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Gemigliptin, a novel dipeptidyl peptidase-4 inhibitor, exhibits potent anti-glycation properties *in vitro* and *in vivo*

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ABSTRACT

This study evaluated the inhibitory effects of gemigliptin, a highly selective dipeptidyl peptidase-4 inhibitor, on the formation of advanced glycation end products (AGEs) and AGE cross-links with proteins in *in vitro* as well as in type 2 diabetic *db/db* mice. In *in vitro* assay, gemigliptin dose-dependently inhibited methylglyoxal-modified AGE-bovine serum albumin (BSA) formation (IC_{50}=11.69 mM). AGE-collagen cross-linking assays showed that gemigliptin had a potent inhibitory effect (IC_{50}=1.39 mM) on AGE-BSA cross-links to rat tail tendon collagen, and its activity was stronger than aminoguanidine (IC_{50}=26.4 mM). In addition, gemigliptin directly trapped methylglyoxal in a concentration-dependent manner *in vitro*. To determine whether gemigliptin inhibits the *in vivo* glycation processes, gemigliptin (100 mg/kg/day) was orally administered into type 2 diabetic *db/db* mice for 12 weeks. Elevated serum levels of AGEs in *db/db* mice were suppressed by the administration of gemigliptin. These inhibitory effects of gemigliptin on the glycation process in both *in vitro* and *in vivo* suggest its therapeutic potential for ameliorating AGE-related diabetic complications.

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1. Introduction

The glycation process is a spontaneous non-enzymatic chemical reaction (called glycosylation) of sugar molecules with proteins, DNA and lipids to form Amadori product in a biological environment. The Amadori product irreversibly undergoes a variety of dehydration and rearrangement reactions, resulting in the formation of complex group known as advanced glycation end products (AGEs) (Brownlee et al., 1988). AGEs accumulate in all tissues and on plasma lipoproteins and bind to a specific receptor for AGE (RAGE) (Moritoh et al., 2009). Enhanced binding of AGEs to RAGE have been shown in diabetic patients, and the AGE/RAGE interaction has an important role to develop diabetes related complications (Sato et al., 2006). Moreover, AGEs can covalently cross-link with proteins, which changes the biochemical structures and functions of those proteins. Intracellular AGEs are also generated from sugar-derived dicarbonyl precursors. Methylglyoxal, a reactive dicarbonyl metabolite, is physiologically produced as an intermediate in glycolysis. The levels of methylglyoxal are increased in the plasma and tissue of patients and animals with diabetes (Haik et al., 1994). Methylglyoxal is more chemically reactive than blood sugars. Thus, methylglyoxal easily cross-links with free amino acid residues in proteins, which leads to the generation of stable end products (Bourajjaj et al., 2003). Evidence for methylglyoxal-derived modifications in human and animal tissues has been reported (Horan et al., 2007; Miyata et al., 1997; Shamsi et al., 1998).

It has been suggested that suppression of the glycation reaction may prevent the progress of diabetic complications. Aminoguanidine, a nucleophilic hydrazine compound, could inhibit the glycation reaction *in vitro* as well as *in vivo* (Hammes et al., 1991; Kumari et al., 1991). The mechanism of action of aminoguanidine may involve trapping dicarbonyl metabolites, such as methylglyoxal (Lo et al., 1994). The roles of potential glycation reaction

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inhibitors have not yet been investigated thoroughly. Gemigliptin (previously identified as LC15-0444) is a novel and selective dipeptidyl peptidase-4 (DPP-4) inhibitor used for the treatment of type 2 diabetes (Pennock et al., 2013). Although the conformational structure of gemigliptin differs from that of aminoguanidine, a very recent report has shown that another chemically similar DDP-4 inhibitor, sitagliptin, reduces serum levels of glycated albumin in type 2 diabetic subjects (Shima et al., 2014) and reduces the AGE content in lenses in streptozotocin-induced diabetic rats (Pandit et al., 2013). However, it remains unclear whether DPP-4 inhibitors have inhibitory effects on the glycation processes and cross-linking with proteins.

The purpose of this study was to evaluate the inhibitory effects of gemigliptin on the formation of AGEs and the cross-linking between preformed AGE-BSA and proteins *in vitro*. In addition, we also investigated whether the inhibitory activity of gemigliptin on the glycation process is due to its reactivity with reactive dicarbonyl compounds. Moreover, because various classes of structurally different DPP-4 inhibitors are currently available, we also compared the effectiveness of gemigliptin with that of the other DPP-4 inhibitors, vildagliptin and saxagliptin. Subsequently, we compared AGE formation and cross-links in type 2 diabetic *db/db* mice with and without administration of gemigliptin.

