Original

**Effect of Ethylene Diurea on Oxygen-induced
Ischemic Retinopathy in the Neonatal Rat**

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Abstract : This study investigated the effect of N-[2-(2-oxo-1-imidazolidinyl) ethyl]-N-phenylurea (ethylene diurea, EDU) on oxygen-induced ischemic retinopathy (OIR) in a neonatal rat model. OIR was induced by maintaining daily cycles of 80% oxygen (20.5 h), ambient air (0.5 h), and a progressive return to 80% oxygen (3 h) for 12 days (postnatal day: P12). The rats were treated intraperitoneally with EDU (30 mg/kg body weight) or distilled water (DW) from P6 to P17. At P18, the percentage of avascular areas in the total retinal area (%AVA) was measured, and retinal neovascularization (NV) was scored in ADPase-stained retinas. Retinal superoxide dismutase (SOD) activity in the retina was also determined by a chemiluminescence method. The mean %AVA in the EDU-treated group ($9.3 \pm 1.7\%$, $n = 16$) was lower than in the DW group ($18.2 \pm 4.7\%$, $n = 17$). EDU did not significantly affect NV, but significantly increased SOD activity (1.36 ± 0.13 units/mg protein, $n = 4$) compared to DW treatment (1.04 ± 0.01 units/mg protein, $n = 4$, $P = 0.032$) at P18. These results suggest that EDU treatment decreased the %AVA, accompanied by an increase in normal retinal vascular growth and/or a decrease in vessel proliferation. The increased SOD activity observed in the present study is likely to involve the EDU-mediated effects.

Key words : oxygen-induced retinopathy, EDU, superoxide dismutase, neovascularization, retina

Introduction

Oxidative stress has been linked to retinopathy of prematurity (ROP) through several mechanisms related to the oxygenation of retinal tissue^{1,2)}. ROP is one of the most important causes of blindness and visual impairment in children^{3,4)}, and an effective treatment is required to prevent these vision problems⁵⁾. Oxygen-induced ischemic retinopathy (OIR) in the neonatal rat provides a good model system for studying the vascular changes that occur

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in ROP⁶). This animal model has similarities to the oxygen stresses that preterm infants experience in modern-day neonatal intensive care units in that the affected rats develop an appearance similar to stage 3 ROP with intravitreal neovascularization (NV) at the junction of the vascular and avascular retina. Using the OIR rat model, previous studies have shown that a high oxygen concentration inhibits vascular growth in the retina and subsequent normal oxygen concentration in the air promotes NV⁶). During angiogenesis in the OIR retina, several angiogenic factors have been identified including vascular endothelial growth factor (VEGF)⁷⁻⁹, VEGF receptor-1 (VEGFR-1)⁹, insulin-like growth factor 1 (IGF-1)^{9,10}, and matrix metalloproteinases (MMPs)¹¹; these factors are promoted by reactive oxygen species (ROS). Superoxide dismutase (SOD) is involved in the metabolism of ROS, catalyzing the dismutation of O_2^- to H_2O_2 . H_2O_2 is generally metabolized by peroxisomal catalase, while glutathione peroxidase metabolizes mitochondrial or cytosolic H_2O_2 . Therefore, increased SOD activity may inhibit NV in the OIR retina. The experimental chemical, ethylene diurea (EDU: N-[2-(2-oxo-1-imidazolidinyl)ethyl]-N-phenylurea) was reported to protect plants from otherwise damaging doses of the air pollutant, ozone (O_3)^{12,13}. EDU promoted the activity of SOD and catalase in the leaves of a normally O_3 -sensitive snap bean cultivar and protected against acute and chronic foliar injuries due to O_3 ^{14,15}. EDU has also induced SOD and catalase activity both *in vitro* (human gingival fibroblasts)¹⁶ and *in vivo* (rat heart, liver, and lung) without adverse effects¹⁷. This study investigated the effect of EDU on retinal NV in the OIR neonatal rat model.

Materials and Methods

All animals were cared for in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The Institutional Committee of Animal Care and Use at our institution approved the study protocol.

Animal model

Female Sprague-Dawley rats at 14 days gestation were purchased from CREA Japan Co., Ltd. (Tokyo, Japan). Rats were divided into two groups: room-air and oxygen-exposed. In both groups, 13 or 14 neonatal rats were kept with a mother in each cage after birth. Mother rats were rotated between the room-air and oxygen-exposed groups every two days during the experimental period. Retinal NV was induced in neonatal rats by our standard protocol⁶. The oxygen-exposed rats were exposed from birth to day 12 (postnatal day: P12) to daily cycles of 80% oxygen (20.5 h), room air (0.5 h), and then a progressive return to 80% oxygen (3 h). On P12, the oxygen-exposed rats were placed in room air until the end of experiments at P18 (Fig. 1).

Treatment schedule

The rats were treated by intraperitoneal (i.p.) injection of either 30 mg/kg EDU or

Fig. 1. The treatment schedule

distilled water (DW) in a constant volume of $50 \mu\text{l} / 10 \text{ g}$ body weight once daily from P6 to P17. Body weight was measured every 2 days from P0 to P18.

