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Eye drop propranolol administration promotes the recovery of oxygen-induced retinopathy in mice

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ABSTRACT

The mouse model of oxygen-induced retinopathy (OIR) is a well-established model of retinopathy of prematurity (ROP), characterized by the abnormal formation of new blood vessels, which is similar to ROP. In this model, we have recently shown that subcutaneous (sc) administration of the non-selective beta-adrenergic receptor (β-AR) blocker propranolol ameliorates angiogenic processes in the retina when its effects are evaluated at postnatal day (PD) 17. In the present study, we investigated whether propranolol application as collyrium can promote the recovery of OIR. After propranolol administration on the eye, mice were first tested for retinal concentrations of propranolol as compared with those measured after sc or *per os* administration. Subsequently, we determined the effects of propranolol ophthalmic solutions, at the optimal dose for delivery, on VEGF, IGF-1, hypoxia-inducible factor (HIF)-1α, signal transducer and activator of transcription 3 (STAT3) and retinal neovascularization as assessed in both the superficial and the deep vascular plexuses. The results showed that 2% topical propranolol has an efficiency (in terms of final propranolol concentration in the retina) comparable to that of 20 mg/kg propranolol sc or *per os* and significantly higher than those observed with doses and administration routes that are currently used with children. Propranolol ophthalmic solutions reduced VEGF and IGF-1 up-regulation in response to hypoxia and drastically inhibited HIF-1α accumulation and STAT3 phosphorylation. As a result of its inhibitory effects on hypoxia-induced proangiogenic factors, propranolol significantly reduced retinal neovascularization in the superficial but not in the deep vascular plexus. An evaluation of retinal neovascularization at PD21 showed that propranolol was still effective in inhibiting OIR. These findings strengthen the hypothesis that β-AR blockade can efficiently counteract OIR and suggest that topical eye application of propranolol can represent an alternative delivery route to systemic administration thus avoiding the risk of associated complications and side effects that could make this drug unsafe in the ROP treatment.

1. Introduction

Retinopathy of prematurity (ROP) is a major cause of blindness and visual impairment in children in both developing and developed countries around the world (Gilbert, 2008). ROP is a multifactorial disease characterized by perturbation of normal vascular development in the retina. The pathogenesis of ROP is hypothesized to consist of two distinct phases of which the second phase is characterized by hypoxia-induced up-regulation of vascular endothelial growth factor (VEGF) and retinal neovascularization (Madan and Penn, 2003). The features of ROP are well depicted in the model of oxygen-induced retinopathy (OIR; Smith et al., 1994). In response to hypoxia, neovascular tufts are formed in the superficial vascular

Abbreviations: β-AR, beta-adrenergic receptor; Ct, cycle threshold; DBS, dried blood spot; ELISA, enzyme-linked immunosorbent assay; HIF-1, hypoxia-inducible factor 1; HIF-1α, α subunit of HIF-1; IGF-1, insulin-like growth factor 1; NE, norepinephrine; OD, optical density; OIR, oxygen-induced retinopathy; PB, phosphate buffer; PD, postnatal day; PVDF, polyvinylidene difluoride; QPCR, quantitative real-time RT-PCR; ROP, retinopathy of prematurity; sc, subcutaneous; STAT3, signal transducer and activator of transcription 3; pSTAT3, phosphorylated form of STAT3; VEGF, vascular endothelial growth factor.

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plexus of the mid-peripheral retina. Both the superficial and the deep vascular plexuses show capillary loss in the central retina (Stahl et al., 2010). The proliferative phase of neovascularization reaches its maximum at PD17. After PD17, spontaneous regression of neovascularization and reduction of the avascular area can be observed (Lange et al., 2009).

As understanding of the heterogeneous pathophysiology of ROP has increased, emphasis has shifted to selective therapies that target components of the angiogenesis cascade. In this respect, there are some indications that the β-adrenergic system may interfere with ROP in infants. For instance, polymorphisms of intracellular effectors linked to beta-adrenergic receptor (β-AR) function existing in many African American infants seem to be responsible of the lower incidence of ROP progression in these infants as compared to non-African American infants (Good et al., 2012). In addition, β-AR blockade with propranolol has been reported to induce involution of infantile hemangioma (Léauté-Labrèze et al., 2008; Sans et al., 2009), the most common tumor of infancy that is often associated with ROP (Praveen et al., 2009), suggesting that β-AR blockers might be effective in ROP as well.

Recently, we have hypothesized that the β-adrenergic system may interfere with ROP by influencing the angiogenic cascade in the retina. In the mouse model of OIR we have demonstrated that β-AR blockade is protective against pathologic retinal angiogenic processes suggesting that β-AR blockers may be efficacious in counteracting ROP (Martini et al., 2011; Ristori et al., 2011). In this respect, in preterm newborns suffering from a precocious phase of ROP, a study protocol aimed at evaluating safety and efficacy of propranolol in counteracting the progression of retinopathy has been recently published (Filippi et al., 2010).

