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Early treatment of recombinant Akt protects the retina from oxygen-induced injury in mice

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Received February 26, 2019, accepted March 30, 2019

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Pharmazie 74: 428-431 (2019) *doi: 10.1691/ph.2019.9416*

Akt, or protein kinase B, is an important signaling molecule that modulates many cellular processes such as cell growth, survival, and metabolism. However, the *in vivo* roles and effectors of Akt in retinal angiogenesis are not explicitly clear. We therefore investigated the effects of recombinant Akt on inhibiting vessels loss and on suppressing experimental retinal neovascularization in this model. We showed that, compared with control groups, administration of the recombinant Akt in the first phase of retinopathy markedly reduced capillary-free areas, and significantly decreased retinal neovascularization. These results indicate that Akt plays a critical role in the pathological process (vessels loss and neovascularization) of oxygen-induced retinopathy in a mouse model, which may provide a valuable therapeutic tool for ischemic-induced retinal diseases.

1. Introduction

Oxygen therapy is administered to decrease tissue hypoxia and to relieve arterial hypoxemia in patients with acute and chronic cardiovascular diseases and to premature babies with respiratory distress syndrome. Retinopathy of Prematurity (ROP) is a disorder of the developing retina, and one of the leading causes of blindness in infants (Dave et al. 2012). There are many risk factors leading to progression of ROP including low birth weight, gestational age, supplemental oxygen therapy, sepsis, blood transfusions, respiratory distress syndrome and genetics (Chen et al. 2011; Hakeem et al. 2012; Alpay et al. 2012; Giusti et al. 2012). However, gestational age, low- birth weight and supplemental oxygen are the most widely accepted major risk factors. ROP has been described as a two-phase disease, beginning with delayed vascular growth after premature birth (phase I – hyperoxic phase), followed by the release of hypoxia stimulated factors to stimulate new blood vessel growth (Phase II – hypoxic phase) (Smith et al. 1994). In the first phase, hyperoxia induces cessation of normal vessel growth and regression of existing vessels, subsequently causing obliteration of normal retinal vessels. In the second phase, along with hyperoxia-induced vessel loss and subsequent relative hypoxia, the overexpression of pro-angiogenic substances including VEGF, angiopoietin, NO, EPO, IGF-1, TNF, etc, causes the development of retinal neovascularization, which is thought to occur in response to ischemic and hypoxic insult (Frank et al. 1991), This leads to alterations in the existing vasculature and compensatory, albeit pathological, new capillary growth (Grant et al. 1986; Limb et al. 1996). That is to say, the insufficient blood supply resulting from early vessel loss causes tissue ischemia and hypoxia, which determines the severity of subsequent pathological retinal vessel growth.

Akt, also named protein kinase B, is activated by several growth factors and cytokines, and serves as a multifunctional regulator of cell biology, glucose metabolism and protein synthesis (Franke et al.1995; Hemmings 1997a, b). Numerous studies have demonstrated that Akt activation plays an important role in inhibiting cell apoptosis in fibroblasts, epithelial and lymphoid cell lines, and neuronal cells (Ahmed et al. 1997; Kulik et al. 1997; Kennedy et al. 1997; Kauffmann-Zeh et al. 1997; Dudek et al. 1997; Hemmings 1997c). However, these studies with Akt have been carried out *in vitro* and the *in vivo* significance of this pathway in protection from cell death induced by different stimuli including hyperoxic stress remains to be investigated.

Previous treatments of ROP focused on phase II (hypoxic phase, retinal neovascularization), including laser photocoagulation and intravitreal ranibizumab injection, but these measures cannot prevent progression of this aberrant angiogenesis and usually causes inflammation and tissue destruction (Bandello et al. 2001). We hypothesise that correcting retinal Akt deficiency during the first phase of retinopathy (phase I – hyperoxic phase) with exogenous Akt could protect the postnatal mouse retina from hyperoxia-induced vessel loss, thereby reducing retinal neovascularization in phase II of ROP.

Here we demonstrate that a constitutively recombinant Akt introduced intravitreally into the mice with oxygen-induced retinopathy (OIR) in phase I – hyperoxic phase protects mice from oxygen-induced retinal neovascularization.

