Captopril Improves Retinal Neovascularization via Endothelin-1

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PURPOSE. The purpose of this study was to determine the effect of an angiotensin converting enzyme inhibitor, captopril, on oxygen-induced retinopathy (OIR) in the mouse. Endothelin-1 (ET-1) expression is assessed in a mouse model of OIR.

METHODS. OIR was produced in C57BL6 mice. Captopril (0.5mg/kg/d SC) was given from P7 (post natal day 7) for 5 days. Retinopathy was assessed by a retinal scoring system and by quantification of extra retinal neovascular nuclei on retinal sections at P17 to P20. The expression of ET-1 was determined using a reverse transcriptase polymerase chain reaction.

RESULTS. Pups treated with captopril during hyperoxia had a lower median retinopathy score of 4.5 (25th, 75th quartile: 3, 6.4) compared with animals exposed to hyperoxia alone with median score 9.5 (25th, 75th quartile: 7.1, 10.4; P < 0.001). The pups treated with captopril during hyperoxia had significant reduction in number of nuclei extending beyond the inner limiting membrane (15.8 ± 16.7, mean ± SD) when compared with the animals exposed to hyperoxia only (50.4 ± 28.0; P < 0.01). ET-1 expression in the retina increased 4.1-fold from P7 to P12 and a 1.9-fold increase from P12 to P17. Overall, there was an 8-fold increase in ET-1 expression from P7 to P17. Hyperoxia increased ET-1 expression by 2.1-fold at P12 over room air-reared animals. At P17, there was a 2.9-fold increase in retinal ET-1 expression when compared with room air. At P17, there was a 6.2-fold suppression in ET-1 expression in captopril-treated animals when compared with the oxygen-only-treated animals.

CONCLUSIONS. Captopril reduces retinal neovascularization in a mouse model of oxygen-induced retinopathy. ET-1 expression is increased from P7 to P17, altered by hyperoxia exposure and relative hypoxic recovery and modulated by captopril in a mouse model of OIR. (Invest Ophthalmol Vis Sci. 2001;42:1807–1872)

Retinopathy of prematurity (ROP) is a vasoproliferative disorder that can lead to severe visual impairment and blindness in preterm infants.1 Despite the advances in neonatology, ROP continues to cause severe morbidity in the very low birth weight infant. The causes of ROP are not yet completely understood and are believed to be multifactorial. ROP is initiated by relative hyperoxia (compared with in utero) as a result of preterm birth. Vasoconstriction and delay in the normal development of retinal vasculature occurs, followed by angiogenesis resulting in retinopathy.

Recent reports indicate that angiotensin-converting enzyme (ACE) inhibitor therapy may be protective against retinopathy in patients with diabetes2,3. Captopril is a pharmacological agent that inhibits ACE. ACE stimulates the production of endothelin-1 (ET-1) by converting angiotensin I to angiotensin II and subsequently increasing intracellular calcium. Although the mechanism of injury may be different in diabetic retinopathy and ROP, the retinal angiogenesis is similar. ET-1 has been implicated in retinal vessel constriction.4 Higgins5 has shown that captopril, an ACE inhibitor, blocks hyperoxic-induced ET-1 secretion from retinal and adrenal capillary endothelial cells. Hendricks-Munoz6 has shown that captopril downregulates basal ET-1 secretion in large vessel endothelial cells. It has been shown that hyperoxia stimulates ET-1 secretion from endothelial cells.7 The goal of this study is to evaluate the effect of captopril, ET-1 expression, and effect of captopril on ET-1 expression in a mouse model of OIR.

METHODS

Animal Model

This study was approved by the Georgetown University Animal Care and Use Committee and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. C57BL6 mice were obtained from Taconic Laboratories (Germantown, NY). The pups were placed in an infant incubator (Ohmeda Inc., Columbia, MD) with their nursing mother in 75% oxygen from postnatal day 7 (P7) to P12 to produce oxygen-induced retinopathy as described previously.8 Oxygen concentration was measured with Hudson oxygen analyzer (Hudson VENTRONICS, Temecula, CA) and checked at least twice daily while the animals were in oxygen. On P12, the animals were placed in room air and subsequently killed on postnatal days 17 to 20 with a lethal dose of pentobarbital (120 mg/kg; Abbott Laboratories, North Chicago, IL) when maximal neovascularization was observed.7

