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Linagliptin inhibits lipopolysaccharide-stimulated interleukin-6 production, intranuclear p65 expression, and p38 mitogen-activated protein kinase phosphorylation in human umbilical vein endothelial cells

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Abstract

Background: Linagliptin, the only bile-excreted dipeptidyl peptidase-4 (DPP-4) inhibitor, is a therapeutic drug for patients with diabetes receiving hemodialysis, for whom inflammation is a prognosis-related factor, because of its potential anti-inflammatory effects. Although the anti-inflammatory effects of linagliptin in vivo are reported, no study has described these effects in vitro. DPP-4 degrades glucagon-like peptide-1 (GLP-1), which is known to have anti-inflammatory properties. Since GLP-1 is a gut hormone secreted by intestinal L cells, in vivo examination of the GLP-1-independent anti-inflammatory effects of DPP-4 inhibitors is difficult. We evaluated the mitogen-activated protein kinase (MAPK)-dependent, GLP-1-independent, anti-inflammatory effects of linagliptin in lipopolysaccharide (LPS)-stimulated human umbilical vein endothelial cells (HUVECs) which do not secrete GLP-1. Furthermore, to determine whether linagliptin has unique pharmacological actions compared with other DPP-4 inhibitors, we assessed the anti-inflammatory effects of sitagliptin (a DPP-4 inhibitor without xanthine-related skeletal system activity), caffeine (a phosphodiesterase inhibitor), loxoprofen, and diclofenac sodium.

Methods: HUVECs were cultured for 24 h at densities of $1–2 \times 10^5$ cells/mL. We pretreated HUVECs with or without linagliptin (1, 5, 10, 50, and 100 nM), 150 nM sitagliptin, 50 nM caffeine, 17 μM loxoprofen, or 1.3 μM diclofenac sodium for 1 h prior to incubation with LPS. The concentration of LPS used (1 μg/mL) was sufficient to induce an inflammatory response in HUVECs. Five hours after incubation with LPS, culture media was evaluated for interleukin (IL)-6 expression. Intranuclear p65 (a subunit of nuclear factor kappa-B (NFκB)) levels were measured 5 h after treatment with LPS and 50 nM linagliptin, while phosphorylated p38 MAPK levels were measured in the cytosolic fractions obtained 30 min after treatment with LPS and 50 nM linagliptin.

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Results: Linagliptin significantly inhibited LPS-stimulated IL-6 production, intranuclear p65 expression, and p38 MAPK phosphorylation. Treatment with sitagliptin, caffeine, loxoprofen, and diclofenac sodium significantly inhibited LPS-stimulated IL-6 production.

Conclusions: The results of this study demonstrate the GLP-1-independent anti-inflammatory effects of linagliptin via MAPK-dependent mechanisms in vitro. Our findings suggest that linagliptin will play a crucial role in the treatment of hemodialysis (HD) patients with diabetes and chronic inflammation.

Keywords: Linagliptin, Human umbilical vein endothelial cells, Lipopolysaccharide, Interleukin-6, Intranuclear p65, p38 mitogen-activated protein kinase, Sitagliptin, Caffeine, Loxoprofen, Diclofenac sodium

Background

The prevalence of diabetes mellitus (DM) is increasing worldwide [1]. DM is the most common cause of kidney failure [2, 3], and glycemic control is necessary for the prevention of renal insufficiency. Moreover, glycemic control to improve hyper- or hypoglycemia is an important factor in the overall prognosis of patients with end-stage renal disease receiving hemodialysis (HD) [4, 5].

Dipeptidyl peptidase-4 (DPP-4) degrades hormones such as glucagon-like peptide-1 (GLP-1) [6], which is a gut hormone secreted by the intestinal L cells and affects glucose metabolism. DPP-4 inhibitors decrease blood glucose levels by inhibiting DPP-4 enzymes [7]. Although nine types of DPP-4 inhibitors have been developed, their anti-diabetes effects are similar. Therefore, it is important to understand the underlying pharmacological mechanisms of each inhibitor to facilitate their context-dependent use in patients.

Linagliptin is the only bile-excreted, anti-diabetic oral DPP-4 inhibitor; therefore, its dose reduction is not necessary [8–10]. Moreover, some studies reported that linagliptin decreased the risk of cardiovascular and cerebrovascular diseases, which are associated with systemic atherosclerosis and related prognostic factors [11–13]. We previously reported the anti-inflammatory effects of linagliptin in HD patients with diabetes [14] and, on the basis of reports from other groups, proposed three possible underlying mechanisms: increased GLP-1 activity (including an anti-diabetic effect) [15–20], inhibition of DPP-4 (CD26) [21–23], or xanthine-related skeletal system activity [24, 25]. Therefore, linagliptin is a critical therapeutic drug for this patient population, for which inflammation is a prognosis-related factor [26].

