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The anti-ALS drug riluzole attenuates pericyte loss in the diabetic retinopathy of streptozotocin-treated mice

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ABSTRACT

Loss of pericytes, considered an early hallmark of diabetic retinopathy, is thought to involve abnormal activation of protein kinase C (PKC). We previously showed that the anti-amyotrophic lateral sclerosis (ALS) drug riluzole functions as a PKC inhibitor. Here, we examined the effects of riluzole on pathological changes in diabetic retinopathy. Pathological endpoints examined in vivo included the number of pericytes and integrity of retinal vessels in streptozotocin (STZ)-induced diabetic mice. In addition, PKC activation and the induction of monocyte chemotactic protein (MCP1) were assessed in diabetic mice and in human retinal pericytes exposed to advanced glycation end product (AGE) or modified low-density lipoprotein (mLDL). The diameter of retinal vessels and the number of pericytes were severely reduced, and the levels of MCP1 and PKC were increased in STZ-induced diabetic mice. Administration of riluzole reversed all of these changes. Furthermore, the increased expression of MCP1 in AGE- or mLDL-treated cultured retinal pericytes was inhibited by treatment with riluzole or the PKC inhibitor GF109203X. In silico modeling showed that riluzole fits well within the catalytic pocket of PKC. Taken together, our results demonstrate that riluzole attenuates both MCP1 induction and pericyte loss in diabetic retinopathy, likely through its direct inhibitory effect on PKC.

1. Introduction

Diabetic retinopathy (DR), a retinal microvascular disease, is one of the leading causes of severe visual loss among the working-age population (Praidou et al., 2010; Yau et al., 2012; Bandello et al., 2013; Ruta et al., 2013). DR is a multifactorial disease whose pathogenesis remains incompletely understood. Nearly all patients of type 1 diabetes and more than 60% of patients with type 2 diabetes suffers of some degree of DR around 15–20 years after diagnosis (Williams et al., 2004). DR can be broadly divided into two clinical stages: nonproliferative DR (NPDR) and proliferative DR (PDR). During the NPDR phase, abnormal permeability and/or nonperfusion of retinal capillaries result in the earliest visible sign of retinal damage followed by the formation of microaneurysms (Williams et al., 2004). In PDR, proliferation of new but fragile blood vessels on the retinal surface (neovascularization) is the hallmark finding (Williams et al., 2004). Among newly diagnosed patients with type 2 diabetes, 22% with no DR at baseline developed NPDR at 6 years and 29% of patients with baseline DR showed progression of DR of two or more steps on the Early Treatment Diabetic Retinopathy Study scale after 6 years' disease duration (Williams et al., 2004). Streptozotocin (STZ)-induced diabetic mice typically present features of NPDR, while oxygen-induced retinopathy mouse models and Kimba (trVEGF029) transgenic mouse models are commonly used to investigate PDR (Lai and Lo, 2013).

An early event in DR that occurs prior to the development of microaneurysm formation or neovascularization is the breakdown of the blood-retinal barrier (BRB), leading to the increased permeability of retinal capillaries followed by the increased secretion of

Abbreviations: AGE, advanced glycation end products; BRB, blood-retinal barrier; BSA, bovine serum albumin; DR, diabetic retinopathy; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; mLDL, modified low-density lipoprotein; MCP1, monocyte chemoattractant protein-1; NPDR, nonproliferative diabetic retinopathy; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PDGFR-β, platelet-derived growth factor receptor-β; PDR, proliferative diabetic retinopathy; PKC, protein kinase C; STZ, streptozotocin; VEGF, vascular endothelial growth factor.

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inflammatory cytokines (Kaur et al., 2008; Praidou et al., 2010; Chew et al., 2014). The functional abnormalities and eventual loss of pericytes may play a critical role in this process (Praidou et al., 2010; Fu et al., 2012; Du et al., 2013; Lai and Lo, 2013). Hyperglycemia per se may directly lead to vasoregression, or it may do so indirectly via the action of advanced glycation end products (AGEs), which are non-enzymatically formed in a hyperglycemic environment (Chen et al., 2006; Singh et al., 2014; Cai and McGinnis, 2016). Modified low-density lipoprotein (mLDL) is also formed in diabetic patients through oxidation and/or glycation, and is known to cause accelerated vascular changes in diabetes (Sonoki et al., 2002; Renier et al., 2003; Zhang et al., 2008). The decrease in pericyte numbers is associated with a subsequent increase in vascular permeability, decrease in perfusion, and induction of angiogenic factors such as vascular endothelial growth factor (VEGF) (Motiejunaite and Kazlauskas, 2008; Praidou et al., 2010).

Protein kinase C (PKC) plays a key role in VEGF signaling, and aberrant PKC signaling, specifically that of the PKCβ isoenzyme, is thought to be involved in the pathogenesis of DR (Aiello, 2002; Budhiraja and Singh, 2008; Praidou et al., 2010). Accordingly, PKCβ has recently been suggested as a potential target in the treatment of both early and late diabetic vascular complications (Aiello, 2002; Menne et al., 2013; Koya, 2014). Other factors that have been causally linked to DR, including inflammatory cytokines such as monocyte chemoattractant protein-1 (MCP1), are also modulated by the PKC pathway (Kim et al., 2012). We have previously found that riluzole, the FDA-approved drug for amyotrophic lateral sclerosis (ALS), is an inhibitor of PKCβ, showing that riluzole attenuates pathological changes in oxygen-induced retinopathy, a surrogate model of DR (Sims, 1986). Since PKC is involved in the early stage of disease progression (Sims, 1986; Noh et al., 2000), these findings suggest the possibility that riluzole might also have an inhibitory effect on DR.

