Hyperoxia/Normoxia-Driven Retinal Angiogenesis in Mice: A Role for Angiotensin II

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PURPOSE. To examine a possible role for the angiotensin system in a rodent model of retinopathy of prematurity.

METHODS. A previously described model was used in which oxygen cycling (5 days hyperoxia and 5 days hypoxia) induced retinal alterations in newborn mice. An angiotensin-converting enzyme inhibitor (perindopril), or angiotensin receptor antagonists AT1 (losartan) or AT2 (PD123319) were administered subcutaneously for 5 days after the hyperoxia exposure. According to histologic methods, the endothelial cell count within the anterior part of the ganglion cell layer was used for the evaluation of the compound effect.

RESULTS. Histologic evaluation showed an increased number of endothelial cells in retinas of hypoxic pups compared with hyperoxic or normoxic pups. Hypoxic animals treated with perindopril (4 mg/kg) showed a significant decrease (29%, \( P \leq 0.001 \)) in endothelial cell number (163 ± 7) compared with hypoxic control animals (231 ± 10). Losartan also decreased the endothelial cell number (14%, \( P \leq 0.05 \)), whereas the AT2 antagonist had no effect.

CONCLUSIONS. The data showed a protective effect of an angiotensin-converting enzyme inhibitor and of an AT1 receptor antagonist on hyperoxia- and normoxia-induced neovascularization in newborn mice. The results suggest a role for the angiotensin system in this model and that such compounds may be of interest in the prevention of proliferative retinopathies such as proliferative diabetic retinopathy. (Invest Ophthalmol Vis Sci. 2001;42:429–432)

The discovery of new drugs for the treatment of diabetic retinopathy is hampered by the lack of understanding of the initial process and of suitable animal models. The major causative factor for the development of diabetic retinopathy is hyperglycemia, which leads to vascular changes, such as endothelial cell proliferation or neovascularization. In humans these changes occur clinically after 10 to 15 years of diabetes, but in animal models of diabetes these changes require a year or more and consist only of pericyte death and the loss of capillaries.1

Both the human and the experimental model of retinopathy of prematurity (ROP) in the rodent are related, at least in part, to an overproduction of vascular endothelial growth factor (VEGF), a mechanism that ROP shares with proliferative diabetic retinopathy (PDR). Neovascularization is the final common pathway of both PDR and ROP.

Angiotensin II (AII) plays a role in the development of many cardiovascular and renal diseases. Because AII induces an increase in VEGF mRNA,2 this peptide could locally increase permeability, growth, and alter the function of microvessels in the retina. It has also been suggested that AII may potentiate VEGF-induced angiogenic activity in the retina through an increase in expression of the VEGF receptor Flk-1/KDR.3

Recently, lisinopril, an angiotensin-converting enzyme inhibitor (ACEi), has been shown to decrease the progression of retinopathy in nonhypertensive patients with insulin-dependent diabetes mellitus type I (IDDM).4 All these observations prompted us to examine whether an ACEi and/or an angiotensin receptor antagonist (AT1 or AT2) is an effective compound in a rodent model of ROP mimicking the retinal degenerative and proliferative process of PDR.

For the first time, this study shows a preventive effect of an ACEi and of an AT1 receptor antagonist in an in vivo newborn mouse model of retinal neovascularization.

METHODS

Animal Model

This model was first described by Smith et al.5 Briefly, one-week-old C57BL/6j mice (Ifa Credo, L’Arbresle, France) and their mothers were exposed to 75% ± 2% oxygen for 5 days (hyperoxia) and then returned to normoxic conditions for another 5 days, inducing relative hypoxic conditions. Unexposed control animals were kept in room air. The animals were maintained at a constant temperature of 21 ± 1°C and on a 12-hour light–dark cycle. Oxygen concentration was measured with an oximeter (Toptronic, Milan, Italy). At the end of the oxygen exposure (day 12) and 5 days after return to normoxic conditions (day 17), the pups were killed, and retinal alterations were observed using the two criteria detailed later.

Endothelial Cell Count within the Anterior Part of the Ganglion Cell Layer of the Retina

After enucleation, eyes were fixed in 2.5% glutaraldehyde for 1 hour and embedded in glycol-methacrylate. Serial sections (4 µm) of whole eyes, at eight different levels, were cut sagittally through the cornea and parallel to the optic nerve. Sections were stained with periodic acid–Schiff (PAS) and hematoxylin. Nuclei, easily distinguishable under a white-light microscope (DMLS: Leica, Wetzlar, Germany), were counted in the anterior part of the ganglion cell layer and on the inner limiting membrane of the retina by a person blinded to the sample identity. Cross-sections that included the optic nerve were excluded. Quantitative histology was expressed as the endothelial cell number per section per eye. Sections were photographed with a video camera (Sony, Tokyo, Japan), and an image analyzer (Visiolab 1000; Biocom, Les Ulis, France).