The anti-inflammatory effects of linagliptin have been elucidated in vivo [14, 27–30]. However, to the best of our knowledge, no study has examined these effects in vitro. Furthermore, as mentioned earlier, GLP-1 is a gut hormone secreted by intestinal L cells. Therefore, it is difficult to examine the GLP-1-independent effects of DPP-4 inhibitors in vivo (experimental models in mice, rats, and humans) in the gastrointestinal tract, although several studies have examined the anti-inflammatory effects of GLP-1 using its human analogs (exenatide or liraglutide) [15–20]. Because the factors involved in the complex process of anti-inflammation overlap and cannot be independently analyzed, particularly in vivo, the pharmacological anti-inflammatory effects of DPP-4 inhibitors remain unclear. Zeng et al. reported the anti-inflammatory effects of sitagliptin, a DPP-4 inhibitor, via mitogen-activated protein kinase (MAPK)-dependent mechanisms in apolipoprotein-E knockout mice [31]. However, the study does not shed light on the mechanisms underlying these anti-inflammatory effects, and it remains unclear whether they are related to the independent pharmacological actions of sitagliptin or increased GLP-1 activity.

Among the nine types of DPP-4 inhibitors, linagliptin is the only one with a xanthine-related skeletal system. Xanthine-related skeletal systems, also exhibited by caffeine and theophylline, are involved in anti-inflammatory effects [24]. Drugs based on these compounds promote cyclic adenosine monophosphate (cAMP) production through the inhibition of phosphodiesterase. This increased cAMP production may prevent MAPK phosphorylation by activating protein kinase A (PKA) [25].

Lipopolysaccharide (LPS) is found in the outer membrane of gram-negative bacteria and elicits strong immune responses. It is the most important quality marker for the dialysate fluid used in dialysis treatment. Moreover, LPS is strongly associated with extracorporeal circulation treatment (e.g., direct hemoperfusion with polymyxin B-immobilized fiber). Because studies have reported that 1 μg/mL of LPS can significantly induce inflammation in human umbilical vein endothelial cells (HUVECs) [32–35], we decided to use this compound for our study.

On the basis of the abovementioned perspectives, we investigated the GLP-1-independent anti-inflammatory effects of linagliptin via MAPK-dependent mechanisms following stimulation of HUVECs, which do not secrete GLP-1, with LPS in vitro. We analyzed three important markers: interleukin (IL)-6, which is the most typical inflammatory marker; intranuclear p65 (a subunit of nuclear factor kappa-B (NFκB)), which is a transcription factor that induces inflammatory responses; and phosphorylated p38 MAPK, which ultimately activates

transcription control factors. These three markers, particularly p38 MAPK, are important markers in LPS- and PKA-mediated signaling pathways of inflammation. Elucidation of the anti-inflammatory effects of linagliptin on these three markers will benefit HD patients with diabetes, for whom inflammation is a prognosis-related factor [26]. Furthermore, to determine whether linagliptin has unique pharmacological actions compared with other DPP-4 inhibitors, we assessed the anti-inflammatory effects of sitagliptin (a DPP-4 inhibitor without xanthine-related skeletal system activity), caffeine (a phosphodiesterase inhibitor), loxoprofen, and diclofenac sodium.

Methods

Study materials and cell culture

Linagliptin and sitagliptin compounds were provided by Boehringer Ingelheim Pharmaceuticals, Inc. (Ingelheim am Rhein, Rhineland-Palatinate, Germany). Caffeine was purchased from Mylan Inc. (Tokyo, Japan); loxoprofen and diclofenac sodium from Wako Pure Chemical Industries Ltd. (Osaka, Japan); and LPS, indoxyl sulfate (IS), and H89 (PKA inhibitor) from Sigma-Aldrich (St. Louis, MO, USA). All chemicals used in this study were of the purest grade available commercially. The HUVEC line, EGM™ BulletKit™ medium, and EGM™ SingleQuots™ Kit were purchased from Lonza (Walkersville, MD, USA). HUVECs were cultured in endothelial basal medium supplemented with 10.0 mL fetal bovine serum (FBS), 2.0 mL bovine brain extracts, 0.5 mL human epidermal growth factor, 0.5 mL hydrocortisone, 50 mg/mL gentamicin, 50 μg/mL amphotericin-B, and 0.5 mL ascorbic acid according to the supplier's instructions (EGM™ BulletKit™ medium and EGM™ SingleQuots™ Kit). HUVECs were cultured in a humidified atmosphere of 5 % CO_2 and 95 % air at 37 °C.

Drug treatment

All drug treatments were carried out under aseptic conditions in medium lacking FBS, bovine brain extracts, and hydrocortisone to prevent an increase in the spectrophotometric absorbance through binding with coating antibodies.

