Retinal Protective Effects of Resveratrol via Modulation of Nitric Oxide Synthase on Oxygen-induced Retinopathy

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Purpose: Retinopathy of prematurity (ROP) is one of the leading causes of blindness, with retinal detachment occurring due to oxygen toxicity in preterm infants. Recently, advances in neonatal care have led to improved survival rates for preterm infants, and ROP has increased in incidence. In the present study, we aimed to determine whether or not resveratrol exhibits protective effects in an animal model of ROP and in primary retinal cell cultures of neonatal rat via nitric oxide (NO)-modulating actions using western blotting and real-time PCR with inducible nitric oxide synthase (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS) antibodies and mRNAs.

Methods: In an in vivo oxygen-induced retinopathy (OIR) model, cyclic hyperoxia was induced with 80% O₂ for one day and 21% O₂ for one day from P1 to P14 in newborn Sprague-Dawley (SD) rats. Resveratrol was injected intravitreally for seven days and rats were sacrificed at P21. In vitro OIR primary retinal cell culture was performed using P0-2 SD rats. Hyperoxia injuries were induced through 100% O₂ exposure for six hours. Western blotting and real-time PCR using iNOS, eNOS, nNOS antibodies and primers were performed in the rat model of ROP and the dispersed retinal cell culture.

Results: In both in vivo and in vitro OIR, the expression of iNOS antibody and mRNA was increased and of eNOS and nNOS were reduced in the resveratrol-treated group.

Conclusions: In conclusion, resveratrol appeared to exert retinal protective effects via modulation of NO-mediated mechanism in in vivo and in vitro OIR models.

Key Words: Nitric oxide synthase, Retinopathy of prematurity, Resveratrol

Retinopathy of prematurity (ROP) is one of the leading causes of blindness and a complex disease of the immature retina in premature infants. This disease affects approximately 80% of babies born with birthweights less than 1000 g [1]. Preservation of visual acuity in ROP usually requires ablation of the peripheral retina via methods such as cryotherapy and laser photocoagulation therapy [2]. Because of these treatments the incidence of blindness was reduced by 25%. Clinical manifestations of ROP range from mild transient changes of the peripheral retina to severe progressive vasoproliferation resulting in scarring, retinal detachment, and potential blindness. ROP includes all stages of disease such as acute (early) or chronic (late or cicatricial) stages, as well as late sequelae such as high myopia, strabismus, amblopia, diplopia, glaucoma, cataract, viterous hemorrhage, and low vision [3]. Retrolental fibroplasia (RLF), the previous name for this disease, describes only the cicatricial stages. Given the current understanding of ROP, it is recommended that all infants born at less than 1500 g receive regular eye examinations starting at four weeks chronologic age or 31 weeks postconceptional age, whichever is later [4].

After premature delivery, oxygen levels in postnatal tissue are significantly increased compared to those in utero. Additionally, oxygen therapy further increases oxygen levels in the developing retina. This hyperoxia results in vaso-obliteration and destruction of normal retinal vascular development. At that time, low insulin-like growth factor (IGF)-1 levels decrease and hypoxia inducible factor (HIF), as transcriptional factor for vascular endothelial growth factor (VEGF), and VEGF are also reduced. After the return to
normal oxygen levels after the cessation of oxygen therapy, the nonperfused portions of the retina become hypoxic. Retinal hypoxia stimulates IGF-1, HIF, and VEGF before neovascularization. VEGF causes retinal neovascularization and retinal proliferation. VEGF results in extraretinal angiogenesis—stage 3 ROP at the vitreoretinal interface of the ROP ridge. Stage 3 ROP may resolve spontaneously, or may progress to traction retinal detachment and blindness. Further inhibition of IGF-1, HIF, and VEGF decreases retinal neovascularization [5].

In the present study, an in vivo oxygen-induced retinopathy (OIR) model was designed using a rat model of ROP induced by cyclic hyperoxia, exposed to 80% O2 for one day and 21% O2 for another day from P1 to P14 of newborn Sprague-Dawley (SD) rats, as described by Penn et al. [6] Retinal detachment was identified using Hematoxylin and eosin (H&E) staining. An in vitro OIR model was designed using dispersed retinal cell cultures, as described by Seigel [7]. All cells were damaged by oxygen exposure for six hours. Photoreceptors, the major population of neuronal cells in retinal cell culture, were immunolabeled with interphotoreceptor retinoid-binding protein (IRBP) antibody.

