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# Ruboxistaurin, a PKCβ inhibitor, inhibits retinal neovascularization *via* suppression of phosphorylation of ERK1/2 and Akt

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ABSTRACT

Ruboxistaurin, a protein kinase C (PKC) β inhibitor, exhibits significant anti-angiogenic activity that reduces the response of vascular endothelial cells to stimulation by vascular endothelial growth factor (VEGF). In addition, the activation of PKC, specifically PKCβ, plays a key role in the retinal neovascularization. However, the effect of ruboxistaurin on oxygen-induced retinopathy (OIR), an experimental murine model of proliferative retinopathy, has not yet been investigated. In this study, we assessed the efficacy of ruboxistaurin both *in vitro* and *in vivo* and also evaluated its anti-angiogenic mechanisms. Ruboxistaurin inhibited formation, proliferation, and migration of VEGF-induced human umbilical vein endothelial cells (HUVECs) in a concentration-dependent manner. It also inhibited the VEGF-induced phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) and serine/threonine protein kinase family protein kinase B (Akt). In *in vivo* retinal neovasuclarization experiments, induced in neonatal mice by returning the retina to normoxia (21% $O_2$) after exposure to hyperoxia (75% $O_2$), ruboxistaurin given subcutaneously significantly reduced pathologic vascular changes. No effect was seen on revascularization of the capillary-free area. These findings indicate that ruboxistaurin has anti-angiogenic effects both *in vitro* and *in vivo* that are exerted partly *via* suppressing the phosphorylation of ERK1/2 and Akt. Ruboxistaurin may be a candidate for treatment of angiogenesis in retinal neovascularization diseases.



## 1. Introduction

Angiogenesis, termed neovascularization, is the formation of new blood vessels from a pre-existing vascular network, with continued expansion of a vascular tree in response to an increase in tissue mass (Risau, 1997; Tripathi et al., 1998; Carmeliet and Tessier-Lavigne, 2005). Retinal neovascularization is a major pathologic process that leads to blindness in several ocular disease processes, including diabetic retinopathy, retinopathy of prematurity, and neovascular glaucoma (Smith et al., 1994; Barinapa, 1995). In these diseases, hypoxia is the underlying common condition, and the induction of a potent angiogenic factors by hypoxia, such as vascular endothelial growth factor (VEGF), triggers the neovascular proliferation (Miller et al., 1994; Thieme et al., 1995). In retinal neovascularization, experimental and clinical evidence suggests that VEGF plays a dominant role in this process (Miller et al., 1997).

Although the pathogenesis of diabetic-related microvascular complications is complex, several putative pathways have been implicated, including increased formation of nonenzymatic advanced glycosylation end products and increased activation of the PKC pathway (Nishikawa et al., 2000). In this context, it has been reported that PKC, especially PKCβ, plays an important role in diabetic retinopathy (Aiello, 2002). Furthermore, many biochemical abnormalities have been identified in the retina of diabetic patients and animals, including elevated oxidative stress, increased PKC activity, nonenzymatic glycations, polyol pathway activation, and elevated nitric oxide levels (Kador et al., 1990; Ishii et al., 1996; Kowluru et al., 1996, 1999, 2000; Stitt et al., 1997; Kowluru et al., 1998). Experimental studies have further demonstrated that while the intracellular signaling process for VEGF is complex, one critical component, particularly in terms of its mitogenic and permeability-inducing effects, is the activation of PKCβ (Xia et al., 1996; Aiello et al., 1997).

The binding of VEGF to its cognate receptors induces dimerization and subsequent phosphorylation of the receptors, leading to the activation of several intracellular signaling molecules such as phosphatidylinositol 3-kinase, phospholipase C γ (PLCγ), PKC, nitric oxide synthase, mitogen-activated protein kinases (MAPKs), and focal adhesion kinases (Karkkainen and Petrova, 2000; Takahashi et al., 2001; Claesson-Welsh, 2003; Lawson et al., 2003; Zachary,

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2003; Sakurai et al., 2005). Accordingly, a PKCβ inhibitor, ruboxistaurin, would be expected to function as a therapeutic agent to counteract retinal neovascularization. In fact, ruboxistaurin has been shown to inhibit intraocular neovascularization caused by ischemia in pigs (Danis et al., 1998). In addition, it also appears to ameliorate the visual acuity changes associated with diabetic macular edema in humans (Davis et al., 2009). To confirm ruboxistaurin as an agent targeted against ocular diseases caused by retinal neovascularization, it would be necessary to evaluate anti-angiogenic effect with a further *in vivo* model.

Evaluation of the anti-angiogenic effects of ruboxistaurin has not yet been reported using a murine oxygen-induced retinopathy (OIR) model. The aim of this study was to incorporate a widely used animal model of retinal neovascularization diseases (Madan and Penn, 2003) to evaluate the anti-angiogenic effect of ruboxistaurin. An objective and quantitative method that can be performed using imaging software on the whole retina in a mouse OIR model (Chikaraishi et al., 2007) was adopted. We examined retinal neovascularization in mice *in vivo* as well as tube formation, cell proliferation, and migration *in vitro*. The mechanism underlying the observed changes was examined through studies of signal transduction related to angiogenesis, namely phosphorylation of signal-regulated kinase 1/2 (ERK1/2) and serine/threonine protein kinase family protein kinase B (Akt) induced by VEGF.