HUVECs were cultured for 24 h at densities of 1–2 × 10^5 cells/mL and were pretreated with 1, 5, 10, 50, and 100 nM linagliptin; 150 nM sitagliptin; 50 nM caffeine; 17 nM loxoprofen; or 1.3 μM diclofenac sodium for 1 h or left untreated. Then, the media were replaced, and the cells were treated with 1 μg/mL LPS or 1 mM IS together with 1, 5, 10, 50, and 100 nM linagliptin; 150 nM sitagliptin; 50 nM caffeine; 17 μM loxoprofen; 1.3 μM diclofenac sodium; or 10 nM H89 or left untreated. The concentration of LPS (1 μg/mL) or IS (1 mM) was sufficient to induce an inflammatory response in HUVECs. For IL-6 measurements with LPS, the above procedure was performed using 1, 5, 10, 50, and 100 nM linagliptin; 150 nM sitagliptin; 50 nM caffeine; 17 μM loxoprofen; 1.3 μM diclofenac sodium; or 10 nM H89, while for NFκB and p38 MAPK measurements with LPS, only 50 nM linagliptin was used. For IL-6 measurements with IS, the above procedure was performed using only 50 nM linagliptin. The same number of cells ($N = 1–2 \times 10^5$ cells/mL) were used in all treatment groups for standardization. The passage number of HUVECs in this study ranged from two to seven. There was no change in the cell characteristics in each experiment. Each experiment was repeated at least three times.

Measurement of plasma IL-6 levels in the supernatant

We measured IL-6 levels using an IL-6 enzyme-linked immunosorbent assay (ELISA) kit (Bender Medsystems, Vienna, Austria). First, we adsorbed the anti-IL-6 coating antibody onto microplate wells. Then, the IL-6 in the culture media and standard solutions provided in the assay kit was bound to these antibodies. Thereafter, we treated the solution with the following compounds in order: biotin-conjugated anti-IL-6 antibody, streptavidin-horse radish peroxidase (HRP), amplification reagents I (biotinyl-tyramide) and II (streptavidin-HRP), and a substrate solution reactive with HRP. We created a colored product in proportion to the amount of IL-6 present in the sample or standard. The addition of acid terminated the reaction, and the absorbance was determined at 405 nm using the Spectra Max 340 pc (Molecular Devices Co., CA, USA). The results of IL-6 measurements are shown according to the amount of protein in 1 mL of medium.

Measurement of intranuclear NFκB levels

Cells were harvested, and their nuclei were extracted using the commercially available Nuclear/Cytosol Fractionation kit (BioVision Inc., Milpitas, CA, USA). For quantitative determination of intranuclear p65 levels, the NFκB/p65 ActivElisa™ Kit (Nevus Biologicals, Littleton, CO, USA) was also used according to the manufacturer's instructions.

Detection of p38 MAPK phosphorylation in cytosolic fractions

We evaluated the phosphorylation of p38 MAPK using the Cell-Based p38 MAPK (Thr^{180}/Tyr^{182}) ELISA kit (RayBiotech, Inc. Norcross, CA, USA). Briefly, the cells were fixed, blocked, and incubated for 2 h at 15–25 °C with anti-phospho-p38 (Thr^{180}/Tyr^{182}) and anti-p38 antibodies. After incubation, the cells were washed with wash buffer before incubation for 1 h with HRP-conjugated anti-mouse IgG at 37 °C. Then, the cells were washed again with wash buffer, followed by treatment with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution

to achieve a color proportional to the amount of protein. The addition of Stop Solution ceased the reaction, resulting in a change of color from blue to yellow. We examined the intensity of the color at 405 nm.

Statistical analysis

The results are expressed as means ± standard errors of means (SEM). The effects of various treatments were compared with those of no treatment using one-way analysis of variance (ANOVA) and Bonferroni post hoc analysis. A one-sided P value of <0.05 was considered statistically significant.

Results

Effects of linagliptin on LPS-induced IL-6 production

IL-6 is the most typical inflammatory marker. We performed preliminary experiments to determine the most effective extraction time to detect IL-6. One, 5, and 24 h after treatment with LPS, the IL-6 levels were 1.36 ± 0.15, 5.45 ± 0.45, and 3.45 ± 0.57 times those in the control cells, with the highest increase at 5 h after treatment. Accordingly, IL-6 levels were measured 5 h after simultaneous treatment with LPS and the different compounds.

There was no significant difference in IL-6 production between linagliptin-treated cells (at all concentrations) and the untreated controls. Further, LPS alone significantly increased IL-6 production compared with the control treatment. The treatment of cells with linagliptin at all concentrations significantly inhibited the LPS-stimulated IL-6 production (Fig. 1).

Effects of linagliptin on the translocation of NFκB/p65 into the nuclei of LPS-stimulated HUVECs

NFκB is an important transcription factor involved in inflammatory responses, typically present in the cytoplasm by virtue of being bound to an inhibitory protein (IκB). Upon activation by an inflammatory stimulus, IκB is rapidly phosphorylated, ubiquitinated, and broken down by proteasomes. Degradation of IκB then leads to the release of NFκB, which translocates to the nucleus to transcriptionally induce the expression of inflammation-related genes. We performed preliminary experiments to determine the most effective extraction time to detect NFκB. One, 5, and 24 h after treatment with LPS, the NFκB levels were 0.87 ± 0.18, 1.66 ± 0.59, and 0.50 ± 0.17 times those in the control cells, with the highest increase observed at 5 h after treatment. Thereafter, to monitor the nuclear translocation of p65, its levels were measured 5 h after simultaneous treatment with LPS and linagliptin.

