Graefe's Arch Clin Exp Ophthalmol (1997)
235:388–392 

LABORATORY INVESTIGATION

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# Effect of oxygen on relaxation of retinal pericytes by sodium nitroprusside

Received: 27 September 1996
Revised version received: 21 January 1997
Accepted: 4 February 1997

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**Abstract** ● Background: This study addresses whether oxygen modulates the relaxation induced in retinal pericytes by sodium nitroprusside (SNP), a nitric oxide (NO) donor that stimulates the NO/guanylate cyclase pathway. ● Methods:Bovine retinal pericytes were cultured on silicone. On the silicone surface, basal pericyte contractile tone induces wrinkles. Drug-induced changes in pericyte contractile tone were assessed by changes in the number of wrinkles. The effects of 100% nitrogen (hypoxia) and 100% oxygen (hyperoxia) were studied on: (a) the basal tone of quiescent pericytes, (b) the relaxation to 3 and 10 μM SNP or 1 μM forskolin, and (c) the recontraction that followed the washout of 3 μM SNP or 1 μM forskolin. ● Results: Neither hypoxia nor hyperoxia had any apparent influence on pericyte basal tone, on forskolin-induced relaxation, or on pericyte recontraction after a forskolin-induced relaxation. In hypoxia, relaxations to SNP 3 μM ($P<0.05$) and 10 μM ($P<0.01$) were significantly more pronounced than in hyperoxia. Hypoxia also reduced the recontraction after an SNP-induced relaxation ($P<0.001$). ● Conclusion: Oxygen modulates the relaxation of bovine retinal pericytes evoked by SNP (guanylate cyclase-mediated), but not the relaxation induced by forskolin (adenylate cyclase-mediated). These results suggest that in the retinal capillary circulation an interaction between oxygen and the NO/guanylate cyclase pathway modulates pericyte tone, and thus potentially blood flow.

## Introduction

Capillaries of the retina and optic nerve head are interconnected in a dense and continuous network [2, 3, 5]. They are made of an endothelium layer surrounded by pericytes of a mesenchymal origin [10, 11].

Pericytes are contractile cells which are relaxed by nitric oxide (NO) [15, 17]. They can modify the diameter of a retinal capillary [24] and thus potentially flow within such a vessel [18, 22].

Blood flow in the retina and the optic nerve head is increased and decreased in response to hypoxia and hyperoxia, respectively [12]. NO, which is rapidly degraded by oxygen [25], maintains the ophthalmic circulation in a constant state of mid-vasodilation [8, 13, 14]. Oxygen, by modulating the relaxing effect of NO, could help control retinal capillary blood flow in keeping with local metabolic needs, as reflected in local partial pressures of oxygen [6].

Therefore, in this study, we investigated the effect of 100% nitrogen (hypoxia) and 100% oxygen (hyperoxia) on the relaxation/recontraction of bovine retinal microcirculation pericytes in culture. We showed an effect of oxygen tension when the NO donor, sodium nitroprusside (SNP), was present.

## Materials and methods

### Pericyte isolation and identification

Retinal bovine pericytes were grown in culture and identified according to the method described by D'Amore [6, 7, 15]. In agreement with the principles of laboratory animal care, as well as specific national laws for the use of animals in experimentation, bovine eyes were obtained from Aries Scientific (Richardson, Tex.) and shipped to our laboratory on ice by an overnight express service. Thirty to forty retinas were dissected free, minced, and incubated for 1 h in phosphate-buffered saline containing 0.2% collagenase and 0.2% bovine serum albumin. After filtration through a Nitex mesh, cells were retrieved and rinsed by serial centrifugation (800 *g*), resuspended, and cultured in 75-cm square flasks in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, 50 mg/ml fungizone, and 1.25 μg/ml gentamicin. The cells cultured had the typical morphological appearance of pericytes [7] and stained positively for the antiganglioside antibody 3G5, which marks pericytes but not vascular smooth muscle cells [20]. These cultured pericytes did not express the glial fibrillar acidic protein found in cultured astrocytes [1]. In contrast to retinal pigment epithelium cells, they lacked the ability to phagocytize rod outer segments [19]. In contrast to endothelial cells, the main potential contaminant of retinal pericyte culture, they did not take up fluorescein-tagged low-density lipoprotein (LDL) [23]. After these general features of the pericytes cultured had been demonstrated [6], each lot of cells was checked by documenting binding of the antiganglioside 3G5 and the absence of cells that take up LDL.

