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Effects of simvastatin on CAT-1-mediated arginine transport and NO level under high glucose conditions in conditionally immortalized rat inner blood-retinal barrier cell lines (TR-iBRB)

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ABSTRACT

Objective: Hyperglycemia causes the breakdown of the blood-retinal barrier by impairing endothelial nitric oxide synthase (*e*NOS) function. Statins have many pleiotropic effects such as improving endothelial barrier permeability and increasing *e*NOS mRNA stability. The objective of this study was to determine effect of simvastatin on L-arginine transport and NO production under high-glucose conditions in conditionally immortalized rat retinal capillary endothelial cell line (TR-iBRB).

Methods: Changes in L-arginine transport uptake and, expression levels of cationic amino acid transporter 1 (CAT-1) and eNOS mRNA were investigated after pre-treatment with simvastatin and NOS inhibitors (L-NMMA and L-NAME) under high-glucose conditions using TR-iBRB, an *in vitro* model of iBRB. The NO level released from TR-iBRB cells was examined using Griess reagents.

Results: Under high glucose conditions, [^3H]L-arginine uptake was decreased in TR-iBRB cells. Simvastatin pre-treatment elevated [^3H]L-arginine uptake, the expression levels of CAT-1 and *e*NOS mRNA, and NO production under high-glucose conditions. Moreover, the co-treatment with simvastatin and NOS inhibitors reduced [^3H]L-arginine uptake compared to pretreatment with simvastatin alone.

Conclusion: Our results suggest that, in the presence of high-glucose levels, increased L-arginine uptake due to simvastatin treatment was associated with increased CAT-1 and eNOS mRNA levels, leading to higher NO production in TR-iBRB cells. Thus, simvastatin might be a good modulator for diabetic retinopathy therapy by increasing of the L-arginine uptake and improving endothelial function in retinal capillary endothelial cells.

1. Introduction

Hyperglycemia, hypertension, and dyslipidemia are risk factors for ischemic retinopathies such as diabetic retinopathy (DR), an ocular complication of diabetes and a leading cause of blindness worldwide (Zhang et al., 2011; Gardner et al., 2011; Cai and Boulton, 2002). Hyperglycemia causes damage to endothelial cells by impairing the function of endothelial nitric oxide synthase (*e*NOS) and causing the breakdown of the inner blood-retinal barrier (iBRB) which is maintained by endothelial cell (Awata et al., 2004). The BRB consists of retinal pigment epithelial cells (outer RPE) and retinal capillary endothelial cells (inner BRB) complex (Hosoya and Tomi, 2005; Runkle and Antonetti, 2010; Kubo et al., 2012). It possesses a well-developed junction complex (Hosoya and Tomi, 2005; Runkle and Antonetti, 2010; Kubo et al., 2012). NO derived from extracellular L-arginine by *e*NOS maintains arteriolar vasodilation in the retina (Tomi et al., 2009). It plays an important role as a regulator of vascular tone for appropriate endothelial function (Albrecht et al., 2003). Reduction of NO availability in pathological conditions can result in endothelial dysfunction. One may argue that L-arginine is important for regulating NO synthesis and NO action. L-Arginine uptake is mediated by cationic amino acid transporter 1 (CAT-1), a Na^+ independent, and saturable process with Michaelis-Menten constants of 11.2 μM and 530 μM in inner BRB cells under normal condition (Tomi et al., 2009). In diabetes-induced coronary endothelial dysfunction, L-arginine uptake is decreased (Tawfik et al., 2006). Therefore, it is important to investigate the L-arginine transport under high-glucose conditions.

Simvastatin is a member of statins, also known as the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (HMG-CoA). It is an enzyme in the mevalonate pathway (Takemoto and Liao, 2001). Simvastatin has beneficial effects for atherosclerosis by increasing *e*NOS activity, leading to decreased serum cholesterol levels but enhanced endothelial NO production (Laufs et al., 1998a, 1998b; Ii and Losordo, 2007). It exerts pleiotropic effects by improving endothelial function while decreasing oxidative stress and vascular inflammation (Kim et al., 2012; El-Azab et al., 2011). Simvastatin also improves endothelial

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barrier permeability in the aorta of Watanabe heritable hyperlipidemic rabbits (Geerten et al., 2000) and endothelial barrier function in cerebral, retina, and cardiac muscles (Mooradian et al., 2005). In addition, simvastatin can increase the NO bioavailability by inducing *e*NOS expression in coronary arteries (Tawfik et al., 2006). Moreover, atorvastatin (also a statin) can restore endothelial cell dysfunction by modulating CAT-1 protein and *e*NOS activity as well as increasing chronic renal failure-induced L-arginine uptake (Schwartz et al., 2006). However, the effect of simvastatin on L-arginine uptake in iBRB cells under high-glucose conditions has not been investigated.

Therefore, the objective of this study was to determine the effect of simvastatin on L-arginine uptake, CAT-1 expression, *e*NOS expression and NO production under high-glucose conditions using an immobilized rat retinal capillary endothelial cell lines (TR-iBRB) as an in vitro model (Fig. 1).