We observed no significant differences between the intranuclear p65 levels in linagliptin-treated cells and those in untreated cells. However, LPS alone significantly increased the intranuclear p65 levels compared with the control treatment. Pretreatment with 50 nM linagliptin significantly inhibited LPS-stimulated intranuclear p65 production (Fig. 2).

Effects of linagliptin on the phosphorylation of p38 MAPK in LPS-stimulated HUVECs

Exogenous stimuli also induce the phosphorylation of p38 MAPK by an upstream kinase of MAPK, ultimately activating transcription control factors such as NFκB. The production of proinflammatory cytokines such as IL-6 is induced via the MAPK signaling pathway. We performed preliminary experiments to determine the most effective extraction time to detect phosphorylated p38 MAPK. Phosphorylation of p38 MAPK occurs upstream of the reaction cascade that leads to activation of the transcription factors. Therefore, we estimated that the peak LPS-stimulated reaction would occur within 5 h. Fifteen minutes, 30 min, 1 h, and 5 h after treatment with LPS, the phosphorylated p38 MAPK levels were 0.88 ± 0.04, 1.20 ± 0.08, 0.96 ± 0.08, and 0.92 ± 0.02 times those in the control cells, with the increase being the highest at 30 min after treatment. Thereafter, p38 MAPK phosphorylation was measured in cytosolic fractions 30 min after simultaneous treatment with LPS and linagliptin.

No significant differences were observed between the linagliptin-treated and untreated cells with regard to p38 MAPK phosphorylation. However, we observed significant upregulation of phosphorylated p38 MAPK with LPS stimulation, but not with the control treatment. Pretreatment with 50 nM linagliptin significantly inhibited LPS-stimulated p38 MAPK phosphorylation (Fig. 3).

IL-6 production after sitagliptin, caffeine, loxoprofen, and diclofenac sodium treatments and linagliptin treatment

For these experiments, we chose the maximum blood concentration achieved with a single administration of the drug in healthy volunteers. Accordingly, 5 nM linagliptin, 150 nM sitagliptin, 50 nM caffeine, 17 μM loxoprofen, and 1.3 μM diclofenac sodium were used. Sitagliptin, caffeine, loxoprofen, and diclofenac sodium inhibited LPS-stimulated IL-6 production to the same extent as linagliptin (Fig. 4).

IL-6 production after linagliptin and H89 treatment

There was no significant difference in IL-6 production between linagliptin- and H89-treated cells and the untreated controls. The treatment of cells with 50 nM linagliptin significantly inhibited LPS-stimulated IL-6 production. Furthermore, H89 significantly increased linagliptin-inhibited IL-6 production (Fig. 5).

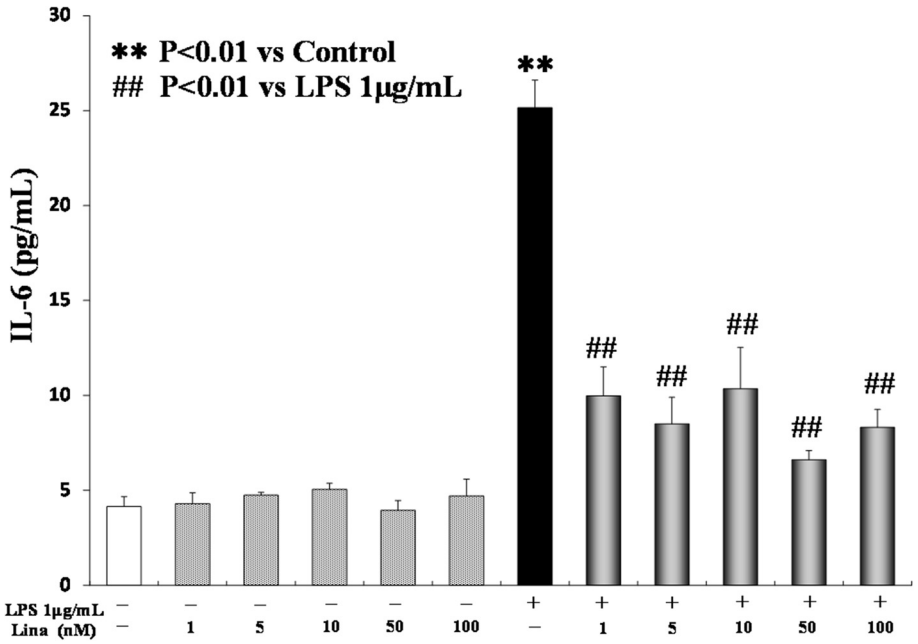

Fig. 1 Effects of linagliptin on lipopolysaccharide (LPS)-induced interleukin (IL)-6 production. Human umbilical vein endothelial cells (HUVECs) were treated with LPS and/or linagliptin. IL-6 levels in the supernatants were determined by ELISA after 5 h of treatment. $^{**}P < 0.01$ vs. control; $^{\#\#}P < 0.01$ vs. LPS 1 μg/mL. *Lina* linagliptin

Effects of linagliptin on IS-induced IL-6 production

There was no significant difference in IL-6 production between linagliptin-treated cells and untreated controls. Furthermore, IS alone resulted in a significant increase in IL-6 production compared with the control treatment. The treatment of cells with 50 nM linagliptin significantly inhibited IS-stimulated IL-6 production (Fig. 6).