### Culture of pericytes on silicone membrane

First and second passages of bovine retinal pericytes were grown in DMEM for 3–7 days on a thin membrane of silicone, coating the bottom of a petri dish. When growing on the surface of the silicone these contractile cells induced small wrinkles that were visualized with a phase-contrast, inverted microscope (Zeiss, Oberkochen, Germany) connected to a video camera system (Panasonic, Osaka, Japan) and a printer (Mitsubishi, Cypress, Calif.) [15, 16]. In these cultures, pericytes spontaneously exhibited contractile tone that produced a wrinkled silicone surface beneath them.

### Observation of pericytes

To study the contractile function of the pericytes, the whole petri dish was placed into a closed transparent chamber that maintained the cells at a constant 37° C while allowing multiple fluid exchanges by suction-perfusion. In certain experiments the atmosphere in the chamber (ambient air) was saturated with oxygen or nitrogen. Once the dish was 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 5 mM; HEPES sodium salt 5 mM; glucose 10 mM), a solution that was then used in all subsequent experiments.

After 20 min of adaptation in this solution, pericytes were relaxed with either SNP (3 and 10 μM) or forskolin (1 μM), two substances that stimulate the guanylate cyclase [15] and the adenylate cyclase pathway, respectively [9]. SNP and forskolin were used at concentrations that were about or slightly higher than the half maximal relaxing concentration [9, 15]. The effect of oxygen concentration on the relaxation was studied.

In separate experiments, the spontaneous recontraction of pericytes after exposure and relaxation to 3 μM SNP or 1 μM forskolin (in normoxia) was studied. In these experiments, pericytes were first relaxed with 3 μM SNP or 10 μM forskolin. After 10 min, when the maximal relaxing effect of drugs was reached, SNP and forskolin solutions were washed out and replaced by plain HEPES-buffered solution. The recovery of pericyte contractile tone was then observed, and the effect of oxygen concentration on the recovery was tested.

### Changes in oxygen concentrations

Hypoxic and hyperoxic conditions were obtained by bubbling the HEPES-buffered solution for 10 min with 100% nitrogen and 100% oxygen, respectively, prior to its infusion in the petri dish. Once in the petri dish, the atmosphere surrounding the solution was immediately saturated with either 100% nitrogen or 100% oxygen. In preliminary experiments it was shown that, after bubbling for 10 min the petri dish solution with 100% nitrogen or 100% oxygen, the partial pressure of oxygen in the solution was 2% and 97%, respectively, conditions which are clearly hypoxic and hyperoxic. Our experiments, specifically studied the effect of hypoxic, normoxic and hyperoxic conditions on the basal resting tone of pericytes, on the relaxation induced by either SNP or forskolin, and on the recontraction of pericytes after the wash-out of either SNP or forskolin.

### Assessment of the data

Changes in pericyte contractile tone were quantified by counting the changes in the number of the wrinkles [15]. Measurements were usually taken at 2, 5, and 10 min. In all experimental protocols, maximal effect was reached after 10 min; therefore, only measurements taken at 10 min were compared. Results are expressed either as percentage loss of the number of wrinkles of pericyte's basal tone or as percentage recovery of the number of wrinkles after a relaxation induced by SNP (3 μM) or forskolin (1 μM). The data, which were normally distributed, are given as the mean and the standard error of the mean (mean ± SEM), and the results are compared using an unpaired Student's *t*-test, with $P < 0.05$ considered significant.

### Drugs

Sodium nitroprusside, forskolin, fungizone, collagenase, HEPES, dimethylpolysiloxane, and bovine serum albumin were obtained from Sigma Chemical (St Louis, Mo.). Gentamicin and the fetal bovine serum were purchased at Gibco (Gaithersburg, Md.), and the oxygen and the nitrogen at Liquid Carbonics (Miami, Fla.). SNP was diluted in distilled water and made daily. Forskolin was diluted in alcohol and stored in stock solution (–20 C).

## Results

### Effect of oxygen on pericyte basal tone

Exposing quiescent pericytes for 10 min to hypoxic or hyperoxic conditions had virtually no effect on pericyte basal contractile tone. Indeed, hypoxia or hyperoxia only evoked a slight and no significant decrease in the number of wrinkles of 1 ± 6% ($n = 5$), and 5 ± 3% ($n = 4$), respectively (Fig. 1).