Commonly used streptozotocin (STZ) or alloxan-induced rodent models exhibit rapid onset of hyperglycemia and several symptoms of NPDR such as retinal pericyte loss and capillaries, thickening of vascular basement membrane, and increased vascular permeability (Cai and McGinnis, 2016). Apoptosis of retinal ganglion cells and vascular cells can be identified since 6 weeks of hyperglycemia (Lai and Lo, 2013), while retinal pericyte loss starts between 4 and 8 weeks of diabetes (Hammes, 2005). Accordingly, in the present study, we examined the effects of riluzole on the induction of cytokines in STZ-induced diabetic mice and by putative mediators of DR in cultured human retinal pericytes.

2. Methods

2.1. Chemicals

STZ, dimethyl sulfoxide (DMSO), riluzole, and GF109203X were purchased from Sigma (St. Louis, MO, USA). GF109203X, a pan-PKC inhibitor, was selected as positive control. AGE was purchased from Bio Vision (Milpitas, CA, USA). mLDL was prepared by diluting low-density lipoprotein, obtained from Calbiochem (La Jolla, CA, USA), in phosphate-buffered saline (PBS) and incubating with 10 μM $CuCl_2$ at 37 °C for 24 h (Jenkins et al., 2000; Lyons et al., 2000).

2.2. Animals

The animal experimental protocol was approved by the Internal Review Board for Animal Experiments of Asan Life Science Institute, University of Ulsan College of Medicine (Seoul, Korea). The animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. Male, 8-week-old (22–25 g) C57BL/6NCrSlc mice were obtained from Japan SLC, Inc. (Hamamatsu, Japan) and maintained at 24 °C ± 0.5 °C under a 12-hour light/dark cycle. Animals were injected intraperitoneally with STZ (150 mg/kg body weight in 50 mM citrate buffer, pH 4.5) or citrate buffer alone (control) after 4 h of fasting (Lai and Lo, 2013; Leskova et al., 2013). Diabetes was confirmed using Super Glucocard II from Dongbang International Inc. (Seoul, Korea), and animals with blood glucose levels higher than 300 mg/dl 1 week after STZ injection were considered to be diabetic. This is one of the standard protocols recommended by the Animal Models of Diabetic Complications Consortium (Lai and Lo, 2013). Mice were injected intraperitoneally with riluzole (1 mg/kg body weight per day) or 1% DMSO in saline daily for 4 weeks. The number of mice used for experimental setup and treatment is described in Table S1.

2.3. Human retinal pericyte culture

A human retinal pericyte cell line was obtained from Applied Cell Biology Research Institute (Kirkland, WA, USA) and cultured in Dulbecco's Modified Eagle's Medium with 1 g/l glucose (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS; Invitrogen), 1% penicillin-streptomycin (Lonza, Allendale, NJ, USA), and 2 mM glutamine (Sigma) at 37 °C in a humidified 5% CO_2 incubator. Cells between passage 5 and 10 were used for experiments after reaching approximately 80% confluence.

2.4. Fluorescein angiography

Eight weeks after STZ injection, mice were anesthetized by isoflurane (1.5%) inhalation, and fluorescein angiography was performed using a MICRON III retinal imaging system (Phoenix Research Laboratories, Inc., Pleasanton, CA, USA). Photographs were obtained using a mouse-specific contact lens in MICRON III after intraperitoneal injection of 0.2 ml of 2% fluorescein sodium (Alcon Laboratories, Inc., Fort Worth, TX, USA) (Choi et al., 2013). Fluorescein angiographs of the early phase were taken at 3 min after fluorescein injection, while those of the late phase were taken at 15 min. Quantification of fluorescence was evaluated as follows: fluorescein intensity was measured at regular distances from the optic disc between major retinal vessels by Image J software (NIH, Bethesda, MD, USA), and 5 measurements of intensity were averaged for each eye.

2.5. Fluorescein isothiocyanate (FITC) staining of retinal flat-mounts

All mice were injected intramuscularly with 0.3 ml of Zoletil (diluted 1:5), and cardiac perfusion was performed with 0.5 ml of 15 mg/ml dextran (2000 kDa, #FD2000S, Sigma) in saline. After 5 min, eyes were fixed in 4% paraformaldehyde for 1 h at room temperature and transferred to a culture dish filled with PBS. Retinal tissues were dissected and flat-mounted on slides with coverslips. Capillary intensity was measured as the fluorescein intensity of capillaries by Image J software, and 5 measurements of capillary intensity were averaged for each retina.

2.6. Immunohistochemistry

The presence of pericytes was assessed by immunostaining retinal tissues with an antibody against platelet-derived growth factor receptor-β (PDGFR-β, 1:100; #ab32570, Epitomics, Burlingame, CA, USA). Co-staining with CD31 antibody (1:500, #MAB1398Z, Millipore, MA, USA), an endothelial cell marker, was performed to verify the presence of retinal vessels, while Hoechst 33342 (1:5000, #H3570, Molecular Probes™, MA, USA) was also used to confirm the loss of cells. After fixing in 4% paraformaldehyde, retinas were washed in PBS and incubated in a permeabilizing and blocking solution consisting of PBS containing 0.2% Triton X-100 and 1% bovine serum albumin (BSA). After incubation with a primary antibody at 4 °C for 7 days, tissues were washed in PBS three times for 10 min. They were further incubated with Alexa Fluor-conjugated secondary antibodies at 4 °C for 1 day (1:500; Alexa Fluor 555-donkey anti-rabbit IgG or Alexa Fluor 647-goat anti-hamster IgG, Invitrogen). After incubation with a secondary antibody, tissues were