Captopril Dosage

The animals were divided into four groups: room air, room air and captopril, oxygen, and oxygen and captopril. Captopril (Sigma Chemical Co., St. Louis, MO) dissolved in normal saline (Abbott Laboratories) was given SC (0.5 mg/kg/d) in the nape of the neck from P7 for 5 days. A group of pups were given sham injection, and litters were routinely divided to have drug-treated, sham-treated, and control animals in each group. All pups were placed in room air from postnatal day 12 until they were killed. Retinal neovascularization were assessed by fluorescein-conjugated dextran angiography9 by a retinopathy scoring system10 and by quantification of extraretinal neovascularization by counting the extraretinal nuclei beyond the inner limiting membrane11 on periodic acid-Schiff (PAS)-stained retinal sections.

Fluorescein Dextran Perfusion of the Retinal Blood Vessels

After the animals were given lethal dose of pentobarbital, a median sternotomy was performed, and the pulsating left ventricle was identified. The left ventricle was perfused with 1 ml of a 50 mg/ml solution of high molecular weight (MW, 2,000,000) fluorescein-conjugated dex-
Tran (Sigma Chemical Co.) in 4% paraformaldehyde (Sigma Chemical Co.) as previously described. The eyes were then enucleated and placed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 2 to 24 hours. The retinas were dissected, radial cuts were made, retinas were mounted on a slide with a drop of 2% gelatin (Sigma Chemical Co.), and coverslips were applied. The coverslip was sealed with clear nail polish. Fluorescent microscopy was used to score the retinal whole mount. A previously described retinopathy scoring system (Table 1) was used to evaluate the retina, and the average retinopathy score for each animal was used for analysis. Scoring was done by two individuals in a masked fashion.

PAS Stain of Retinal Sections

After a lethal dose of sodium pentobarbital, a median sternotomy was performed, and the pulsating left ventricle was perfused with 4% paraformaldehyde in PBS. The eyes were then enucleated, placed in optimal cutting temperature (OCT) embedding compound (Sakura Fine Tek, Inc., Torrance, CA), and frozen at −70°C. Serial sections through the cornea parallel to the optic disc with thickness of 7 to 9 μm were made using a cryostat. The sections were stained with PAS reagent and hematoxylin. Multiple sections from each eye (minimum of six sections at least 50 μm apart) were scored in a masked fashion using light microscopy by counting the number of nuclei extending beyond the inner limiting membrane on the vitreous as previously described. The average number of neovascular nuclei from each eye was used for the statistical analysis.

ET-1 RT-PCR

For detection of ET-1 mRNA levels, RT-PCR analysis was performed. Total RNA extracted from freshly obtained retinas from P7 (before oxygen exposure), P12 (just after oxygen exposure), and P17 (at maximal neovascularization) with TRIzol reagent (Life Technologies, Rockville, MD) as described by the manufacturer. The isolated 10 μg RNA and 2 μM oligo(dT)16 (total volume, 22 μl) were heated at 68°C for 2 minutes and then cooled on ice. First-strand synthesis was performed by incubating the RNA and oligo(dT)16 in a reaction mixture (total volume, 50 μl) containing 50 mM Tris-HCl, pH 8.5, 40 mM KCl, 8 mM MgCl2, 2 mM DTT, 50 U reverse transcriptase, and 0.8 mM each of dATP, dCTP, dGTP, and dTTP. The mixture was incubated at 42°C for 1 hour, then 99°C for 5 minutes, and 4°C for 5 minutes. The resultant cDNA was diluted with 100 μl of H2O and stored at −20°C until PCR was performed.

