by another activator. For this purpose, we exposed the cells to atrial natriuretic peptide (ANP), known to activate directly a membrane-bound guanylate cyclase, and compared the results with the effect of 3-morpholino-sydnonimine (SIN-1), a donor of NO, which activates a cytosolic-soluble guanylate cyclase.

The effects of hyperoxia, normoxia, and hypoxia on pericyte relaxations with SIN-1 and ANP were compared.

**MATERIAL AND METHODS**

**Pericyte Culture**

As described earlier, pericytes were cultured from bovine retinas. In agreement with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, 30 to 40 bovine eyes were obtained from Aries Scientific (Richardson, TX) and shipped to our laboratory on ice by an overnight express service. Retinas were then dissected free, minced, and incubated for 1 hour in phosphate-buffered saline (PBS) containing 0.2% collagenase and 0.2% bovine serum albumin. After filtration through a Spectramesh (Spectrum Medical Industries, Terminal Annex, LA) cells were retrieved and rinsed by serial centrifugation (800g), resuspended, and cultured in 75-cm² square flasks in Dulbecco’s modified Eagle’s medium (DMEM), containing 10% fetal bovine serum, 50 mg/ml fungizone, and 1.25 µg/ml gentamicin.

**Pericyte Identification**

The cells cultured had the typical morphologic appearance of pericytes and stained positively for the antiganglioside antibody 3G5, which marks pericytes but not vascular smooth muscle cells. These cultured pericytes did not express the glial fibrillary acidic protein found in cultured astrocytes, they lacked the retinal pigment epithelium’s ability to phagocytose rod outer segments, and they also lacked the ability to take up fluorescein-labeled low-density lipoprotein (LDL)—a characteristic feature of endothelial cells, which are the main potential contaminants of pericyte cultures obtained from the retina. After demonstrating these general features in the pericytes we cultured, we ensured the identification of cells and the absence of endothelium for each lot of cells used in our experiments by documenting binding of the antiganglioside 3G5 and the absence of cells that take up low-density lipoprotein, respectively.

**Silicone Membrane Cell Culture Substrate**

First and second passages of bovine retinal pericytes were grown in DMEM for 3 to 7 days on a thin silicone membrane coating the bottom of a petri dish (dimethylpolysiloxane, 60,000 centipoise viscosity silicone fluid surface hardened by flame). When these contractile cells attach to the silicone membrane and grow on its surface, they place the underlying elastic silicone membrane under tension, which results in a series of small wrinkles that can be visualized with a phase-contrast inverted microscope (Zeiss, Oberkochen, Germany). To enhance the reproducibility of this method, every experiment was matched with a control conducted with the same batch of cells and the same batch of silicone membranes.

**Observation of Pericytes**

To enable observation of the cells and the wrinkles, the whole petri dish was placed on the stage of the microscope, enclosed in a transparent chamber that maintained the cells at a constant 37°C temperature while allowing multiple fluid exchanges by a suction-perfusion system. Within the chamber the room-air atmosphere could be saturated by insulating oxygen or nitrogen into the chamber under positive pressure. The microscope was connected to a video camera system (Panasonic, Osaka, Japan) and a printer (Mitsubishi, Cypress, CA), and the number of wrinkles appearing on the prints were tabulated with a Zeiss Videoplan 2 Image Analysis System (Kontron, Eching, Germany). Fields of view were selected in which the cells were sufficiently separated, not in a cluster, so that the wrinkles related to individual cells could be easily analyzed (1–5 cells per field of view).

**Experimental Conditions**

Once the petri dish had been placed in the chamber, the DMEM solution was immediately replaced with a Hepes (pH 7.4)-buffered solution (NaCl, 140 mM; KCl, 5 mM; CaCl₂, 1 mM; MgCl₂, 1.5 mM; Hepes sodium salt, 5 mM; Hepes sodium salt, 5 mM; glucose, 10 mM), a solution that was then used in all subsequent experimental manipulations. After 20 minutes of adaptation in this solution, the pericytes, which tend to have a high level of basal contractile tone, were exposed to SIN-1 or to ANP in the presence or in the absence of methylene blue (MB) or hemoglobin, or under different conditions of oxygenation. In all cases, control runs alternated with experimental runs.