2. Investigations and results

2.1. Use of exogenous Akt in the first phase can prevents retinal vessel loss and retinal neovascularization

We examined whether exogenous Akt treatment during the first phase of oxygen-induced retinopathy helps to prevent retinal vessel loss and subsequent retinal angiogenesis. At P17, mice treated with recombinant Akt had a capillary-free/total retinal area of 7.2±0.8 % compared with 31.4±0.3 % in PBS-injected littermate controls (P≤0.001; Fig. 1A, B), suggesting that recombinant Akt treatment had a protective effect. Retinal neovascularization was also evaluated by measuring the neovascular tufts area. At P17, mice treated with recombinant Akt had a pathologic neovascular tufts (4.7±0.4 %) compared with PBS control (14.0±0.8 %; P≤0.001; Fig. 1A, C). Our data suggest that recombinant Akt treatment during the first phase of retinopathy protects the retina from vessel loss and subsequent pathological proliferation.

3. Discussion

The focus of the current study was to test the effects of exogenous Akt in preventing retinal vessel loss and neovascularization *in vivo*.

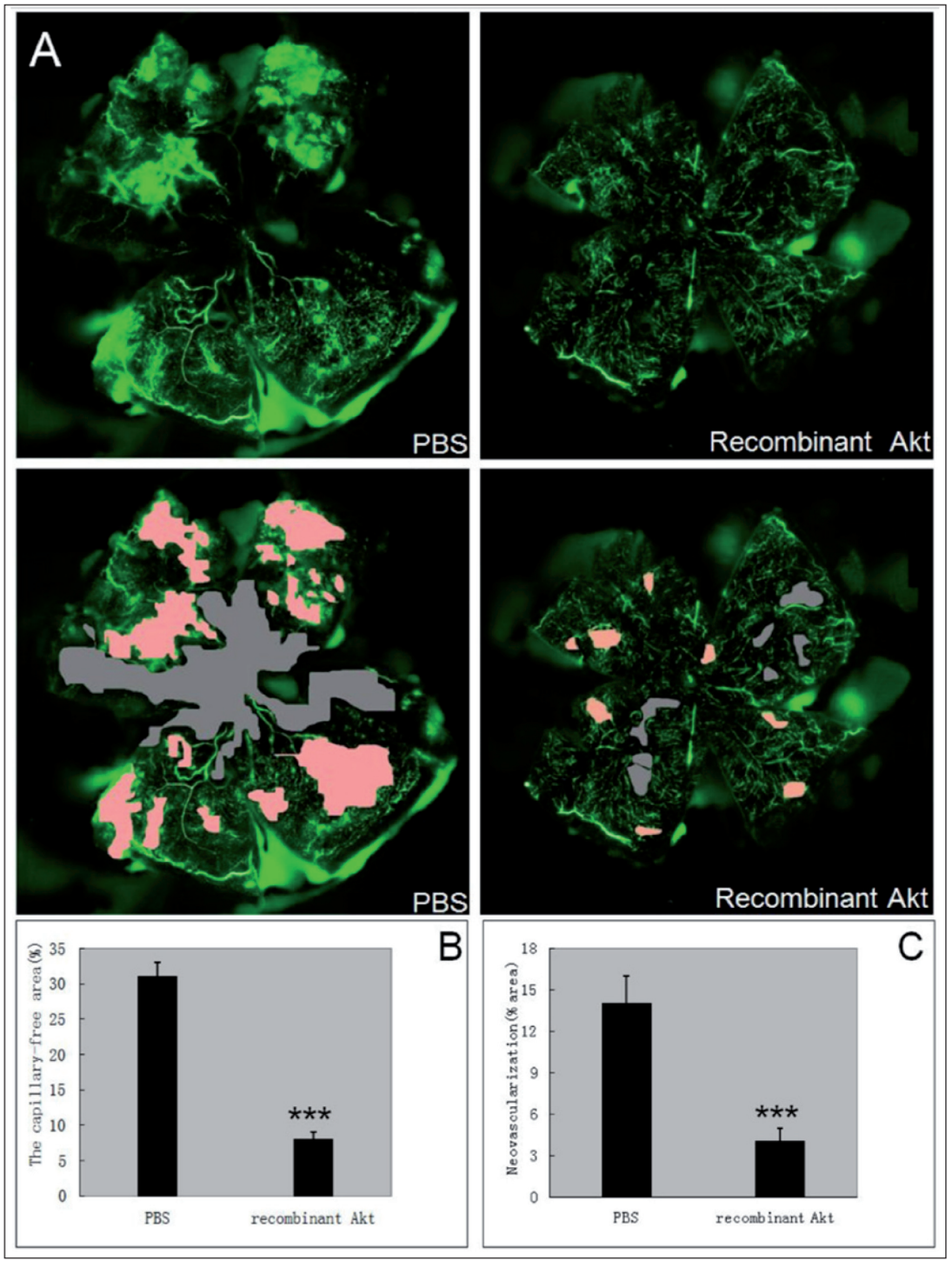

Fig. 1: Recombinant Akt treatment suppressed retinal vessel loss and neovascularization in a murine oxygen-induced retinopathy model. (A) Representative retinal whole-mounts showing area of capillary-free and neovascularization after 5 days of relative oxygen deficiency exposure and intravitreal injections (P6 and P7) of recombinant Akt and PBS. Areas of capillary-free (gray) and neovascularization (pink) were quantified. Original magnification×5. (B)The capillary-free areas and the total areas were quantified. Data are shown as mean±SEM. (PBS, n=10; recombinant Akt, n=15; ***P≤0.001). (C)The areas of neovascularization and the total areas were quantified. Data are shown as mean±SEM. (PBS, n=10; recombinant Akt, n=15; ***P≤0.001).