This previous report of β-AR blockade protective effects against OIR has been contradicted by recent results produced in the same model, but in a different mouse strain in which propranolol fails to suppress retinopathy development (Chen et al., 2012a).

Aim of the present study was to re-evaluate the efficacy of propranolol treatment in retinopathy using topical propranolol as an alternative to systemic propranolol and avoiding high risk of associated complications that could make this drug unsafe for use in premature infants (see for review Starkey and Shahidullah, 2011). There is evidence that administration of propranolol as opthalmic solutions is the preferred route to deliver the drug to the ocular anterior tissues (Hao et al., 2011) while no data are reported about its delivery to the posterior segment of the eye. To the best of our knowledge, this is the first study in which propranolol ophthalmic solutions are used to target the retina. We chose to develop a topical therapy using propranolol dissolved in commercially available artificial tears. This agent was first tested for the optimal dose for delivery by comparing the retinal concentrations of propranolol after topical application to those obtained after subcutaneous (sc) or *per os* administration. Subsequently, the effects of propranolol ophthalmic solutions on proangiogenic factors and retinal neovascularization were determined.

2. Materials and methods

2.1. Reagents

Propranolol hydrochloride (propranolol; 99% purity), the mouse monoclonal antibody directed to β-actin and the rabbit anti-mouse horseradish peroxidase-labeled secondary antibody were purchased from Sigma–Aldrich (St. Louis, MO). Commercially available artificial tears were from Farmigea SpA (Pisa, Italy). The labeled standard D_7-propranolol (99% purity) was obtained from GC-TMS (Roma, Italy). The filter paper used in the measurement of plasma propranolol was from Whatman (Dassel, Germany). The RNeasy Mini Kit, the QuantiTect Reverse Transcription Kit and the SYBR Green PCR Kit were from Qiagen (Valencia, CA). The ELISA kits for the detection of VEGF and insulin-like growth factor 1 (IGF-1) were obtained from R&D Systems (Minneapolis, MN). The protease inhibitor cocktail Complete and the phosphatase inhibitor cocktail PhosStop were from Roche Applied Science (Indianapolis, IN). Polyvinylidene difluoride (PVDF) membrane was obtained from GE Healthcare (Piscataway, NJ). The mouse monoclonal antibodies directed to the α subunit of hypoxia-inducible factor-1 (HIF-1α) or the phosphorylated form of the signal transducer and activator of transcription 3 (pSTAT3), the rabbit polyclonal antibody directed to STAT3 and the mouse anti-rabbit horseradish peroxidase-labeled secondary antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). The enhanced chemiluminescence reagent was from Millipore (Billerica, MA). The rat monoclonal antibody directed to CD31 was obtained from BD Pharmingen (San Diego, CA). The secondary antibody Alexa Fluor 488 was from Molecular Probes (Eugene, OR). All other chemicals and solvents were obtained from Sigma–Aldrich.

2.2. Animals

Procedures involving animals were carried out in agreement with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and in compliance with the Italian guidelines for animal care (DL 116/92) and the EU Directive (2010/63/EU). Procedures were approved by the Ethics Committee in Animal Experiments of the University of Pisa. All efforts were made to reduce both animal suffering and the number of animals used. Two month-old male and female mice (C57BL/6J strain) were originally purchased from Charles River Laboratories Italy (Calco, Italy) and were mated in our breeding colony. Experiments were performed on a total of 72 mouse pups of both sexes. Mice were sacrificed at PD17 (5.87 ± 0.64 g body weight). In some experiments, mice at PD21 were also used (10 animals; 6.59 ± 0.55 g body weight). Animals were kept in a regulated environment (23 ± 1 °C, 50 ± 5% humidity) with a 12-h light/dark cycle (lights on at 8 AM) with food and water ad lib. In all experiments, mice were anesthetized with halothane (4%), sacrificed by cervical dislocation and the eyes were enucleated.

2.3. Model of oxygen-induced retinopathy

In a typical model of OIR (Smith et al., 1994), litters of mice pups with their nursing mothers were exposed in an infant incubator to high oxygen concentration (75% ± 2%) between PD7 and PD12, prior to return to room air between PD12 and PD17. Oxygen was checked twice daily with an oxygen analyzer (Pro-Custom Elettronica, Milano, Italy). Individual litters were either oxygen or room air reared. Topical administration of propranolol was performed in animals anesthetized by i.p. injection of Avertin (1.2% tribromoethanol and 2.4% amylene hydrate in distilled water, 0.02 mL/g body weight). All experiments were performed at the same time of day to exclude possible circadian influences. The data were collected from both males and females and the results combined as there was no apparent gender difference.

2.4. Pharmacological treatment

Propranolol is a non-selective β-AR blocker. It is used at doses up to about 2 mg kg^{-1} day^{-1} to treat hemangiomas with considerable success in promoting tumor regression, although its mechanism of action is unclear (Starkey and Shahidullah, 2011). To date there are few reports of controlled prospective trials of propranolol safety and efficacy (Hogeling et al., 2011). In addition, only few data on the application of propranolol as a collyrium have been reported (Hao