Retinal processing and analysis

At P18, the rats were sacrificed by i.p. injection of 0.3 ml sodium pentobarbital (50 mg/ml) and enucleated eyes were fixed in 4% paraformaldehyde in cacodylate buffer (0.1 M, pH7.2) for 48 hours, stained with ADPase, and flat-mounted⁶⁾. Digital images of the ADPase-stained retinas were obtained using a camera and scanner (Nikon, Tokyo, Japan), with retinal NV scored using the method of Hasebe *et al*¹⁸⁾. The intensity of NV in each retina was scored as thickened vascular long ridge: 4, short ridge: 3, five or more glomerular buds: 2, less than five glomerular buds: 1, and none observed: 0. Avascular areas (AVAs) were also measured in the ADPase-stained retinas, with the avascular and total retinal areas measured using NIH image software (NIH, Bethesda, MD). The mean percentages of AVAs per total retinal area (%AVAs) were calculated.

Quantification of vascular endothelial growth factor (VEGF) protein and SOD activity

Retinal samples were collected from the sacrificed rats and immersed in $100 \mu\text{l}$ of tissue protein-extraction reagent (T-PER, Thermo Fisher Scientific Inc, Rockford, IL) containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO; 1:100), and then stored at -40°C until analysis. The samples were then cut into small pieces with scissors, sonicated in 5-s bursts at 40 W (Branson Sonifier Cell Disruptor 200, Branson, Rochester Hills, MI), and centrifuged at 3000 rpm for 10 min. VEGF in the supernatant was assayed using a rat VEGF immunoassay kit (R&D Systems, Minneapolis, MN). SOD activity was determined by a chemiluminescence method¹⁹⁾, based on the inhibition of integrated light intensity

(A) DW treatment (B) EDU treatment

Fig. 2. Rat oxygen-induced ischemic retinopathy (OIR) model. The typical samples of flatmounted ADPase stained retinas at P18. (A) DW group, NV score is 9, %AVA is 25.9. (B) 50 mg/kg EDU-treated group, NV score is 7, %AVA is 11.7.

generated by O_2^- production in biological systems. The reaction mixture contained 25 mM 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo [1,2-a] pyrazin-3-one hydrochloride (MCLA : Fluka, Tokyo), 25 mM hypoxanthine (Sigma-Aldrich), and 3.38×10^{-6} units xanthine oxidase (Sigma-Aldrich) in 42.5 mM Tris buffer (Wako, Osaka), pH 7.4. Reactions were carried out at 25°C and the chemiluminescence was measured using a Luminescence reader BLR-301 (Aloka, Tokyo).

Protein concentrations were determined using a BCA protein assay reagent kit (Thermo Fisher Scientific, Rockford, IL) using bovine serum albumin as the standard.

Statistical Analysis

Statistical analysis was performed using the Mann-Whitney U-Test. Results were considered statistically significant when the *P* value was less than 0.05.

Results

The body weight of rats treated with EDU increased similar to the DW-treated controls in both the room-air exposed and OIR groups. Normal retinal vascular development was observed in all animals in the room-air groups (NVs were not observed in the DW- and EDU-treated groups). OIR retinas exhibited neovascular changes and retained avascular areas (Fig. 2). In the DW- and EDU-treated groups, %AVAs were $18.2 \pm 4.7\%$ ($n = 17$) and $9.3 \pm 1.7\%$ ($n = 16$), respectively (Fig. 3), while NV scores were 6.0 ± 0.96 and 5.94 ± 0.82 , respectively (Fig. 4). The EDU treatment therefore suppressed %AVA, but did not influence the NV extent in the OIR rat retina.

The quantity of VEGF protein in the OIR retinas increased significantly from P12 to P13 : from 71.7 ± 15.0 pg/mg protein to 588.2 ± 229.4 pg/mg protein in the DW group and

Fig. 3. Effect of 50 mg/kg EDU-treatment on %AVA in the OIR. The open column shows %AVA in DW group (n = 17) and the closed column shows %AVA in EDU treated group (n = 16). The data represent mean ± SE (*: P < 0.05).

Fig. 4. Effect of 50 mg/kg EDU-treatment on NV score in the OIR. The open column shows NV score in DW group (n = 17) and the closed column shows NV score in EDU treated group (n = 16). The data represent mean ± SE.

from 59.9 ± 16.1 pg/mg protein to 493.7 ± 92.9 pg/mg protein in the EDU-treated group, and at P18, it decreased to 136.7 ± 52.3 pg/mg protein in DW group and 136.2 ± 47.5 pg/mg protein in the EDU-treated group (Fig. 5). In the room-air group, VEGF levels were lower than in OIR animals at each time point. In the EDU-treated group, VEGF levels were 272 ± 6.3 pg/mg protein at P12, 28.0 ± 2.9 pg/mg protein at P13, and 32.7 ± 4.3 pg/mg protein at P18, while in DW-treated rats the comparable values were 23.5 ± 2.7 , 24.2 ± 14.6 and 33.7 ± 3.5 pg/mg protein, respectively. Thus, EDU had no effect on VEGF levels in either the OIR or room-air groups.