Fluorescein-Dextran–Perfused Retinas

Pups were anesthetized (Forene inhalation; Abbott Labs, Queensborough, UK) and then underwent a cardiac injection (0.03 ml/kg of body weight) of fluorescein-conjugated dextran (molecular weight: 2 × 10^6; Sigma, St. Louis, MO) dissolved in phosphate-buffered saline (PBS; 50 mg/ml). The eyes were enucleated, fixed for 1 hour in 4% buffered paraformaldehyde, and the retinas were flat mounted with citifluor (Oxford Instruments, Orsay, France), observed by fluorescence microscopy (Orthoplan; Leitz), and photographed on color slide film (Elite 400; Kodak, Rochester, NY).

Treatment

After 75% oxygen exposure, pups from each mother were divided into three or four subgroups. The first group received vehicle (control...
group), and the other groups received perindopril (Coversyl; Servier Research Group, Neuilly sur Seine, France) or a stereoisomer devoid of ACE inhibitory activity (S11803). In another experiment, a pup from each mother was treated with vehicle (control group), another pup with losartan (AT1 antagonist), another with PD123319 (AT2 antagonist), and another with a mixture of the two antagonists. All compounds were solubilized in sterile water and administered subcutaneously (0.02 ml/g of body weight), once a day, for the 5 days after the return to normoxic conditions after hyperoxia exposure.

All studies were approved by the ethics committee and performed in accordance with Principles of Laboratory Animals Care (NIH publication 83-25, revised 1985) and French law regulating animal experiments (Decree 87-848, October 19, 1987, and the Ministerial Decrees of April 19, 1988). All work adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Statistical Analysis
Results were expressed as the mean ± SEM. Treatment effects were performed using a one-way analysis of variance (ANOVA) followed by a comparison of treated groups versus control groups using Dunnett’s test. The significance threshold was 5%.

RESULTS
Effect of Perindopril at 2 and 4 mg/kg
At day 17, in control animals (vehicle treated) subjected to the hyperoxia-normoxia cycle (called relative hypoxic pups), the endothelial cell number (196 ± 10) was significantly higher (P ≤ 0.001) than that seen in the retinas of animals that remained in normal room air during the same time (75 ± 7). Animals treated with perindopril (2 mg/kg) for 5 days after their transfer from hyperoxic to normoxic conditions demonstrated a tendency toward a decreased (12%, nonsignificant [NS]) endothelial cell number (172 ± 11). The treated group with perindopril (4 mg/kg) showed a significant decrease (22%, P ≤ 0.01) in endothelial cell number (151 ± 6).

Comparative Effect of Perindopril and S11803 at 4 mg/kg
At day 17, in control animals (vehicle treated) subjected to the hyperoxia-normoxia cycle (called relative hypoxic pups), the sagittal cross section of eyes showed penetrating capillaries across the plexiform plate and endothelial cell tufts on the inner limiting membrane of the retina extending into the vitreous, participating in the neovascularization process (Fig. 1A).

![Image](image.png)

**Figure 1.** Histologic alterations induced by hyperoxia and hypoxia in the retina of newborn mice. Comparative effect of perindopril and S11803 at 4 mg/kg. Animals were subjected to 7 days in normoxia, followed by 5 days in hyperoxia, and then by 5 days in normoxia. Treatment was administered during the last 5 days. (A, C, and E) Sagittal cross sections of eye observed under white light after PAS and hematoxylin staining. (B, D, and F) Whole isolated retinas, flat mounted and observed under UV light after cardiac injection of fluorescein-conjugated dextran (molecular weight, 2 × 10⁶). (A, B) Retinas of animals that received water only; (C, D) perindopril (4 mg/kg); (E, F) ACE inactive isomer S11803 (4 mg/kg). c, penetrating capillary; ilm, internal limiting membrane, nt, neovascularization tuft. Bar, (A, C, and E) 40 μm; (B, D, and F) 570 μm.
Compared with that of a hypoxic animal treated with water only (day 17), the retina of an animal treated with perindopril showed a reduction of endothelial cell proliferation, with a reduction of penetrating capillaries and with fewer and smaller endothelial cell tufts (Fig. 1C). In contrast, animals treated with S11803 were not significantly different from control animals (Fig. 1E).

Figure 2A shows that the endothelial cell count within the anterior part of the ganglion cell layer of the retina of the relative hypoxic pups was 231 ± 10. In the same conditions as described earlier, pups treated with perindopril (4 mg/kg) showed a significant decrease of 29% in the endothelial cell number (165 ± 7, P ≤ 0.001). In contrast, S11803 did not produce any effect on endothelial cell number.

