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Myricetin inhibits advanced glycation end product (AGE)-induced migration of retinal pericytes through phosphorylation of ERK1/2, FAK-1, and paxillin *in vitro* and *in vivo*

Young Sook Kim [1], Junghyun Kim [1], Ki Mo Kim, Dong Ho Jung, Sojin Choi, Chan-Sik Kim, Jin Sook Kim *

Korean Medicine-Based Herbal Drug Development Group, Herbal Medicine Research Division, Korea Institute of Oriental Medicine (KIOM), 1672 Yuseongdae-ro, Yuseong-gu, Daejeon, South Korea

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ABSTRACT

Advanced glycation end products (AGE) have been implicated in the development of diabetic retinopathy. Characterization of the early stages of diabetic retinopathy is retinal pericytes loss, which is the result of pericytes migration. In this study, we investigated the pathological mechanisms of AGE on the migration of retinal pericytes and confirmed the inhibitory effect of myricetin on migration *in vitro* and *in vivo*. Migration assays of bovine retinal pericytes (BRP) were induced using AGE-BSA and phosphorylation of Src, ERK1/2, focal adhesion kinase (FAK-1) and paxillin were determined using immunoblot analysis. Sprague-Dawley rats (6 weeks old) were injected intravitreally with AGE-BSA and morphological and immunohistochemical analysis of p-FAK-1 and p-paxillin were performed in the rat retina. Immunoblot analysis and siRNA transfection were used to study the molecular mechanism of myricetin on BRP migration. AGE-BSA increased BRP migration in a dose-dependent manner via receptor for AGEs (RAGE)-dependent activation of the Src kinase-ERK1/2 pathway. AGE-BSA-induced migration was inhibited by an ERK1/2 specific inhibitor (PD98059), but not by p38 and Jun N-terminal kinase inhibitors. AGE-BSA increased FAK-1 and paxillin phosphorylation in a dose- and time-dependent manner. These increases were attenuated by PD98059 and ERK1/2 siRNA. Phosphorylation of FAK-1 and paxillin was increased in response to AGE-BSA-induced migration of rat retinal pericytes. Myricetin strongly inhibited ERK1/2 phosphorylation and significantly suppressed pericytes migration in AGE-BSA-injected rats. Our results demonstrate that AGE-BSA participated in the pathophysiology of retinal pericytes migration likely through the RAGE-Src-ERK1/2-FAK-1-paxillin signaling pathway. Furthermore, myricetin suppressed phosphorylation of ERK 1/2-FAK-1-paxillin and inhibited pericytes migration.

1. Introduction

Advanced glycation end products (AGE) are generated from early glycation products, such as Schiff's bases, or their derivative Amadori products in which amino acids in proteins undergo a non-enzymatic reaction with glucose and other reducing sugars [1]. The rate of AGE accumulation is increased in diabetes mellitus [2,3]. Increased AGE act on various cells including endothelial cells, pericytes, and mesangial cells, through receptors for AGE (RAGE); and they lead to chronic cellular activation with cytokine production and tissue damage [4–6]. This cellular activation is associated with increased expression of extracellular matrix proteins, vascular adhesion molecules, cytokines and growth factors, and is associated with chemotaxis, angiogenesis, oxidative stress, cell proliferation and/or programed cell death depending on the cell type and concurrent signaling [7].

Diabetic retinopathy is one of the most frequent complications of diabetes and is the leading cause of vision loss in adults under 40 years from developed countries [8]. Early diabetic retinopathy is characterized by increased vascular permeability, microaneurysm formation and loss of retinal pericytes [9]. Retinal pericytes have contractile functions, provide vascular stability [10] and accumulate AGE during diabetes. AGE not only induce growth retardation and apoptotic cell death, but they also exert an immediate toxicity to retinal pericytes *in vitro* [11,12]. The elevated AGE levels in the

* Corresponding author.
E-mail addresses: ykim@kiom.re.kr (Y.S. Kim), jskim@kiom.re.kr (J.S. Kim).
[1] These authors contributed equally to this work.

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retinal vessels of diabetic patients are correlated with serum AGE levels and with the severity of retinopathy by inducing a blood–retinal barrier dysfunction [13]. Pericyte migration is a key cellular feature of a wide variety of physiological processes, including the repair of blood vessel endothelial damage [9]. Cell motility requires cytoskeletal reorganization and involves cytoskeleton-associated tyrosine kinase phosphorylation and the formation of focal adhesion kinase (FAK-1) complexes [14]. FAK-1 undergoes autophosphorylation on a single tyrosine residue, which creates a binding site for SH2-containing proteins. Inhibiting FAK-1 activity by expression of its carboxyl terminus decreases cell motility, and cells from FAK-1 deficient mice also show reduced migration [15,16]. Paxillin is a focal adhesion protein that is also phosphorylated on a tyrosine residue by a number of stimuli [17]. Paxillin interacts with FAK-1 at two sites in the amino and carboxyl termini, which are not required for targeting paxillin to focal adhesions [18]. Most of the studies concerning the effects of AGE-BSA on blood vessels have been conducted on vascular endothelial cells and smooth muscle cells [19,20]. However, the mechanisms underlying AGE-BSA influence on retinal pericyte migration are not well known.