Fig. 5. Effect of EDU treatment on VEGF contents in retina. The content of VEGF significantly increased at P13 in OIR retina (open column: DW group, closed column: EDU-treated group). The spotted and the slanting line column show the contents of VEGF in DW and EDU-treated group in the room-air group. The data represent mean $\pm$ SE pg/mg protein ($n = 3$ for each point). *: $P < 0.05$ compared to the value at P12 in each group.

SOD activity was significantly increased in the OIR group at P18 following EDU treatment (1.36 ± 0.13 units/mg protein, $n = 3$) compared with those treated with DW (1.04 ± 0.01 units/mg protein, $n = 3$). In the room-air group, the SOD activity was 2.09 ± 0.26 units/mg protein in the EDU-treated group and 1.79 ± 0.51 units/mg protein in the DW group at P18 (Fig. 6).

Discussion

In the present study, both %AVAs and NV scores were significantly higher in the oxygen-exposed rat retinas compared to those in room air. These findings confirmed that our OIR neonatal rat model is a suitable model for the present study^{8,20}.

Oxidative stress has been linked to ROP through several mechanisms related to oxygenation of retinal tissue and O_2^- production¹. The retina is susceptible to oxidative damage given its high metabolic rate and rapid rate of oxygen consumption²¹. In addition, the premature infant has a reduced ability to scavenge ROS²², increasing its vulnerability to oxidative stress, which could also increase apoptosis and damage retinal tissues²³. The major source of O_2^- in cells results from NAD(P)H oxidase activity. Saito *et al*²⁴ reported that the NAD(P)H oxidase inhibitor, apocyanin, reduced avascularity and apoptosis in the OIR

Fig. 6. Effect of EDU treatment on SOD activity in OIR and the room-air control retina. The columns are expressed as same as Fig. 5. The abscissa shows SOD activity in the retina (unit / mg protein). The data represent mean $\pm$ SE units / mg protein ($n = 4$ for each point, *: $P < 0.05$).

rat retina through pathways triggered by ROS generation²⁴). The cause of the increased %AVA in this model remains unclear. However, cleaved caspase-3 was observed at vessels in the OIR retina, which may activate apoptosis²²), thereby inhibiting blood vessel growth, and increasing the %AVA.

Exogenously administered SOD and catalase are protective in animal models of oxygen-derived free radical tissue injury^{25,26}). In this study, SOD activity in the OIR rat retinas was increased to 131% by the EDU treatment and catalyzed O_2^- dismutation in the retina. These data suggested that the decreased %AVA in EDU-treated animals was due to increased O_2^- catalysis. On the other hand, increased SOD activity leads to increased H_2O_2 generation, and thus might trigger angiogenesis²⁷⁻²⁹). However, NV was not observed with EDU treatment in the room air-exposed rats in this study. Hence, the noted increase in SOD activity did not enhance VEGF synthesis. We speculate therefore that the increased H_2O_2 was immediately metabolized by catalase and glutathione peroxidase in the retina in this study.

ROP progresses in two phases. The first phase begins with delayed retinal vascular growth after birth in a relatively hyperoxic condition and partial regression of existing vessels, followed by a second phase of hypoxia-induced pathological vessel growth¹). We speculate that the oxygen exposure period from P1 to P12 in our OIR neonatal rat model simulated the first phase, and the subsequent normal oxygen concentration in the air period

from P12 to P18 simulated the second phase of ROP progression. During the first phase, retinal vascular growth after birth might be enhanced by EDU treatment, due to the catalysis of O_2^- dismutation; thus, the %AVA in EDU-treated rats was lower than in the DW group. At P13, the levels of VEGF significantly increased in both the DW- and EDU-treatment groups by the relatively hypoxic conditions, but VEGF expression was not influenced by EDU treatment in the OIR rat retina. This phenomenon might also have resulted in the unchanged NV scores between the treatment groups. During the second phase, NV is stimulated by triggering VEGF signaling, inflammatory pathways, and TNF-alpha or other cytokines³⁰⁾, with subsequent vitreous migration. Bleeding easily occurs in these immature blood vessels and wound-healing NV starts³¹⁾.

VEGF-A was also recently shown to differentially regulate angioblast sprouting behavior by promoting the expression of Notch ligand *delta-like 4*^{32,33)}, which regulates the formation of the appropriate tip-cell numbers to control vessel sprouting and branching in the mouse retina³²⁾. Notch-inhibitory gamma-secretase inhibitors raise tip-cell numbers, leading to excessive vascular sprouting and fusion without VEGF-A expression change³²⁾. Future studies should therefore focus on the cellular steps comprising the first and the second phases of ROP in the OIR model to resolve a causative mechanism.

The mechanism by which EDU induces SOD and catalase activities is unclear. EDU does not act as a scavenger of O_2^- in the hypoxanthine and xanthine oxidase generation system in this experiment. It is interesting that EDU treatment has the potential to reduce the retinal avascular area and lead to increased retinal development in ROP.

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