Discussion

In this study, pretreatment of HUVECs with linagliptin significantly inhibited IL-6 production, intranuclear p65 production, and p38 MAPK phosphorylation stimulated by LPS. To the best of our knowledge, this study is the first to investigate the anti-inflammatory effects of linagliptin in vitro using HUVECs.

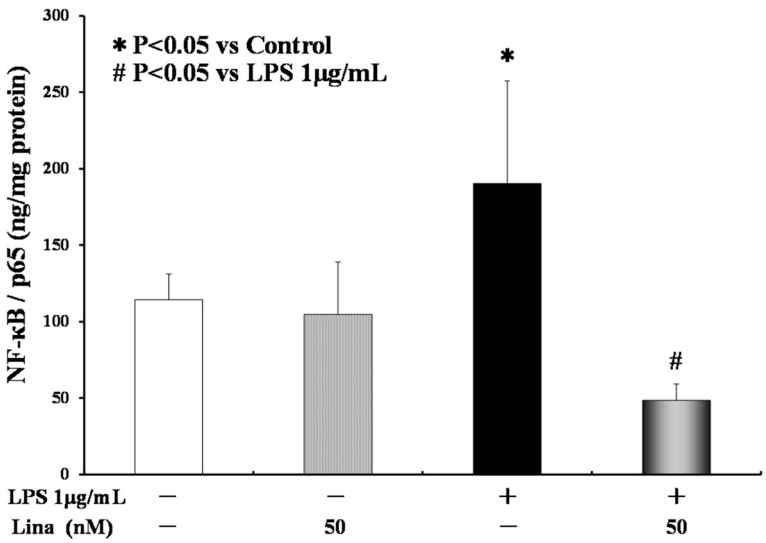

Fig. 2 Effects of linagliptin on nuclear factor kappa-B (NFκB)/p65 translocation into lipopolysaccharide (LPS)-stimulated human umbilical vein endothelial cell (HUVEC) nuclei. HUVECs were treated with LPS and/or linagliptin. NFκB/p65 levels in the nuclear fraction were determined by ELISA after 5 h of treatment. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. LPS 1 μg/mL. *Lina* linagliptin

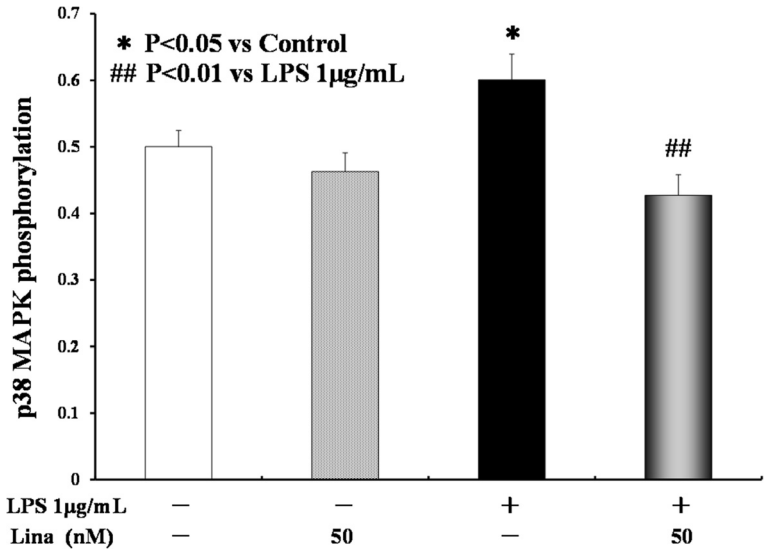

Fig. 3 Effects of linagliptin on p38 mitogen-activated protein kinase (MAPK) phosphorylation in lipopolysaccharide (LPS)-stimulated human umbilical vein endothelial cells (HUVECs). HUVECs were treated with LPS and/or linagliptin. The phosphorylation of p38 MAPK in the cytosol fraction was determined by ELISA after 30 min of treatment. *$P < 0.05$ vs. control; ##$P < 0.01$ vs. LPS 1 μg/mL. *Lina* linagliptin