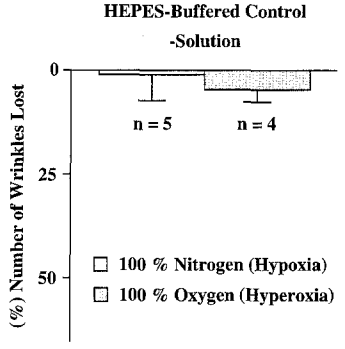


**Fig. 1** Effect of 100% nitrogen (hypoxia) vs 100% oxygen (hyperoxia) on the basal tone of quiescent pericytes in a HEPES-buffered control solution. Neither hypoxia nor hyperoxia had an influence on pericyte basal tone

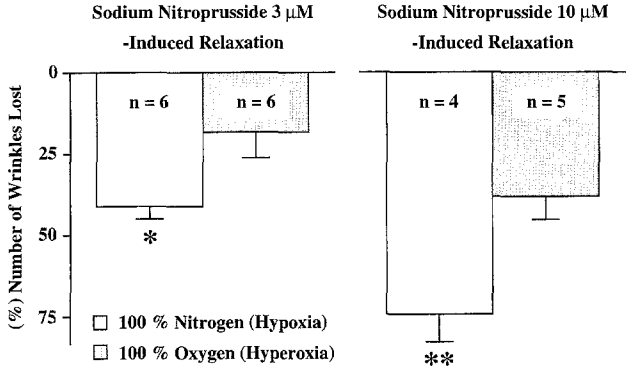


**Fig. 2** Effect of 100% nitrogen (hypoxia) vs 100% oxygen (hyperoxia) on the relaxation of bovine retinal microcirculation pericytes to SNP 3 μM and 10 μM. Relaxations were significantly greater in hypoxia than in hyperoxia. Unpaired Student's *t*-test, hypoxia vs hyperoxia: $^{*}P < 0.05$, $^{**}P < 0.01$

## Effect of hypoxia vs hyperoxia on pericyte relaxations to SNP or forskolin

In comparison to hyperoxic conditions, in hypoxia relaxations to both SNP 3 μM ($P < 0.05$) and SNP 10 μM ($P < 0.01$) were significantly more pronounced (Fig. 2). In hypoxia, SNP 3 μM evoked 41 ± 4% ($n = 6$), and SNP 10 μM evoked 74 ± 8% ($n = 4$) relaxations, while in hyperoxia relaxations to SNP 3 μM and 10 μM were only 19 ± 8% ($n = 6$) and 38 ± 7% ($n = 5$), respectively. In contrast, no significant difference could be detected between relaxations induced by forskolin 1 μM in hypoxic and hyperoxic conditions (Fig. 3); in hypoxia forskolin 10 μM evoked 44 ± 7% ($n = 9$) and in hyperoxia, 43 ± 9% ($n = 4$), relaxations. Likewise, the oxygen level had no effect on the basal contractile tone (Fig. 1).

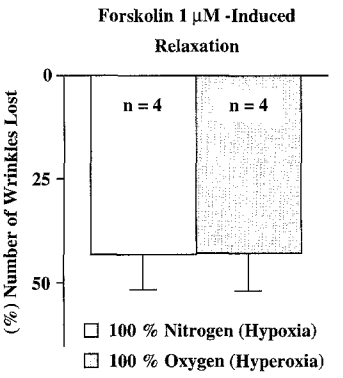


**Fig. 3** Effect of 100% nitrogen (hypoxia) vs 100% oxygen (hyperoxia) on the relaxation of bovine retinal microcirculation pericytes to forskolin 1 μM. No difference was seen between hypoxia and hyperoxia

