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● *Original Contribution*

LOW-INTENSITY ULTRASOUND REDUCES HIGH GLUCOSE-INDUCED NITRIC OXIDE GENERATION IN RETINAL PIGMENT EPITHELIAL CELLS

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Abstract—Diabetic retinopathy (DR) is a severe micro-vascular complication of diabetes. High glucose (HG)-evoked nitric oxide (NO) production mediated by increased oxidative stress is a key factor in DR pathogenesis. In this study, we examined whether low-intensity ultrasound (LIUS) stimulation can reduce HG-induced NO generation. We determined that LIUS stimulation decreased the HG-induced NO generation possibly *via* inhibition of reactive oxygen species (ROS) and subsequently diminished the associated pro-inflammatory pathway involving the induced expression of inducible nitric oxide synthase, cyclooxygenase-2 and vascular endothelial growth factor. In addition, we determined that LIUS stimulation reduced the quantity of NO produced by N-acetylcysteine, which was not mediated by ROS. These results indicate that LIUS can inhibit both ROS-dependent and -independent NO generation processes in ARPE-19 cells. We envision LIUS as a potential therapeutic alternative to treat DR. Further studies are required to understand the underlying mechanism of the LIUS-induced reduction of NO generation for DR therapy. (E-mail: bryan@inha.ac.kr)

Key Words: **Low-intensity ultrasound (LIUS), High glucose (HG), N-acetylcysteine (NAC), Nitric oxide (NO), Reactive oxygen species (ROS).**

INTRODUCTION

Diabetic retinopathy (DR), one of the most common micro-vascular complications of diabetes, is a progressive disease that results from vascular injury because of chronic hyperglycemia. DR remains one of the chief causes of blindness worldwide in working-age adults (Fante et al. 2010). Recent clinical and laboratory investigations identified high glucose (HG)-evoked inflammatory pathway as a crucial molecular mechanism in the development and progression of DR (Rangasamy et al. 2012). Pathologically, DR is characterized by an excessive vessel growth and increased vessel permeability. DR involves an abnormal pathology of major retinal cells, including retinal pigment epithelium (RPE), micro-aneurysms, inter-retinal edema, hemorrhage and intraocular neovascularization (Song et al. 2012). A growing body of evidence indicates RPE to be closely associated with DR pathogenesis (Simo et al. 2010). One of the characteristic features of DR is the leakage of the blood content through the RPE barrier, causing macular edema, which constitutes the prime cause of DR-induced vision loss (Xu et al. 2011). RPE cells have been widely used as a functional model to study DR pathophysiology (Ablonczy et al. 2011; Gambhir et al. 2012; Xu et al. 2011).

Inflammatory pathways are crucial in the DR pathogenesis (Zhang et al. 2011). Several studies have demonstrated that HG insults increase the expression of pro-inflammatory proteins such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX2) and vascular endothelial growth factor (VEGF) (Adela et al. 2015; Heimsath et al. 2006; Madonna et al. 2016; Rangasamy et al. 2012; Song et al. 2012). In particular, using cultured human RPE cell line, it has been demonstrated that HG exposure induced a significant increase in the expressions of iNOS in the RPE, resulting in DR (Zhang et al. 2012). Therefore, HG-induced inflammation and injury of the RPE remains a major field of research in DR pathology.

With widespread metabolic, vascular and cellular effects, nitric oxide (NO) plays crucial roles in the inflammatory pathways associated with DR (Tessari et al.

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2010; Yuan et al. 2009). Diabetes-induced vascular dilation and inflammation are correlated to and regulated by NO generation (Shin et al. 2014). HG-evoked nitrosative stress is one of the key factors for the disease's progression to result in vascular complications (Cosentino et al. 1997). Along with the HG-induced NO generation, excessive generation of reactive oxygen species (ROS) associated with the HG insults has been adequately documented. HG-induced ROS production and the associated mitochondrial dysfunction and deoxyribonucleic acid damage have been observed in DR (Kowluru and Chan 2007). However, in terms of therapeutic application, the beneficial effects of antioxidants in diabetic complications are rather limited and elusive (Johansen et al. 2005). Although increased ROS is considered a causal link between elevated glucose levels and the associated diabetic complications and notwithstanding the fact that a number of animal studies have demonstrated the beneficial effects of antioxidants on the development of retinopathy, the results obtained from clinical trials are rather ambiguous (Madsen-Bouterse and Kowluru 2008). In particular, it has been demonstrated that the damage or dysfunction caused by oxidative stress persisted even after glycaemia has been normalized. In this context, ROS scavenging is not likely be an effective strategy in DR therapy (Wu et al. 2014).