Resveratrol (trans-3,5,4′-trihydroxystilbene) is a phytoalexin produced by a variety of plants such as grapes, peanuts, and berries in response to stress, injury, ultraviolet irradiation, and fungal infection [8]. Resveratrol can be detected in the leaf epidermis and the skin of grapes [9]. The “French paradox,” the low incidence of coronary heart diseases in spite of a diet rich in saturated fats has been attributed to a number of contained polyphenols, including resveratrol [10]. Resveratrol has some physiological effects, including prevention of lipid peroxidation in human LDL [11], inhibition of arachidonate acid metabolism [12], inhibition of platelet activity [13], and stimulation of NO production in endothelial cells to exert vasodilatory effect on blood vessels [14].

We investigated resveratrol as a nitric oxide (NO)-mechanism modulator to evaluate the mechanisms of ROP based on molecular biology and pharmacological treatments in the in vivo OIR model, the rat model of ROP, and the in vitro OIR model, the hyperoxic injury of cultured dispersed retinal cells. Recent reports reveal that retinal damage also occurs via NO-mediated mechanisms. Previously, we recognized that resveratrol exhibits neuroprotective effects and cardioprotective effects via modulation of NO-mediated mechanisms [15,16].

In the present study, the protective ability of resveratrol was explored in a rat model of ROP and in primary retinal cell cultures of neonatal rat retinas. We attempted to better define whether resveratrol is a promising treatment of ROP and has preventive mechanisms via NO-modulating actions using western blotting and real-time PCR with inducible nitric oxide synthase (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS) antibodies and mRNAs.

### Materials and Methods

#### Materials

Resveratrol, papain, glucose, and poly-D-lysine were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). Rabbit polyclonal IRBP and secondary goat anti-mouse or rabbit IgG-HRP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal iNOS, rabbit polyclonal eNOS, and rabbit polyclonal nNOS antibodies were purchased from Assay Designs (Stressgen, Ann Arbor, MI, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was purchased from Duchefa (Haarlem, Amsterdam, Netherlands). Hanks’ balanced salt solution (HBSS), Eagle’s Minimal Essential Medium (MEM), fetal bovine serum (FBS), and gentamicin were purchased from Gibco BRL (Grand Island, NY, USA). DNase I was obtained from Takara (Otsu, Shiga, Japan). Complete protease inhibitor cocktail tablets were purchased from Roche Applied Science (Mannheim, Germany). Enhanced chemiluminescence (ECL) and a western blotting detection system were purchased from Amersham Biosciences (Piscataway, NJ, USA). SUPEX was purchased from Neuronex (Pohang, Korea).

#### Animal model of ROP (in vivo OIR)

Postnatal day 1 SD rats were obtained from Samtako (Osan, Korea) or HyoChang Science (Daegu, Korea). We implemented a cyclic oxygen exposure protocol that was modified from previous rat oxygen-induced retinopathy studies [6]. Hyperoxic experiments were conducted in an airtight polypropylene container 295×230×84 mm (3.9 L volume; Lock & Lock, Yongin, Korea) equipped with inlet and outlet ports. The inlet port received 100% medical grade oxygen and the airflow from the outlet was monitored for oxygen content using an oxygen monitor (Hudson RCI, Temecula, NC, USA). The oxygen levels remained above 98% throughout the entire exposure period. The interior of the chamber was maintained at room temperature. Control animals were maintained in room air. The cyclic hyperoxic conditions were performed at 80% O2 for one day and 21% O2 for another day from P1 to P14 in newborn SD rats. The drug was injected intravitreally (into the vitreous humour of the eye) once a day for seven days and the rats were sacrificed at P21.

The animals were divided into three groups. Group 1 (normoxia control, N, n=7) was not exposed to hyperoxia. Group 2 (hyperoxia only, H, n=8) was subjected to hyperoxia without treatment. Group 3 (hyperoxia+resveratrol, HR, n=8) was administered resveratrol (30 mg/kg, intraperitoneal injection).

#### H&E stain

Histologic studies were performed seven days after hyperoxic insult. After sacrifice and intracardiac perfusion...