HD patients with diabetes are highly susceptible to inflammation because of hyperglycemia [36, 37], LPS associated with extracorporeal circulation treatment, and atherosclerosis. Moreover, inflammation is an important prognostic factor in HD patients [26]. Linagliptin is the only bile-excreted, anti-diabetic oral DPP-4 inhibitor; therefore, its dose reduction is not necessary. Therefore, this population was considered suitable for examination of the anti-inflammatory effects of linagliptin, and further elucidation of the anti-inflammatory effects of linagliptin will benefit HD patients with diabetes. We previously reported the anti-inflammatory effects of linagliptin in HD patients with diabetes [14] and, on the basis of reports from other groups, proposed three possible underlying mechanisms: increased GLP-1 activity (including an anti-diabetic effect) [15–20], inhibition of DPP-4 (CD26) [21–23], or xanthine-related skeletal system activity [24, 25]. However, anti-inflammation is a

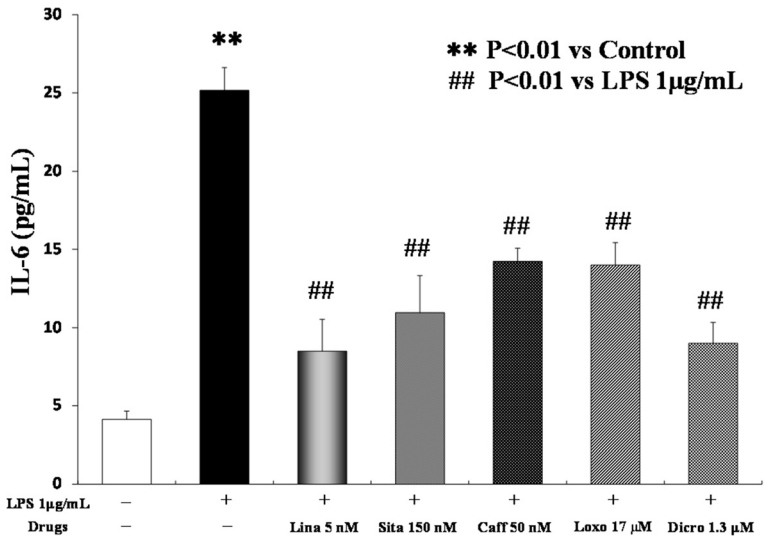

Fig. 4 Interleukin (IL)-6 production after treatment of human umbilical vein endothelial cells (HUVECs) with linagliptin, sitagliptin, caffeine, loxoprofen, and diclofenac sodium. HUVECs were treated with lipopolysaccharide and/or linagliptin, sitagliptin, caffeine, loxoprofen, and diclofenac sodium. IL-6 levels in the supernatants were determined by ELISA after 5 h of treatment. **$P < 0.01$ vs. control; ##$P < 0.01$ vs. LPS 1 μg/mL. *Lina* linagliptin, *Sita* sitagliptin, *Caff* caffeine, *Loxo* loxoprofen, *Diclo* diclofenac sodium

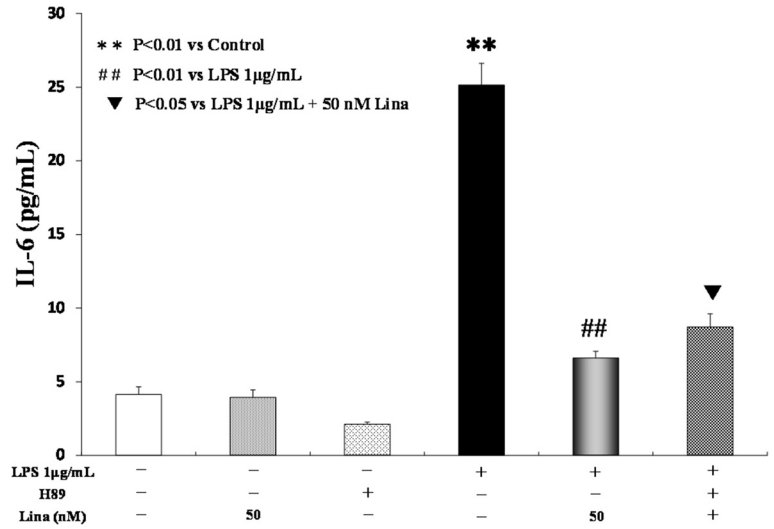

Fig. 5 Interleukin (IL)-6 production after linagliptin and protein kinase A inhibitor (H89) treatment. Human umbilical vein endothelial cells (HUVECs) were treated with lipopolysaccharide and/or linagliptin and H89. IL-6 levels in the supernatants were determined by ELISA after 5 h of treatment. $^{**}P < 0.01$ vs. control; $^{\#\#}P < 0.01$ vs. LPS 1 μg/mL; $^{\blacktriangledown}P < 0.05$ vs. LPS 1 μg/mL and 50 nM linagliptin. *Lina* linagliptin

complex phenomenon to study in vivo because of various overlapping factors that cannot be independently analyzed. In our study, however, we did not use either GLP-1 or its analogs, and all experiments were performed in HUVECs, which do not secrete GLP-1. Therefore, the anti-inflammatory effects of linagliptin observed in this study were possibly brought about by the independent pharmacological actions of linagliptin, not by increased GLP-1 activity. Another reason for selecting HUVECs was the association with CD26. DPP-4 is expressed as CD26 on various cell membranes, where it acts as an inflammatory mediator; therefore, inflammation can be modulated by DPP-4 inhibition [21–23]. Among the various cells, leukocytes are believed to be most relevant to inflammation. Therefore, we did not use leukocytes in our study.