2. Materials and methods

2.1. Materials

[^3H]L-Arginine (specific activity 45.1 Ci/mmol) was purchased from American Radiolabeled Chemical, Inc. (St. Louis, MO, USA). Bovine serum albumin and Bio-Rad DC protein assay kit were obtained from Bio-Rad Laboratories (Hercules, CA, USA). High Capacity RNA-to-cDNA kit, TaqMan Gene Expression Master Mix, gene-specific oligonucleotide primers, probes for CAT-1, *e*NOS and endogenous control GAPDH were purchased from Applied Biosystems (Foster City, CA, USA). Griess Reagent System was purchased from Promega (Madison, WI, USA). Simvastatin, L-arginine, N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME), N^{ω}-methyl-L-arginine acetate salt (L-NMMA), geranylgeraniol (GGOH), and other chemicals were purchased from Sigma Chemical (St. Louis, MO, USA). All other chemicals and reagents were commercial products of reagent grade. They were obtained from commercial sources.

2.2. Cell culture

TR-iBRB cell lines (conditionally immortalized rat retinal capillary endothelial cell lines) (Tomi et al., 2007; Tomi et al., 2009; Lee et al., 2016) were cultured at 33°C in a 5% CO_2/air humidified incubator on rat tail collagen type 1-coated tissue dishes (Iwaki, Tokyo, Japan). Because TR-iBRB cells were isolated from transgenic rat harboring a temperature-sensitive simian virus 40 (SV 40) large T-antigen gene (Tg rat) and ts SV 40 large T-antigen is inactivated at 37 °C (Hosoya et al., 2001; Terasaki and Hosoya, 2001). These cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 15 μg/mL endothelial cell growth factor, 100 U/mL penicillin and 100 μg/mL streptomycin (Lee and Kang, 2013). For uptake studies, TR-iBRB cells were cultured at 33 °C for 2 days and seeded at 1×10^5 cells per well into rat tail collagen type 1-coated 24 well culture plates until confluence. The high-glucose conditions comprised cell culture medium containing 25 mM glucose while normal glucose condition contained 5 mM glucose.

2.3. Uptake study of [^3H]L-arginine uptake

The [^3H]L-arginine uptake study was performed according to a previous report (Lee et al., 2012). Briefly, TR-iBRB cells in 24-well culture plates were washed three times per each well with 1 mL extracellular fluid (ECF) buffer (pH 7.4) at 37°C. They were then incubated with 0.2 mL of ECF buffer containing [^3H]L-arginine (11 nM) from an initial uptake until 30 min. After the incubation, ECF buffer containing [^3H]L-arginine was removed and the cells were washed three times with ice-cold ECF buffer to terminate the uptake. Cells were then dissolved with 1 N NaOH solution and incubated over-night at room temperature. Aliquots (0.5 mL) were collected from each well and mixed with 4.5 mL Ultima gold reagent. Their radioactivities were counted using a liquid scintillation counter (LS6500; Beckman, Fullerton, CA, USA). Protein levels were evaluated using a DC protein assay kit with bovine serum albumin as a standard. [^3H]L-Arginine uptake was expressed as cell-to-medium (μL/mg protein) ratio as follows: radioactivity (dpm/μL) in the sample per milligram cell protein (dpm/mg protein).

To assess Na^+ dependency, NaCl in transport buffer was replaced with LiCl, choline chloride or $CoCl_2$ while $NaHCO_3$ replaced with K_2HCO_3. To determine the effect of inhibitors, ECF buffer containing [^3H]L-arginine was mixed with 1 mM L-arginine, 1 mM L-NMMA. We also used 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH), L-type amino acid transporter 1 (LAT-1) substrate, as a negative control. To test the effect of simvastatin on [^3H]L-arginine uptake under-high glucose conditions, cells were pretreated with 0.5 mM L-NAME, 0.5 mM L-NMMA, 10 μM GGOH (a mevalonate pathway intermediate), 0.5 μM simvastatin, or co-treatment of each of these compounds with simvastatin. The uptake was performed for 5 min.

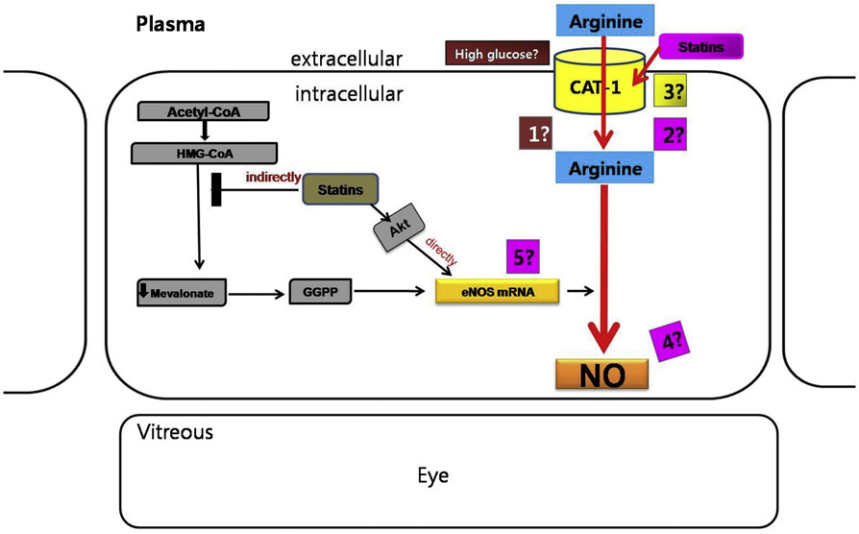

Fig. 1. The proposed experimental process related to pathway for arginine uptake, NO production and the effects of statins. This experiment was performed to investigate the mechanism of L-arginine uptake (1) and the effects of simvastatin on L-arginine uptake (2), CAT-1 expression (3), NO production (4) and *e*NOS expression (5) under high-glucose conditions in TR-iBRB cells.