**Hemoglobin Preparation**

Bovine hemoglobin (crystallized, dialyzed, and lyophilized) was placed in PBS (pH 7.4), converted to oxymoglobin with sodium hydrosulfite, redialized, and deoxygenated by bubbling with 100% nitrogen. Small aliquots were stored at −80°C until thawed for use. Low concentrations of methylhemoglobin were assured by spectrophotometric assay at the time of the experiment.
Oxygen and Pericyte Relaxations With SIN-1 and ANP

FIGURE 1. Relaxing effect of 3-morpholino-sydnonimine (SIN-1) and atrial natriuretic peptide (ANP) on bovine retinal microvascular pericytes. Unpaired Student’s t-test: **P < 0.01, ***P < 0.001. □ = time-control experiments.

Hypoxic and Hyperoxic Conditions

Before its infusion into the petri dish the Hepes-buffered solution was bubbled with 100% nitrogen or 100% oxygen for 10 minutes. Drugs were then added to the Hepes-buffered solution and immediately infused into the petri dish. At the same time, the atmosphere surrounding the petri dish within the closed transparent chamber was immediately saturated with either 100% nitrogen or 100% oxygen. Samples of the solution, taken just before and 10 and 20 minutes after its infusion into the petri dish and analyzed with a blood gas analyzer, showed that the partial pressure of oxygen in the petri dish solution was always lower than 4% when bubbled with 100% nitrogen and higher than 97% when bubbled with 100% oxygen.

Assessment of the Data

Measurements were taken at 5 and 10 minutes after drug exposure. There was no difference between wrinkle counts at 5 and 10 minutes. Only the data taken at 10 minutes were used for analysis. Relaxations of pericytes were quantified by counting the decrease in the number of wrinkles from that observed in basal conditions (after 20 minutes of equilibration in the Hepes-buffered solution). Results have been expressed as a percentage of the number of wrinkles lost, and n corresponds to the number of experiments conducted (one preparation per experiment). Data are presented as the mean and the standard error of the mean (mean ± SEM) and were compared by unpaired Student's t-test with P < 0.05 considered significant.

Drugs and Substances

Atrial natriuretic peptide, collagenase, fungizone, Hepes, dimethylpolysiloxane, and bovine hemoglobin were obtained from Sigma Chemical (St. Louis, MO); SIN-1, the active metabolite of molsidomine, was a present from Hoechst Pharmaceuticals (Paris, France); bovine serum albumin, DMEM, fetal bovine serum, and gentamicin were purchased at Gibco (Gaithersburg, MD); and the oxygen and the nitrogen at Liquid Carbonics (Miami, FL). All drugs were dissolved in distilled water and prepared daily.
RESULTS

SIN-1 and ANP Relax Pericytes
The cells were exposed by fluid exchanges to increasing concentrations of SIN-1 (10^{-10} M-10^{-4} M) or ANP (10^{-11} M-10^{-6} M). For each concentration, the exposure time was 10 minutes, allowing the drugs to express their maximal effect. This was demonstrated for all concentrations by the absence of a significant difference between the responses observed after 5 and 10 minutes of drug exposure.

Both SIN-1 and ANP evoked a marked reduction in the number of wrinkles in a concentration-dependent manner (Fig. 1). The half-maximal response (EC50) for both SIN-1 and ANP was reached at concentrations of 10^{-7} M and 10^{-8} M, respectively. In these experiments and at the highest concentrations used, SIN-1 (10^{-4} M) and ANP (10^{-6} M) evoked losses of wrinkles of 74 ± 5% and 95 ± 3%, respectively, an extent that was significantly different (P < 0.001) from that of time-control experiments (experiments performed without SIN-1 and ANP). These results demonstrate that pericytes are relaxed by SIN-1 and ANP.

Methylene Blue Inhibits Relaxations Induced by SIN-1 and ANP
In vascular smooth muscle cells, the relaxation induced by SIN-1 or ANP results from the stimulation of the enzyme guanylate cyclase, responsible for the production of the second messenger, 3',5'-cyclic guanosine monophosphate (cGMP). To determine whether in pericytes the relaxation induced by SIN-1 and ANP also involves the stimulation of guanylate cyclase, the cells were exposed to SIN-1 and to ANP in the absence or in the presence of MB, an inhibitor of guanylate cyclase.5,21-23

Before the exposure to SIN-1 (10^{-7} M) or ANP (10^{-7} M) at a concentration approximately the EC50 of each drug, quiescent pericytes were preincubated with 3 × 10^{-7} M MB for 20 minutes. At a concentration of 3 × 10^{-7} M, MB is known to inhibit pericytes' guanylate cyclase cGMP-production without interfering with their ability to relax after exposure to a stable analog of cGMP (8-bromo-cGMP, for example).5 After preincubation with MB, pericytes were exposed for 10 minutes to SIN-1 or ANP, always in presence of MB. Control experiments were performed without MB. In comparison with those in control experiments, pericyte relaxations in response to SIN-1 and ANP were significantly inhibited (P < 0.001) by MB (Fig. 2).