PCR reaction mixture (diluted volume, 25 μl) was prepared in 0.2 mM each of dATP, dCTP, dGTP, and dTTP; 50 mM KCl; 10 mM Tris-HCl, pH 8.3; 1.5 mM MgCl2; 100 ng each of the ET-1 forward and reverse primers (see below); 0.625 U of Taq DNA polymerase (Perkin-Elmer, Branchburg, NJ); and 1 μl of the diluted resultant cDNA. The mixture was incubated for 4 minutes at 94°C, followed by 30 cycles of 45 seconds at 94°C, 45 seconds at 60°C, and 45 seconds at 72°C, followed by 7 minutes at 72°C, in a PCR apparatus (model 2400, Perkin-Elmer). To verify that equal amounts of RNA were in each PCR reaction within an experiment and to verify a uniform amplification process, β-actin mRNA was also amplified from each sample simultaneously as an internal control. PCR products were separated on a 1.2% agarose gel and were visualized by staining with ethidium bromide. A 100-bp DNA ladder was used as a size marker. The number of cycles versus the intensity of the PCR band was evaluated to determine the optimum number of cycles to be in the linear range. Gels were photographed and scanned for density using QuantityOne program (Biosoft, Ferguson, MO). The RT-PCR was repeated at least three times for each experiment. The PCR products were sequenced for confirmation. The oligonucleotide primers sets used to amplify ET-1 and β-actin (R&D Systems, Minneapolis, MN) were, respectively, 5′-TCG TCC CTG ATG GAT AAA GAG TGT GTC 3′ and 5′-TCG TCC CTG ATG GAT AAA GAG TGT GTC 3′ (forward) and 5′-GCT GAC ATC ATG CTC TTC GGA GGG CTT-3′ (reverse) and 5′-CTA CAA TGA GCT GCG TGT GG-3′ (forward) and 5′-AAG GAA GGC TGG AAC AGT GC-3′ (reverse). The resultant PCR products were 251 bp (ET-1) and 528 bp (β-actin).

**TABLE 1. Retinopathy Scoring System**

<table>
<thead>
<tr>
<th>Score</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
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<tbody>
<tr>
<td>Blood vessel growth</td>
<td>Complete</td>
<td>Incomplete outer third</td>
<td>Incomplete middle third</td>
<td>Incomplete inner third</td>
<td></td>
</tr>
<tr>
<td>Blood vessel tufts</td>
<td>None</td>
<td>Few, scattered &lt;3 clock hours</td>
<td>3–5 clock hours</td>
<td>6–8 clock hours</td>
<td>9–12 clock hours</td>
</tr>
<tr>
<td>Extra retinal neovascularization</td>
<td>None</td>
<td>Mild &lt;3 clock hour</td>
<td>Moderate 3–6 clock hours</td>
<td>Severe &gt;6 clock hours</td>
<td></td>
</tr>
<tr>
<td>Central vasoconstriction</td>
<td>None</td>
<td>Mild early zone 1 (inner 50% of zone 1)</td>
<td>Moderate throughout zone 1 (outer 50% of zone 1)</td>
<td>Severe extending to zone 2</td>
<td></td>
</tr>
<tr>
<td>Retinal hemorrhage</td>
<td>Absent</td>
<td>Present</td>
<td>Moderate 3–6 clock hours</td>
<td>Severe &gt;6 clock hours</td>
<td></td>
</tr>
<tr>
<td>Blood vessel tortuosity</td>
<td>None</td>
<td>Mild &lt;3 clock hours</td>
<td>Moderate 3–6 clock hours</td>
<td>Severe &gt;6 clock hours</td>
<td></td>
</tr>
</tbody>
</table>

RESULTS

Retinopathy Scoring System

Captopril given at a dose of 0.5 mg/kg/d concurrently with 75% oxygen \( (n = 18) \) improved retinopathy with a median (25th, 75th quartile) total retinopathy score of 4.5 (3, 6.4) versus 9.5 (7.1, 10.4) in the animals exposed to hyperoxia only \( (n = 15; P < 0.001) \). There was no difference in the total retinopathy score between animals given captopril while in room air \( (n = 16; \text{score } 0, 0, 0) \) compared with control animals \( (n = 14; \text{score } 0, 0, 0; \text{Fig. 1}) \). Figure 2 shows representative retinal whole mounts. During the course of the experiment, the day animals were killed did not appear to alter the results. For the oxygen-treated animals, there were three animals killed on P17 with a median retinopathy score of 10.5 (7.9, 9.6), three on P18 with a score of 9.5 (8.25, 10.25), seven on P19 with a score of 9 (6.25, 9.5), and one on P20 with a score of 10. In the oxygen plus captopril group, there were two animals killed on P17 with a score of 5.5 (4.3, 6.8), 4 on P18 with a score of 5.4 (3.9, 6.3), 10 on P19 with a score of 4 (2.6, 6.4), and 2 on P20 with a score of 3.9 (3.7, 4.1).