Myricetin (3,5,7,3′,4′,5′-hexahydroxyflavone, Cannabiscetin) is a primitive flavonoid from the *Chrysobalanaceae* family and found in most berries, vegetables, and various medicinal herbs [21]. Several studies suggested that myricetin protects tert-butylhydroperoxide (t-BHP)-induced oxidative stress in erythrocytes from type 2 diabetic patients and restores the renal activity of glutathione peroxidase (GPx) and xanthine oxidase (XO) in diabetic rats [22,23]. Myricetin along with other flavonoids decreases low density lipoprotein (LDL) glycation and electrophoretic mobility, which reduce the atherosclerotic risk of patients with diabetes mellitus [24]. Furthermore, myricetin has also been shown to inhibit aldose reductase (AR) and AGE, which accelerate the progression of diabetic complications [24,25].

In the present study, we investigated whether AGE-BSA induced phosphorylation of FAK-1 and its substrate paxillin and whether this signal would mediate pericyte migration *in vivo* and *in vitro*. In addition, we examined whether myricetin inhibited AGE-BSA-induced retinal pericyte migration.

2. Material and methods

2.1. Materials

DMEM was purchased from WelGENE Inc. (Daegu, Korea). Collagenase (#10269638001) and dispase (#11097113001) were obtained from Roche Diagnostics (Mannheim, Germany). The α-smooth muscle actin (α-SMA; sc-130616) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, USA). Desmin (ab8592) antibodies were purchased from Abcam (Cambridge, England), and PD98059, SB203580, SP600125 and PP2 were obtained from Calbiochem (San Diego, USA). Phospho-Src (#2105), phospho-FAK-1 (#3281), phospho-paxillin (#2541), phospho-ERK1/2 (#9101), phospho-38 (#9215), phospho-Jun N-terminal kinase (JNK; #9251), Src (#2109), FAK-1 (#3285), paxillin (#2542), ERK1/2 (#9102), p-38 (#9212) and JNK (#9258) antibodies were acquired from Cell Signaling Technology (Beverly, USA). All other reagents used, including bovine serum albumin (BSA; fraction V) and myricetin (#M6760), were purchased from Sigma-Aldrich (St. Louis, USA).

2.2. Cell preparation and culture

Primary bovine retinal pericytes (BRP) were obtained from bovine retinal microvessels as described previously [27]. Briefly, fresh bovine retinas were dissected and homogenized in PBS (pH 7.4). Retinas were minced into small pieces in PBS buffer. The homogenates were then digested at 37 °C for 30 min in a solution of 0.1% collagenase and dispase. After digestion, microvessel fragments were filtered sequentially through 70-μm and 40-μm filters. BRP were collected by centrifugation and cultured with conditioned DMEM medium containing 2 mM glutamine, 1% penicillin/streptomycin, and 20% FBS. BRP from one or two pooled primary cultures were trypsinized and replated (passage 1) onto a culture dish. BRP were characterized for homogeneity by positive staining for both α-SMA and desmin antibodies.

2.3. Preparation of AGE-BSA

We prepared AGE-BSA *in vitro* by incubating 10 mg/ml BSA with 25 mM D-glucose in PBS buffer (pH 7.4) for 4 weeks under sterile conditions at 37 °C. Control non-glycated BSA (10 mg/ml) was prepared in the same manner but without D-glucose supplementation. AGE formation was characterized using fluorescence spectroscopy (ex 350 nm/em 450 nm). We noted a 7.5-fold increase in fluorescence when AGE-BSA was compared to control BSA. AGE-BSA was purified by PD-10 columns (Bio-Rad, Hercules, USA) and tested for endotoxin using the chromogenic LAL endotoxin assay kit (GenScript, Piscataway, USA). AGE-BSA samples contained less than 0.005 U/ml endotoxin.

2.4. Cell migration assay

Migration assays were performed as previously described [14]. Briefly, the chemotactic motility of BRP was assayed using Transwell plates with 6.5-mm diameter polycarbonate filters (8-μm pore size). The lower surface of the filter was coated with gelatine (10 μg/ml). Fresh DMEM media containing AGE-BSA was placed in the lower wells. BRP were trypsinized and suspended at a final concentration of 1×10^6 cells/ml in DMEM containing 1% FBS. One hundred microliters of the cell suspension were loaded into each of the upper wells. Cells were fixed and stained with hematoxylin and eosin.

2.5. Immunoblot analysis

Immunoblot analysis was performed as previously described [28]. Protein expression levels were determined by analyzing the signals captured on the polyvinylidene fluoride membranes using an image analyzer (Las-3000, Fuji photo, Tokyo, Japan).

2.6. Animals

Adult male Sprague-Dawley (SD) rats (6 weeks old) were purchased from Koatech (Pyeongtaek, Korea) and acclimated for 1 week prior to the study. Rats were fed standard laboratory chow and allowed free access to water in an air-conditioned room with a 12-h light/12-h dark cycle until they were used for the experiment. All experimental protocols for animal care were approved by local ethical boards and animal husbandry and procedures were carried out according to institutional guidelines.

2.7. Intravitreal injection of AGE-BSA

SD rats were randomly divided into three groups: normal control, AGE-BSA-treated, and AGE-BSA/myricetin-treated, with eight animals in each group. Each rat was anesthetized with a 1:1 mixture of xylazine hydrochloride (4 mg/kg) and ketamine hydrochloride (10 mg/kg). Rats received an intravitreal injection of 3 μl sterile PBS containing 1 mg/ml AGE-BSA in one eye and 5 μl sterile PBS in the contralateral eye using a 33-gauge Hamilton needle and syringe. The rats in the AGE-BSA/myricetin group