Our results show that early use of recombinant Akt can prevent retinal vessel loss and also can apparently inhibit retinal neovascularization in mice with oxygen-induced retinopathy.

In the phase I – hyperoxic phase of oxygen-induced retinopathy, hyperoxia-induced vessel loss is caused by apoptosis of vascular endothelial cells, and also hyperoxia suppresses VEGF expression (Mazure et al. 1997) and IGF-1 levels. In our previous studies, we found that Akt mRNA, Akt and p-Akt expression was greatly suppressed under hyperoxia environment (Wang et al. 2011). These data suggest that lack of Akt in the hyperoxic phase might contribute to early vessel loss and subsequent retinal neovascularization. In consideration of the antiapotosis effects of Akt, we treated mice early with recombinant Akt. It helped to prevent the vessel loss and ischemia, thereby suppressing subsequent retinal neovascularization. Early use of Akt protects the retina from hyperoxia-induced vessel loss, which is consistent with previous

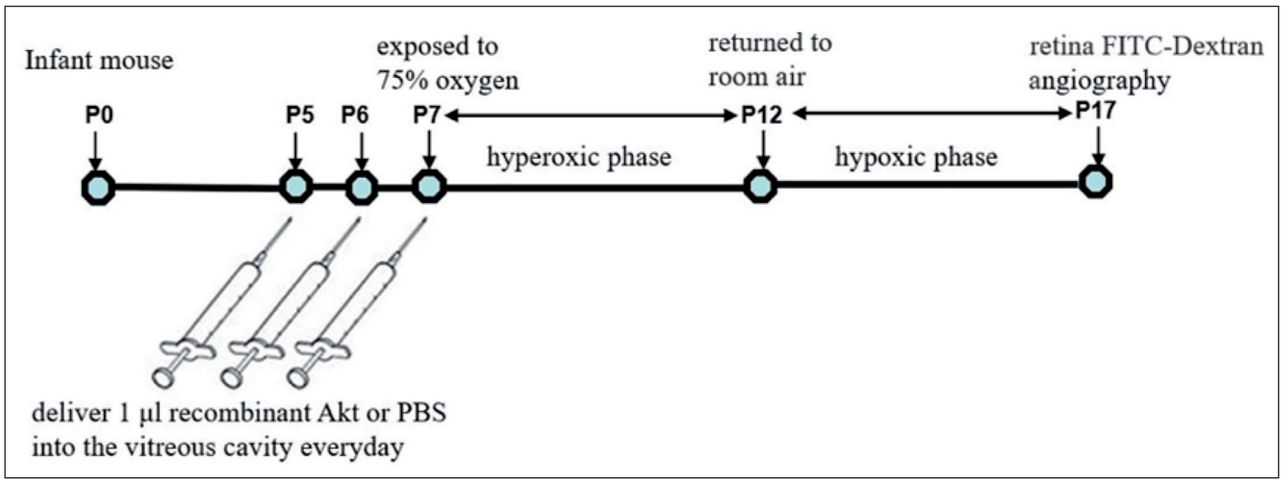

Fig. 2: Simplified diagram of the experimental method.

studies that have found that activated Akt protects the lung from oxidant-induced injury and delays the death of mice (Lu et al. 2001). These findings suggest the important possibility of treating retinopathy patients early with Akt to prevent retinal ischemia without provoking neovascularization.