2. Materials and methods

2.1. In vitro tests

2.1.1. Inhibitory activity on AGEs formation

Bovine serum albumin (Sigma Chemicals, MO, USA) was incubated at 4 °C for 7 days with methylglyoxal (5 mM) in sodium phosphate buffer (0.1 M, pH 7.4). All of the reagent and samples were sterilized by filtration through 0.2 mm membrane filters. The reaction mixture was then mixed with gemigliptin (LG Life Sciences, Seoul, Korea, 99.2% pure by HPLC analysis), vildagliptin (Beijing HuiKang BoYuan Chemical Tech, Beijing, China, 99.4% pure by HPLC) or saxagliptin (Beijing HuiKang BoYuan Chemical Tech, Beijing, China, 99.0% pure by HPLC). Aminoguanidine (Sigma Chemicals, MO, USA) was used as a positive inhibitor. The levels of AGE were determined by measuring AGE-specific fluorescence using a spectrofluorometer (excitation at 370 nm and emission at 440 nm, Synergy HT, BIO-TEK, VT, USA). We calculated the 50% inhibition concentration (IC_{50}) of AGE formation.

2.1.2. AGE cross-linking assay

The ability of DPP-4 inhibitors to inhibit cross-linking of preformed AGE-BSA with collagen was examined. Preformed AGE-BSA (TransGenic Inc, Kobe, Japan) was mixed with DPP-4 inhibitors or aminoguanidine. This mixture was incubated in a rat tail tendon collagen-coated 96-well plate (Sigma, MO, USA) for 4 h at 37 °C. The cross-linked complexes of preformed AGE-BSA-collagen were detected using a mouse anti-AGE antibody (TransGenic, Kobe, Japan) and horseradish peroxidase-conjugated secondary antibody (Santa Cruz, CA, USA). Peroxidase activity was quantified using tetramethylbenzidine. Inhibition of preformed AGE-BSA and collagen cross-linking was expressed as the percentage of optical density.

2.1.3. Scavenging of carbonyl intermediates of AGE formation

We evaluated the ability of DPP-4 inhibitors to interact with methylglyoxal *in vitro* according to our previously reported method (Kim et al., 2011). Aminoguanidine was used as a positive control to determine the relative concentration of the remaining methylglyoxal.

2.2. In vivo tests

2.2.1. Animals

All mice were handled according to the approved procedure (LGMD13-083) by Institutional Animal Care and Use Committee of LG Life Sciences. Seven week-old male C57BL/KsJ-*db/db* mice (*db/db*, SLC, Shizuoka, Japan) and their lean littermates (*db/+*, normal) were randomly assigned to three ($n=10$) groups. One group of *db/db* mice was orally administered gemigliptin (100 mg/kg body weight) and another group was administered the same amount of vehicle *via* oral gavage for 12 weeks. Non-diabetic littermates received the same vehicle treatment. Blood glucose level and body weight were measured weekly.

2.2.2. Quantification of serum AGEs levels

At necropsy, serum samples were collected and serum AGE levels were analyzed using an AGE ELISA kit (MyBioSource Inc, CA, USA) according to the manufacturer's instruction.

2.2.3. RBC-IgG assay

Immunoglobulin G (IgG) is cross-linked to membrane protein of red blood cells (RBCs). RBC-IgG are formed before other AGE cross-links *in vivo*. The amount of RBC–IgG can be used to estimate protein cross-linking levels (Vasan et al., 1996). To test the inhibitory effect of gemigliptin on AGE cross-links, RBCs from heparinized whole blood were collected and RBC-IgG levels were determined using an anti-IgG ELISA.

2.3. Statistical analysis

All results are expressed as the mean ± standard error of the mean (S.E.M.). The IC_{50} values were determined by interpolation from the concentration–inhibition curve. Differences between groups were assessed by Student's *t*-test for single comparisons or by one-way ANOVA followed by Tukey's *post-hoc* test for multiple comparisons. Differences were considered significant at $P<0.01$. The statistical differences and IC_{50} values were determined using Prism 4.0 program (Graphpad, CA, USA).

3. Results

3.1. Inhibitory effect of DPP-4 inhibitors on AGEs formation in vitro

Three DPP-4 inhibitors were examined to evaluate inhibitory effects on AGE-BSA formation. As shown in Fig. 1, gemigliptin dose-dependently inhibited the formation of AGE-BSA ($IC_{50}=11.69\pm0.13$ mM). The inhibitory activity of gemigliptin was less than aminoguanidine ($IC_{50}=2.69\pm0.06$ mM) and other two DPP-4 inhibitors did not show anti-AGE formation activity.

3.2. Inhibitory effect of DPP-4 inhibitors on cross-linking of preformed AGE-BSA with rat tail tendon collagen in vitro

The inhibition of cross-links between preformed AGE-BSA and collagen under various concentrations of DPP-4 inhibitors was examined (Fig. 2). Gemigliptin dose-dependently suppressed the cross-linking of preformed AGE-BSA with rat tail tendon collagen ($IC_{50}=1.39\pm0.10$ mM), and its inhibitory activity was greater than that of aminoguanidine ($IC_{50}=26.4\pm1.20$ mM). In addition, other two DPP-4 inhibitors did not show inhibitory effects on AGE cross-linking with collagen.

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