Furthermore, we demonstrated that the anti-inflammatory effects of linagliptin are attributed to decreased p38 MAPK phosphorylation in HUVECs. Until now, six studies have reported the anti-inflammatory effects of DPP-4 inhibitors other than linagliptin in vitro [38–43], four of which involve leucocytes. Dai et al.

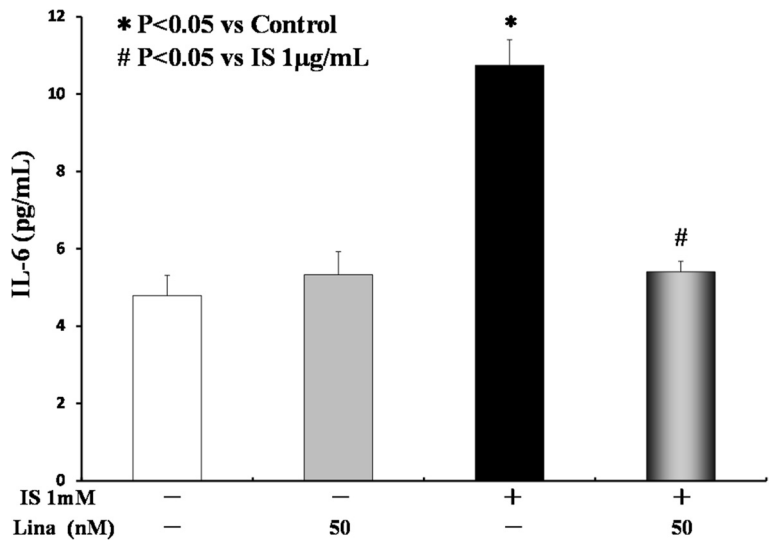

Fig. 6 Effects of linagliptin on indoxyl sulfate (IS)-induced interleukin (IL)-6 production. Human umbilical vein endothelial cells (HUVECs) were treated with IS and/or linagliptin. IL-6 levels in the supernatants were determined by ELISA after 5 h of treatment. $^{**}P < 0.01$ vs. control; $^{\#\#}P < 0.01$ vs. IS 1 μg/mL. *Lina* linagliptin

showed that two DPP-4 inhibitors, sitagliptin and vildagliptin, suppress the expression of Nod-like receptor family pyrin domain containing 3 (NLRP3), toll-like receptor 4 (TLR4), and the proinflammatory cytokine IL-1β in THP-1 macrophages through the inhibition of phosphorylated protein kinase C [38]. Meanwhile, Ikeda et al. reported that sitagliptin inhibits tumor necrosis factor (TNF)-α, extracellular signal-regulated kinase (ERK), c-Fos, NFκB, and the cut-like homeobox 1 expression induced by LPS and soluble CD26 in THP-1 cells and monocytes [39]. Furthermore, Ta et al. found that alogliptin suppresses TLR4-mediated ERK activation and matrix metalloproteinase expression in U937 histiocytes [40]. In addition, Ervinna et al. showed that anagliptin inhibits LPS-induced TNF-α production by inhibiting ERK phosphorylation and the nuclear translocation of NFκB in THP-1 cells [41]. There is also one in vitro study that reported the anti-inflammatory effects of DPP-4 inhibitors in endothelial cells. Hu et al. demonstrated that sitagliptin attenuates TNF-α-mediated induction of NFκB and orphan nuclear receptor NUR77 mRNA expression in vascular endothelial cells. However, there are no reports on the anti-inflammatory mechanisms by which linagliptin inhibits p38 MAPK phosphorylation in HUVECs [42]. Another in vitro study examined proximal tubular cells (human kidney 2 cells) in this context. Wang et al. showed that the anti-apoptotic activity of diprotin A, a DPP-4 inhibitor, ameliorates indoxyl sulfate-induced renal damage, which may be partly attributed to the ROS/p38MAPK/ERK and phosphoinositide 3 kinase (PI3K)-AKT pathways [43]. These in vitro studies raised the possibility that almost all DPP-4 inhibitors, including linagliptin, possess anti-inflammatory functions that are independent of GLP-1. In fact, in our study, sitagliptin was also able to inhibit LPS-stimulated IL-6 production.