The major techniques employed for the treatment of DR include pan-retinal photocoagulation (PRP) therapy, laser treatment and intravitreal injection of anti-VEGF agents. However, in a majority of cases, eyes treated with laser photocoagulation did not display improvement in visual acuity; moreover, with the elapse of time, lesions generally developed into focal areas of chorioretinal atrophy. Furthermore, with anti-VEGF medications also, which are considered superior and more effective than laser treatment therapies, it is necessary to take into account certain risk factors associated with them (*e.g.,* cataract formation and ocular hypertension or glaucoma) (Arden and Ramsey 1995). Therefore, a novel therapeutic approach for DR treatment remains a challenge.

We have previously demonstrated that low-intensity ultrasound (LIUS) stimulation exhibits a therapeutic potential for several disease models *in vitro* or *in vivo*, such as for Parkinson's disease (Karmacharya et al. 2017), brain edema (Karmacharya et al. 2015), arthritis (Chung et al. 2012) and age-related macular degeneration (Kim et al. 2015). Our earlier studies demonstrated that LIUS stimulation reduces mitochondrial oxidative stress in a number of models such as 1-methyl-4-phenylpyridinium ion (MPP^+)-treated PC12 cells (Karmacharya et al. 2017) and L-buthionine-(S,R)-sulfoximine (BSO)-treated ARPE-19 cells (Kim et al. 2015). In these studies, we revealed that LIUS stimulation is protective against the oxidative damage caused by excessive ROS production. Although the underlying mechanism is yet to be adequately understood, these observations evidently indicated that LIUS stimulation directly or indirectly influences the cellular mitochondrial function and reduces ROS generation. In this context, we further explored LIUS stimulation and examined its therapeutic effects on the ROS- and NO-mediated DR pathology in ARPE-19 cells. Here, we used HG-treated ARPE-19 cells as a DR model and reported that LIUS stimulation decreases ROS and/or NO generation and reduces the expression of the downstream pro-inflammatory proteins, namely, iNOS, COX2 and VEGF.

MATERIALS AND METHODS

Chemicals and antibodies

D-glucose, D-mannitol, uric acid (UA), N-acetyl-L-cysteine (NAC), 2,7-dichlorodihydrofluorescein diacetate (DCFHDA) and Protease Inhibitor Cocktail were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-iNOS antibody (mouse polyclonal) and anti-COX2 antibody (rabbit polyclonal) and anti-VEGF antibody (rabbit polyclonal) were purchased from Abcam (Cambridge, MA, USA). Anti-β-actin antibody (mouse monoclonal) and horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture and chemical treatment

Human retinal pigmented epithelial cells (ARPE-19 cells) have been widely used to study DR pathophysiology (Chen et al. 2013; Garcia-Ramírez et al. 2011). ARPE-19 cells were purchased from American Type Cell Collection (Manassas, VA, USA). ARPE-19 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM:F12) supplemented with heat-inactivated horse serum (Gibco, Waltham, MA, USA) to a final concentration of 10%, fetal bovine serum (Gibco) to a final concentration of 5%, 100 units/mL penicillin and 100 μg/mL streptomycin in a water-saturated atmosphere of 5% CO_2 at 37 °C. The cultured cells were grown in culture dishes and treated with either normal physiologic levels of glucose (5.5 mM glucose, NG), 33 mM glucose (high glucose [HG]) or mannitol (Man) (for osmotic control). Each experiment includes nontreated ARPE-19 cells, referred to as *control*. All experiments were carried out 24 h after the cells were seeded.

LIUS treatment

ARPE-19 cells were stimulated with LIUS by a method similar to that of our previous procedure (Karmacharya et al. 2017). Refer to our previous paper (Kim et al. 2015) for the details of our experimental design