When comparing the retinopathy subscores, there was significant improvement in the blood vessel tufts, extraretinal neovascularization, central vasoconstriction, and blood vessel tortuosity in the animals treated with captopril during hyperoxia when compared with the animals exposed to hyperoxia alone. There were no differences in the blood vessel growth or hemorrhage (Table 2).

Retinal Sections

The animals treated with captopril during hyperoxia \( (n = 11) \) had a significantly decreased \( (P < 0.01) \) number of nuclei extending beyond the inner limiting membrane in to the vitreous \((15.8 \pm 16.7)\) when compared with the animals exposed to hyperoxia \((n = 11)\) only \((50.4 \pm 28.0; \text{Fig. 3})\). This supports the finding from the retinal scoring system that treatment with captopril during the hyperoxia decreases extraretinal neovascularization. No significant difference was noticed in the nuclei count in the animals that remained in room air regardless of captopril treatment \((4.7 \pm 1.7 \text{ vs. } 7.1 \pm 5.3; \text{Fig. 3})\).

ET-1 mRNA Expression

ET-1 is expressed in the mouse retina. Its expression is developmentally regulated, with a 4.1-fold increase from P7 to P12 and with a 1.9-fold increase from P12 to P17 (Fig. 4). Hyperoxia as well as relative hypoxia increases ET-1 expression. At P12 there is a 2.1-fold increase in ET-1 expression when compared with room air-reared animals, and at P17, there is a 2.9-fold increase in ET-1 expression when compared with room air-reared animals (Fig. 4).

Table 2. Retinopathy Subscores

<table>
<thead>
<tr>
<th></th>
<th>Room Air</th>
<th>Room Air + Captopril</th>
<th>Oxygen</th>
<th>Oxygen + Captopril</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood vessel growth</td>
<td>0 (0, 0)</td>
<td>0 (0, 0)</td>
<td>0 (0, 0.5)</td>
<td>0 (0.5)</td>
</tr>
<tr>
<td>Blood vessel tufts</td>
<td>0 (0, 0)</td>
<td>0 (0, 0)</td>
<td>2.8 (2, 3)</td>
<td>0.75 (0, 1.5)‡</td>
</tr>
<tr>
<td>Extra retinal</td>
<td>0 (0, 0)</td>
<td>0 (0, 0)</td>
<td>1 (1.1)</td>
<td>0.5 (0.9)§</td>
</tr>
<tr>
<td>Central vasoconstriction</td>
<td>0 (0, 0)</td>
<td>0 (0, 0)</td>
<td>2 (2, 2.5)</td>
<td>1 (1.9)†</td>
</tr>
<tr>
<td>Hemorrhage</td>
<td>0 (0, 0)</td>
<td>0 (0, 0)</td>
<td>1 (1.1)</td>
<td>1 (0.1, 1)</td>
</tr>
<tr>
<td>Blood vessel</td>
<td>0 (0, 0)</td>
<td>0 (0, 0)</td>
<td>2 (1.6, 2.9)</td>
<td>1 (0.8, 1)‡</td>
</tr>
<tr>
<td>tortuosity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Subscore categories are shown as median scores with 25th and 75th quartile in parentheses for each treatment group.

\* \( P < 0.05 \) when compared with oxygen treatment.

\| \( P < 0.01 \) when compared with oxygen treatment.

\| \( P < 0.001 \) when compared with oxygen treatment.
ET-1 expression is suppressed 1.9-fold in the oxygen plus captopril–treated animals at P12 when compared with the oxygen only–treated animals (Fig. 5). At P17, there is a 6.2-fold suppression in ET-1 expression when compared with the oxygen only–treated animals. Captopril decreased retinal ET-1 expression in room air–reared animals at P12 and P17 (Fig. 5). Figure 6 shows a representative gel photograph of the above results.

Animal Growth

Captopril, an ACE inhibitor, could be postulated to decrease blood pressure and alter renal perfusion and therefore could alter growth by affecting the general health of the animals. The animals were weighed on P7, P12, and P17 to 20. No significant weight difference in all four groups of animals was recorded (Table 3).

DISCUSSION

The data presented in this study show a beneficial effect of captopril on OIR. Animals that were treated with captopril during 75% oxygen exposure had significantly reduced retinopathy scores. There were beneficial effects in the retinopathy subscores of blood vessel tufts, extraretinal neovascularization, central vasoconstriction, and blood vessel tortuosity. The number of nuclei extending beyond the inner limiting membrane into the vitreous was also decreased in the animals treated with captopril during hyperoxia.