The maximum blood concentration of linagliptin was 7.32 and 16.7 nM following single administration of 5 mg of the same in healthy volunteers and patients with diabetes with normal renal function, respectively (Boehringer Ingelheim Pharmaceuticals, Inc. Tradjenta® (linagliptin) tablets). This increased to 22.6 nM after repeated administration of 5 mg linagliptin in patients with diabetes, and a creatinine clearance of ≤30 mL/min (Boehringer Ingelheim Pharmaceuticals, Inc. Tradjenta® (linagliptin) tablets [44]). Because HD patients usually suffer from end-stage renal disease, we assumed that the maximum blood concentration is over 22.6 nM after repeated administration of 5 mg linagliptin in HD patients with diabetes. Therefore, we decided to use 50 nM linagliptin for the measurement of intranuclear p65 and phosphorylated p38 MAPK levels. To demonstrate the results of our clinical study in HD patients, we decided the concentration of linagliptin in vitro.

We also investigated the strength and mechanism of the anti-inflammatory effects of linagliptin by measuring the IL-6 production after linagliptin, caffeine, loxoprofen, and diclofenac sodium treatments. The concentrations of caffeine, loxoprofen, and diclofenac sodium for HD patients remain unknown. Therefore, the working concentrations of all five drugs were selected on the basis of their maximum blood concentrations after single administration in healthy volunteers. Linagliptin, loxoprofen, and diclofenac sodium, the latter two being representative anti-inflammatory drugs, significantly inhibited LPS-stimulated IL-6 production to a similar extent. Caffeine also inhibited LPS-stimulated IL-6 production [24]. Both linagliptin and caffeine are drugs with xanthine-related skeletal system activity. Compounds with xanthine-related skeletal system activity, such as theophylline and aminophylline, promote cAMP production through the inhibition of phosphodiesterase. In turn, the increase in cAMP production induces PKA activation [25]. Therefore, it is possible that linagliptin also inhibits MAPK phosphorylation via PKA activation. In fact, we found that pretreatment with 50 nM linagliptin and 50 nM caffeine significantly promoted PKA by 1.6- and 1.8-fold, respectively, compared with control treatment (unpublished results). Moreover, we examined the influence of H89 on linagliptin-induced anti-inflammatory effects and confirmed that H89 significantly increased linagliptin-inhibited IL-6 production. The small increase in IL-6 production by H89 may be affected by one of the several signaling pathways to the p38 MAPK mediated through AMPK- and ROS-ASK- other than PKA. However, the pharmacological mechanisms underlying the anti-inflammatory effects mediated by xanthine-related skeleton system activity remain unclear, necessitating further research.

This study had some limitations. First, proinflammation with agents other than LPS was not used. However, it is certainly important to mimic the experimental models with agents other than LPS. Therefore, we also investigated the anti-inflammatory effects of linagliptin following stimulation of HUVECs with IS. The treatment of cells with 50 nM linagliptin significantly inhibited IS-stimulated IL-6 production to 50 %. Linagliptin also may prevent the inflammation induced by agents other than LPS. Moreover, it may be more clinically relevant to directly apply IL-6 and/or TNF-α at the blood concentration in HD patients. Second, cascade reactions upstream to p38 activation were not investigated. Third, the detailed anti-inflammatory mechanisms of DPP-4 inhibitors other than linagliptin are still unclear. Moreover, it is important to examine the effects of exogenous GLP-1 and CD26 as well as the knockdown of GLP-1 and CD26 used in combination with linagliptin. However, an in vivo experiment with double knockout of GLP-1 and

DPP-4 (CD26) may be difficult. Although our in vitro study included GLP-1 knockout, further study using cell lines with CD26 knockout are important. In the current Japanese insurance system, we cannot administer DPP-4 inhibitors and GLP-1 analogs at the same time. However, it is certainly important to investigate the physiological relevance between incretin-independent mechanisms and incretin-dependent mechanisms. Moreover, it is interesting to examine the synergistic effects of incretin-independent mechanisms and incretin-dependent mechanisms. However, in order to research their effects, first, the incretin-independent effects of linagliptin must be confirmed. If the incretin-independent effects of linagliptin are not confirmed and if we find anti-inflammatory effects in patients without increasing GLP-1 levels, we cannot guess their mechanism. Further extensive research on the anti-inflammatory effects of DPP-4 inhibitors will enable the use of each of the nine DPP-4 inhibitors according to patient requirements.

Conclusions

We demonstrated the GLP-1-independent anti-inflammatory effects of linagliptin via MAPK-dependent mechanisms in vitro using HUVECs. p38 MAPK is an important marker in LPS- and PKA-mediated signaling pathways of inflammation. The prevention of p38 MAPK phosphorylation by linagliptin is expected to benefit HD patients with diabetes, for whom inflammation is a prognosis-related factor. Our findings suggest that linagliptin will play a crucial role in the treatment of HD patients with diabetes and chronic inflammation.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YN, HH, and MIna devised the study concept and design. YN, HH, and MT collected the data. YN and MT analyzed the data. YN interpreted the data. YN searched the literature. YN drafted the manuscript. YN, TO, MM, HS, KN, MIno, TS, IO, HG, YG, and MIna provided substantial revisions to the manuscript. KO gave the final approval of the manuscript. All authors read and approved the final manuscript.

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