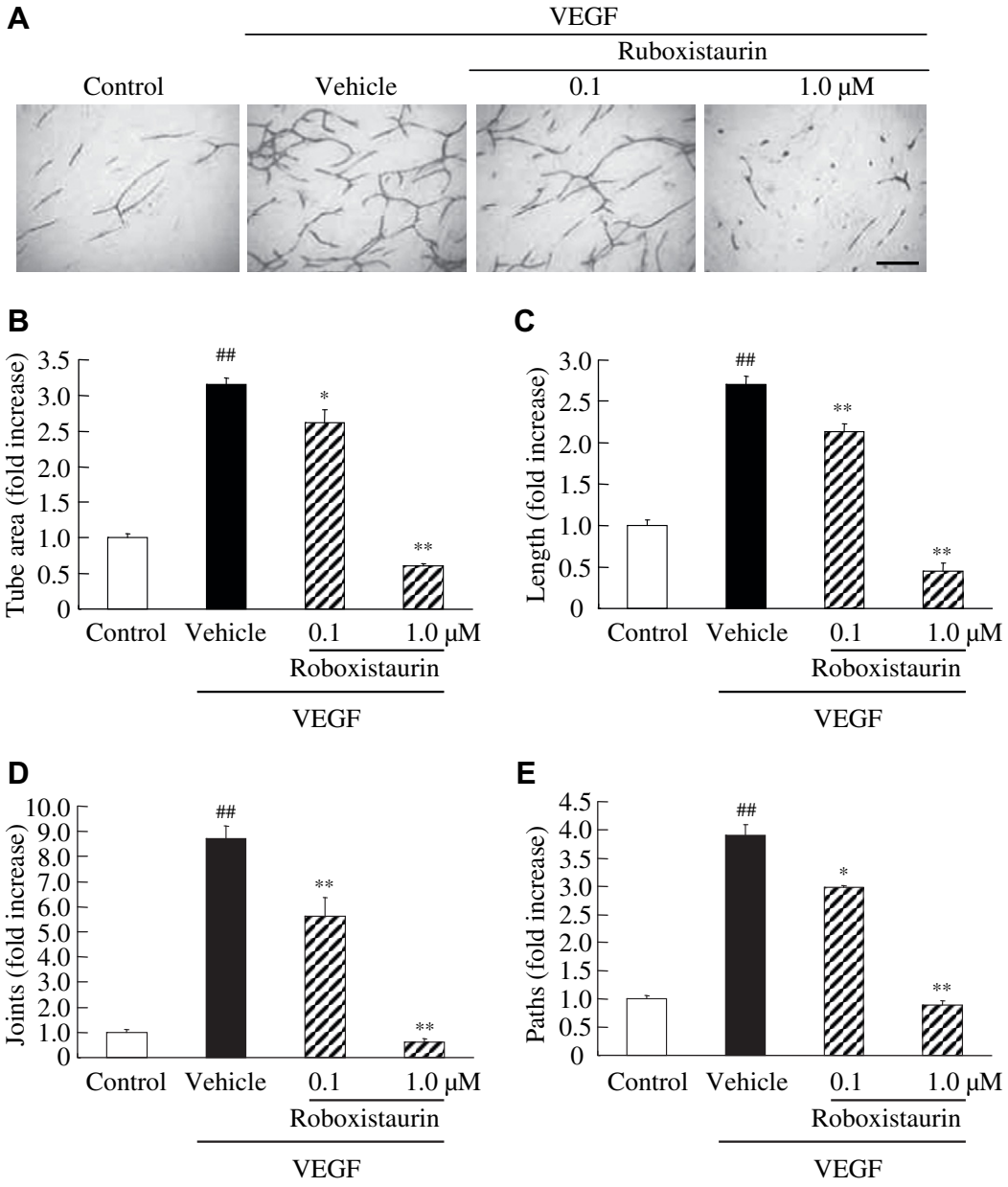


**Fig. 1.** Ruboxistaurin inhibited tube formation induced by VEGF. HUVECs were co-cultured with human fibroblasts, as described in methods. After cultivation for 11 days with or without the indicated concentrations of ruboxistaurin, with the concomitant addition of VEGF (10 ng/ml), HUVECs were stained with anti-CD31 antibody. Representative photographs of tube formation (A). Scale bar = 500 μm. Quantitative analysis of the stained tube-like structures was performed (using an angiogenesis imaging analyzer, version 2) in five different fields for each well, measurements being made of tube area (B), length (C), joints (D), and paths (E). Data are shown as mean ± SEM ($n = 3$). *, $p < 0.05$; **, $p < 0.01$ vs. vehicle (Dunnett's multiple-comparison test). ##, $p < 0.01$ vs. control (Student's *t*-test).