Hemoglobin Inhibits Relaxations Induced by SIN-1 But Not by ANP
To confirm that ANP-induced relaxation (in contrast to that induced by SIN-1) is not mediated by an extracellular release of NO, experiments similar to those just described were repeated, but MB was replaced by hemoglobin (10^{-5} M), a scavenger of NO.24

The relaxation induced by SIN-1 (10^{-7} M) was significantly inhibited (P < 0.001) by the NO scavenger hemoglobin, demonstrating that under our experimental conditions the relaxation of pericytes induced by the NO donor SIN-1 is essentially mediated by the presence of extracellular NO. In contrast, the NO scavenger hemoglobin had no inhibitory effect on the relaxation induced by ANP 10^{-7} M (Fig. 2).

Oxygen Modulates Relaxations Induced by SIN-1 but not by ANP
To examine how oxygen affects guanylate cyclase-mediated pericyte relaxation, the relaxing effect of a 10-minute exposure to SIN-1 (10^{-7} M) or to ANP (10^{-7} M) was assessed in hypoxic (100% nitrogen) and hyperoxic (100% oxygen) conditions. In comparison, the relaxations induced by SIN-1 under normoxic conditions...
were significantly greater ($P < 0.001$) in hypoxia and significantly less ($P = 0.002$) in hyperoxia. This was not the case for ANP, in which neither hypoxic nor hyperoxic conditions influenced relaxation. In addition, different concentrations of oxygen had no effect on the tone of quiescent pericytes (Fig. 3). These results show that pericycle relaxation induced by the NO donor SIN-1 are modulated by different concentrations of oxygen.

DISCUSSION

The results of this study demonstrate that SIN-1 and ANP relax bovine retinal microvascular pericytes (Fig. 1). Relaxations induced by SIN-1 and by ANP were abolished by MB, an inhibitor of guanylate cyclase, providing evidence that relaxations induced by these drugs involve the activation of guanylate cyclase (Fig. 2). The results also show that only guanylate cyclase-mediated relaxations induced by SIN-1 (but not those induced by ANP) involve an extracellular release of NO, because relaxations induced by SIN-1 were abolished by hemoglobin, an extracellular scavenger of NO (Fig. 2). Finally, the results demonstrate that only guanylate cyclase-mediated relaxations induced by SIN-1—which involve an extracellular release of NO—can be modulated by different concentrations of oxygen. This was not the case for guanylate cyclase-mediated relaxations induced by ANP—which did not involve an extracellular release of NO (Fig. 3).

Relaxations induced by SIN-1 and by ANP were abolished by MB, an inhibition that cannot be attributed to an unspecific effect of that substance. Indeed, it has been shown previously that in similar conditions (same concentration of MB, identical experimental set-up, time exposure, and so forth) MB was able to inhibit the guanylate cyclase-cGMP production in pericytes without interfering with the ability of these cells to relax when exposed to a relaxing drug (e.g., 8-bromo-cGMP).5

Atrial natriuretic peptide is known to stimulate guanylate cyclase after binding to a membrane receptor and not through the release of NO. In bovine retinal microvascular pericytes, in contrast to arterial smooth muscle cells, the effect of ANP was inhibited by MB. Similar reports have already been made for different types of cells, even for vascular smooth muscle cells from the lymphatic system, and the inhibition by MB of the ANP-induced guanylate cyclase stimulation has been attributed to the existence of a specific MB-sensitive, ANP-membrane receptor subtype. The current observation made in pericytes shows that within the cardiovascular system, in addition to the known heterogeneity in the vascular responses to ANP (ANP relaxes arteries but not veins), a heterogeneity could also exist in the distribution of different ANP-receptor subtypes.

Retinal pericytes are contractile cells that have the potential to change the lumen diameter of capillaries and thus also the potential to modulate blood flow through such vessels. Rapidly degraded by oxygen, NO has the ability to relax pericytes. Similar to the effect of hypoxia and hyperoxia on retinal and optic nerve head blood flow, the current results show that hypoxia strongly potentiates, whereas hyperoxia markedly inhibits the relaxation of retinal microvascular pericytes induced by the NO donor SIN-1. Because of the constant NO endothelial release in the ophthalmic microcirculation, it appears that an interaction between oxygen and NO might participate in a sectorial blood-flow modulation within the capillary network, according to the local oxygen tension.

Key Words

eye, free radicals, glaucoma, optic nerve head circulation, retina

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References