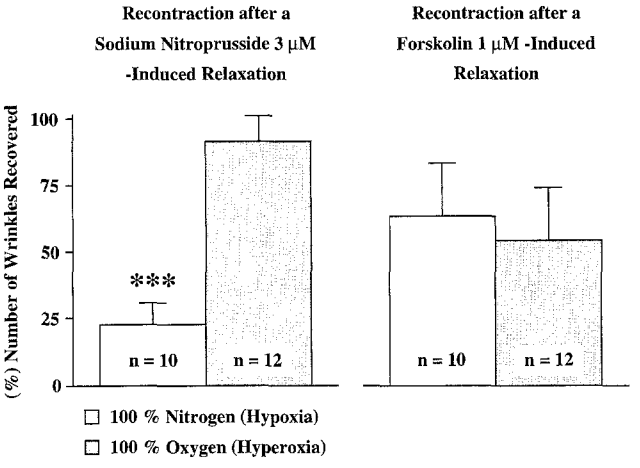


**Fig. 4** Effect of 100% nitrogen (hypoxia) vs 100% oxygen (hyperoxia) on recontraction of bovine retinal microcirculation pericytes following a relaxation induced either by SNP 3 μM or forskolin 1 μM. The level of recontraction was assessed by the amount of wrinkles recovered by pericytes in a HEPES-buffered solution 10 min after the wash-out of SNP or forskolin. In contrast to forskolin, after a SNP-induced relaxation, recontraction was significantly lower in hypoxia than in hyperoxia. Unpaired Student's *t*-test, hypoxia vs hyperoxia: $^{***}P < 0.001$

## Effect of hypoxia vs hyperoxia on pericyte recontractions after SNP or forskolin wash-out

After a 3 μM SNP-induced relaxation and the wash-out of this drug, the spontaneous pericyte recontraction was strongly modulated by different oxygen concentrations (Fig. 4). In hypoxia (23 ± 8%, $n = 10$) the recontraction after an SNP-induced relaxation was significantly lower ($P < 0.001$) than in hyperoxia (91 ± 10%, $n = 12$). Such a difference could not be observed in pericyte recontraction which followed a 1 μM forskolin-induced relaxation (hypoxia 64 ± 20%, $n = 6$; hyperoxia 54 ± 20%, $n = 6$; Fig. 4).

## Discussion

The present study on bovine retinal microcirculation pericytes demonstrates that after exposure to the NO donor SNP, both the relaxation and the recontraction that follows wash-out are strongly modulated by the oxygen concentrations. Indeed, in hypoxia relaxations to SNP were significantly more pronounced, and recontraction significantly less marked, than in hyperoxia (Figs. 2, 4). In contrast, forskolin-evoked relaxation and spontaneous recontraction after forskolin exposure were similar in hypoxia or in hyperoxia (Figs. 3, 4).

The oxygen modulation of SNP pericyte relaxation or recontraction does not appear to result from an unspecific direct relaxing or contracting effect of oxygen, as neither basal nor forskolin-modulated contractile tone of pericytes was affected by hypoxia or hyperoxia (Figs. 1, 3, 4). These results show that the oxygen effect on the relaxation or recovery of pericyte contractile tone (Figs. 2, 4) is directly linked to the pharmacological properties of SNP.

In pericytes, SNP is known to evoke a relaxation through the stimulation of the guanylate cyclase pathway [15], while forskolin relaxes pericytes by the activation of the adenylate cyclase pathway [9]. Therefore, these results demonstrate that it is most likely by interacting with the NO/guanylate cyclase pathway that oxygen modulates the SNP-induced effect on pericytes', contractile tone. As NO is known to be rapidly autoxidized, it is likely that by modulating the half-life of NO, oxygen could indirectly modulate the relaxation of pericytes induced by NO [21, 25]. However, further investigations need to be conducted in order to clarify the exact mechanism by which oxygen modulates the effect of SNP on retinal pericytes.

In conclusion, these results clearly show that the contractile tone of bovine retinal pericytes in response to the NO donor, SNP, is strongly modulated by different concentrations of oxygen. Because the retinal vascular bed is constantly under the influence of NO, an interaction between oxygen and the NO/guanylate cyclase pathway – by modulating pericyte tone – could potentially influence flow within the retinal capillary network [4, 12].

**Acknowledgements** This work was supported in part by US Public Health Service Research Grant RO1 EY 10465, awarded by the National Eye Institute, Bethesda, Maryland, USA. Dr. Douglas R. Anderson has been given a Senior Scientific Investigator's Award by Research to Prevent Blindness, Inc., New York, NY, USA.

Dr. Ivan O. Haefliger was supported by grants of the Société Suisse de Bourses en Médecine et Biologie, the Fondation Florian Verrey, the Fondation Eugenio Litta, and the Swiss National Science Research Foundation (32-42564.94). The authors thank E. Barry Davis for his unique support during the course of this study.

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