Akt plays a very central role in promoting the survival of a wide range of cell types (Zhan and Han 2004), and several studies indicate that Akt activation plays an important role in inhibiting cell apoptosis in fibroblasts, epithelial and lymphoid cell lines, and neuronal cells (Kennedy et al. 1996). In ECs, the majority of growth factor-induced responses are mediated by the activation of the Akt signaling pathway (Shiojima and Walsh 2002). Vascular endothelial growth factor (VEGF) displays multiple biological activities in endothelial cells, including the enhancement of retinal endothelial cell survival (Alon et al. 1995). Thus, activation of Akt seems to be a general antiapoptotic mechanism induced by proangiogenic stimuli. Because apoptosis of endothelial cells can act against angiogenesis, we hypothesised that the Akt signaling pathway leading to apoptosis suppression may significantly contribute to angiogenesis, a process required for the revascularization of ischemic tissue. Akt signaling is important for normal vascular development, since depletion of Akt leads to angiogenic defects (Chen et al. 2005). Akt is expressed in the retina suggesting that it is an endogenous retinal survival factor, and that the Akt pathway can increase endothelial cell proliferation and protect vasculature counteract ischemia and apoptosis.

In summary, we have shown that recombinant Akt has potent inhibitory effects on the vessel loss and the subsequent development of retinal neovascularization. Local treatment can produce less toxicity than systemic delivery, so we chose to deliver recombinant Akt by intravitreal injection. We did not observe toxicity of recombinant Akt in this study. These data suggest that targeting Akt function could provide a novel approach for treatment of angiogenesis in retinal neovascularization diseases.

4. Experimental

4.1. Animal model of proliferative retinopathy

We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during this research. A reproducible model of oxygen-induced retinal neovascularization has been described previously in detail (Smith et al. 1994). Briefly, seven-day-old C57BL/6J (P7) mice (Laboratory Animal Center of Central South University) were exposed to 75±2 % oxygen (hyperoxia) for 5 days with the nursing mothers and then returned to room air for 5 days, producing retinal ischemia and neovascularization by P17. Mice of the same strain and of the same age were kept in room air and used as normoxia controls (Fig. 2).

4.2. Angiography with high-molecular-weight fluorescein-dextran

Mice were deeply anesthetized by intraperitoneal pentobarbital sodium and sacrificed by intracardiac perfusion with PBS containing 1 ml of 50 mg/ml FITC-dextran (molecular weight 2,000,000, Sigma-Aldrich Chemical Co. St Louis, MO, USA). Subsequently, the eyes were enucleated and fixed in 4 % paraformaldehyde for 1 h at room temperature. The retinas were dissected and flat mounted on microscope slides with glycerol gelatin. Images of each of the 4 quadrants of whole mounted retina were taken at ×5 magnifications on a Leica DMI 4000B confocal microscope (Wetzlar, Germany) and imported into Adobe Photoshop software. Retinal segments were merged to produce an image of the entire retina.

4.3. Recombinant Akt intravitreal injections

For evaluation of vessel loss and neovascularization, at P5, P6 and P7, mice were anesthetized by intraperitoneal injection of pentobarbital sodium (Fig. 2). The lid fissure was opened using a No11 scalpel blade and the eye was proposed. Intravitreal injections were performed by first entering the eye with an Ethicon TG140-8 suture needle at the posterior limbus. A 32-gauge Hamilton needle and syringe were used to deliver 1 µl recombinant Akt (Abnova Taiwan Corporation, Taipei City, Taiwan.) into the vitreous cavity. Control PBS was injected into littermates. The eye was then repositioned and the lids were approximated over the cornea. After oxygen exposure and back in room air, eyes from P17 mice were perfused with FITC-dextran. Retinal vessel loss and neovascularization were analyzed with FITC-dextran at P17.

4.4. Statistics

The results were given as mean±SEM. One-way ANOVA followed by the LSD t-test was used to evaluate significant differences. A p value < 0.05 was considered statistically significant.

Conflict of interest: The authors have no conflicts of interest.

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