We show that captopril modulated expression of ET-1 in the neonatal mouse retina. Captopril inhibits the conversion of angiotensin I to angiotensin II, which could block calcium influx into cells, thus decreasing ET-1 expression. ET-1 has been implicated in hyperoxic retinal vascosonstriction,4 and by blocking ET-1 indirectly with an ACE inhibitor, retinopathy is improved. We speculate that captopril may inhibit vasoconstriction (or improve blood flow) during the period of exposure to 75% oxygen, thereby exerting a beneficial effect on the severity of retinopathy.

ET-1 mRNA expression increases with age of the mouse pups. This may be due to an increase in vascularization of the retina over the period from P7 to P17. With increased vascularization, there are more cells to produce ET-1. Thus, ET-1 expression appears to be developmentally upregulated in the mouse retina.

Captopril did not alter growth of the mouse pups. Although blood pressure was not measured in the animals, growth data...
were obtained, and there were no differences observed in captopril-treated versus untreated animals.

Captopril has been shown to decrease retinal ACE levels in streptozotocin-induced diabetic rats, and the authors suggest that captopril may improve retinal complications in diabetes.\(^1\) Captopril has been shown to decrease baseline and hyperoxia-induced endothelin-1 (ET-1) secretion in retinal endothelial cells.\(^3\) ET-1 is a potent vasoconstrictor implicated in regulation of retinal vascular flow.\(^4,13-18\) Our data show that captopril decreases ET-1 mRNA expression as a mechanism for improvement in retinopathy. Retinal vascular tone plays an important role in ocular pathology and modifying vascular tone via ACE inhibition may provide a way to improve outcome in retinopathy.

Captopril has also been described as an antioxidant.\(^19-28\) Oxidant stresses have been described in diabetes,\(^29-30\) particularly in type 1 (or juvenile onset) diabetes.\(^29,30\) Antioxidants have also been implicated in ROP. The thiol group of captopril has been shown by many investigators to scavenge free radicals, and thus may be responsible for the antioxidant effect of captopril. It is possible that captopril exerted an antioxidant effect in the mouse model of OIR.

In addition, captopril has been shown to protect against ischemia–reperfusion injury, especially in myocardial injury.\(^37-43\) The mouse model of OIR has been proposed by other investigators to be a model of ischemia (hypoxia resulting in vascular endothelial growth factor induction after oxygen exposure).\(^44-49\) The improvement in retinopathy observed in the animal model of OIR may also be mediated by captopril’s protection against ischemia–reperfusion in the retina.

Clinical studies showing a beneficial effect of ACE inhibition on diabetic retinopathy have been reported in the literature.\(^2,3,30-52\) The protective effect of ACE inhibition on retinopathy has been postulated to be multifactorial. Improved blood pressure control may delay or halt changes associated with diabetic retinopathy. It has been postulated that vasodilatation with increased blood flow, especially in areas of ischemia may improve ocular outcome.\(^29,30\) Our data may help to explain the protective role of ACE inhibitors in diabetic retinopathy.

ACE inhibitors have been used in premature infants with hypertension. Dosing of captopril (0.1–0.3 mg/kg up to four times per day)\(^53\) is similar to dosing performed in the mice in this study. However, there may be untoward effects such as hypertension and decreased renal perfusion in normotensive premature infants. The mice used in these experiments were born at term and generally healthy as evidenced by the weight data in contrast to the average preterm infant at risk for ROP. Thus, it is premature to investigate systemic captopril therapy for reduction of retinopathy in the extremely low-birth-weight infant.

In summary, captopril may decrease retinal injury and retinal neovascularization by several mechanisms. Captopril may improve retinal blood flow by acting as a vasodilating agent or by inhibiting vasoconstriction. It may decrease oxidative stress by acting as an antioxidant or free radical scavenging agent and reducing tissue injury. Captopril may also protect at a cellular level, by decreasing the injury and/or healing responses of the endothelial cell. The use of an animal model of retinal angiogenesis to clarify clinical observations that ACE inhibition improves retinopathy is particularly important because the clinical studies are often difficult to interpret because of complex medical issues of patients with retinopathy.

References

20. Mak IT, Freedman AM, Dickens BF, Weglicki WB. Protective effects of sulfhydryl-containing angiotensin converting enzyme in...