In control normoxic pups, 12 days after birth, intracardiac injection of fluorescein dextran before death allowed the visualization of the capillary bed spreading all over the retinal tissue. In contrast, after 5 days in 75% oxygen (hypoxic pups), an avascular hypoperfused area was observed in the center of the retina where main vessels were present but appeared to be vasoconstricted. Only the periphery appeared to be perfused. Retinas of the animals that were returned to normal room air after this hyperoxic phase (relative hypoxic pup), showed tortuous vessels, arteriovenous shunts, vasodilation, and endothelial cell sprouting (Fig. 1B). Compared with a relative hypoxic animal treated with water only (day 17) photography of a flat-mounted retina of an animal treated with perindopril showed a decrease in fluorescence, a reduction of the neovascularization process, and fewer endothelial cell tufts (Fig. 1D). In contrast, an inactive stereoisomer of perindopril S11803 did not produce any changes (Fig. 1F).

**Effect of AT1 and AT2 Receptor Antagonists**

Compared with those in relative hypoxic control animals treated with water only (day 17), the retinas of animals treated with an AT1 receptor antagonist (losartan, 10 mg/kg) showed a reduction of endothelial cell proliferation (14%, P ≤ 0.05; Fig. 2B). In contrast, animals treated with an AT2 receptor antagonist (PD123319, 10 mg/kg) were not significantly different from control animals. AT1 and AT2 antagonist coadministration had the same effect as the AT1 antagonist alone (16%, P ≤ 0.05; Fig. 2B).

**DISCUSSION**

The development of PDR is associated with hypoxia and an overproduction of VEGF. Actually, the only prevention of PDR progression is tight control of glycemia, but recently, an angiotensin-converting enzyme-inhibitor (lisinopril) has shown beneficial effects on the progression of human PDR. The present findings demonstrated a beneficial effect of ACE inhibition and AT1 antagonism on the experimental retinopathy of prematurity induced in mice by neonatal exposure to different oxygen concentrations present in inspired air.

Because new vessel formation are not observed in the classic models of experimental diabetes, we used the model of oxygen-induced retinopathy in newborn mice according to Smith et al. Counting the number of endothelial cells was classically used to assess capillary density.

The role of VEGF has been well described in this model, and, in addition, the mRNA level of the KDR/Flk-1 receptor was reported to be higher in the neovascular retina of hypoxic animals than in control animals. Thus, VEGF may represent a link of diabetes and retinal ischemia with PDR neovascularization.

In the present study we demonstrated that the ACE inhibitor perindopril inhibited the neovascularization process induced in neonatal mice exposed to relative hypoxic conditions. The same results were obtained with captopril, another ACE inhibitor (data not shown). Perindopril was of special interest because of the existence of stereoisomers devoid of ACE activity, thus providing another control group, more representative than the vehicle group alone. Thus the beneficial effect of perindopril observed on proliferative retinopathy in neonatal mouse exposed to hypoxic-normoxic cycling conditions was not observed with the inactive stereoisomer S11803. For the first time, we demonstrated also that the AT1 receptor antagonist losartan showed a small but significant effect on the neovascularization process observed in this model. However, an AT2 receptor antagonist was without any effect, which could be explained by either the absence of AT2 receptors in the neonatal mouse retina or by an insufficient dose of PD123319.

These results argued for the participation of AT1 and therefore for the renin–angiotensin system in the induction of proliferative retinopathy in this murine model. Indeed, several reports indicate that in patients with diabetes, the intraocular renin–All system may play a role in diabetic retinopathy, and retinopathy is associated with elevated plasma ACE levels. The components of the renin–angiotensin system are reported to be present in animal ocular tissues, and angiotensinogen mRNA is overexpressed in the retina of rats exposed to hyperoxia-cycling (Bazan et al., personal communication, January 2001).
1999). After ACE inhibition, an increase of bradykinin level could be considered, but a role for bradykinin is unlikely, because no vasodilation or fluorescein leakage was observed under ACEi treatment in our preparations.

How can the effect of All be explained? Receptors for All are present on endothelial cells, where All acts to stimulate endothelial cell growth and upregulate VEGF mRNA expression.\textsuperscript{2} Moreover, All potentiates VEGF-induced angiogenic activity potentially through an increased expression of VEGF receptor KDR/Flk-1.\textsuperscript{3} However, there is no direct evidence for a direct implication of this enzyme in the onset or evolution of diabetic retinopathy.

Therefore, the mechanism of the protection of ACEi or AT1 antagonist against hyperoxia–normoxia neovascularization remains to be clarified. Cardiovascular modifications such as hypotension or vasodilation would be of importance because diltiazem, a calcium channel blocking agent, also reduced (45%) oxygen induced retinopathy in the same animal model.\textsuperscript{4} Other vasodilating agents, nimodipine, dipyridamole, or ginkgobiloba, showed a similar inhibition (50%).\textsuperscript{5} An improvement of the retinal neovascularization process has also been obtained with different compounds such as an amino sterol (squalamine) or a matrix metalloproteinase inhibitor, but a complete inhibition was observed with a nonspecific kinase inhibitor.\textsuperscript{10}

Taken together, these observations suggest that the angiotensin system plays a role in retinal neovascularization and from a therapeutic point of view, inhibition of ACE could be of interest, at least in part, for the prevention or treatment of neovascularization in the retina.

\textbf{References}