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Research Article

Sitagliptin Inhibits the Lipopolysaccharide-Induced Inflammation

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Abstract

Objective: Sitagliptin is an anti-diabetic Dipeptidyl peptidase-4 (DPP-4) inhibitor; used worldwide with a well-established evidence base as an effective anti-diabetic therapy and the lowest cost of all DPP-4 inhibitors. Atherosclerosis and inflammation are more common in diabetic patients than in nondiabetic patients, and progression of atherosclerosis contributes to this inflammation. Therefore, anti-inflammatory therapy is important for the prognosis of diabetic patients. Although several reports have investigated the anti-inflammatory mechanisms of sitagliptin *in vitro*, none of these studies has described its effects on mitogenactivated protein kinase (MAPK) in human umbilical vein endothelial cells (HUVECs) stimulated with lipopolysaccharide (LPS). We assessed the MAPK-dependent anti-inflammatory effects of sitagliptin in HUVECs.

Methods: HUVECs $(1-2 \times 10^5 \text{ cell/mL})$ were either pretreated with different doses of sitagliptin for 1 h or left untreated. Subsequently, HUVECs were either incubated with lipopolysaccharide (LPS) together with sitagliptin (after treatment) or left untreated. Five hours post incubation, the culture medium was sampled for interleukin (IL)-6. Additionally, intranuclear p65 levels were measured 5 h after simultaneous treatment with LPS and sitagliptin. p38 MAPK levels and PKC activity were measured in the cytosolic fractions 30 min after simultaneous treatment with LPS and sitagliptin.

Results: Treatment with LPS alone induced significant IL-6 production compared with untreated control cells. Pretreatment of cells with sitagliptin at all concentrations tested significantly reduced LPS-stimulated IL-6 production. However, after treatment of cells with sitagliptin at any concentration did not inhibit LPS-stimulated IL-6 production. Compared to untreated cells, treatment with 5 nM sitagliptin significantly inhibited LPS-stimulated intranuclear p65 expression, and p38 MAPK phosphorylation. There was no significant difference in PKC activity with LPS or sitagliptin.

Conclusion: In HUVECs, sitagliptin elicits its anti-inflammatory effects through MAPK-dependent mechanisms.

Keywords

Sitagliptin; Inflammation; Interleukin-6; p38 Mitogen-activated protein kinases

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Introduction

The prevalence of diabetes mellitus (DM) continues to increase worldwide [1]. Insufficient control of DM induces macro- or microvascular complications such as coronary heart disease, stroke, kidney disease, amputation, and retinopathy [2,3]. Glycemic control is an important factor in the overall prognosis of DM patients [4,5]. Dipeptidyl peptidase-4 (DPP-4) inactivates incretin hormones such as glucagon-like peptide-1 (GLP-1) that stimulate a decrease in blood glucose levels [6]. GLP-1 is a gut hormone secreted from the intestinal L cells and plays a major role in glucose metabolism. DPP-4 inhibitors reduce blood glucose levels by inhibiting DPP-4, thereby preventing GLP-1 inactivation and maintaining the action of incretins [7]. For older patients with DM, there are limited options for treatment of visual obscuration resulting from diabetic retinopathy. However, DPP-4 inhibitors are oral anti-diabetic drugs, have a lower incidence of hypoglycemia, and provide a good safety profile [7]. Moreover, they do not increase the risk of cardiovascular and cerebrovascular diseases [8]. Therefore, DPP-4 inhibitors represent one option for treatment of this group of patients. Although nine types of DPP-4 inhibitor are available, there are almost no differences in their anti-diabetic effects. Therefore, it is important to understand the underlying mechanisms of the pharmacological actions of each DPP-4 inhibitor, to advance their context-dependent use in patients. Sitagliptin is a renally excreted anti-diabetic DPP-4 inhibitor; used worldwide with a well-established evidence base as an effective antidiabetic therapy and the lowest cost of all DPP-4 inhibitors [9]. Given the near-identical efficacies of DPP-4 inhibitors, sitagliptin is the most attractive treatment option due to its low cost. For medical expenses reduction of diabetes treatment, it is better to select the inexpensive medicine in all DPP-4 inhibitors. Atherosclerosis and inflammation are more common in diabetic patients than in nondiabetic patients, and progression of atherosclerosis contributes to this inflammation. Therefore, anti-inflammatory therapy is important for the prognosis of diabetic patients. The anti-inflammatory mechanisms of sitagliptin have been elucidated in vivo [10,11] and in vitro [12-14]. Zeng reported that the anti-inflammatory effects of sitagliptin occur via mitogen-activated protein kinase (MAPK)-dependent mechanisms in apolipoprotein-E knockout mice [10]. However, these in vivo studies do not shed light on the precise pharmacologic actions of sitagliptin, or the effect of increased GLP-1 on reducing inflammation. Many studies have investigated the anti-inflammatory effect of GLP-1 or analogs of human GLP-1 (exenatide or liraglutide) [15-20]. However, it is difficult to assess these anti-inflammatory effects in vivo in a way that excludes the influence of GLP-1 secreted from the intestinal L cells of the gastrointestinal tract. On the other hand, in three in vitro studies [12-14], although the independent pharmacological effects of GLP-1 or GLP-1 analogs were investigated, the authors did not assess whether the anti-inflammatory effects of these drugs were mediated by MAPK-dependent mechanisms. In this study, we elucidated the anti-inflammatory effects of sitagliptin via MAPKdependent mechanisms following stimulation of human umbilical vein endothelial cells (HUVECs) with lipopolysaccharide (LPS) in vitro. We analyzed four important markers: interleukin (IL)-6, which is a typical inflammatory marker; intranuclear p65 (a subunit of nuclear factor kappa B (NFkB)), which is a transcription factor involved in inflammatory responses; phosphorylated p38 MAPK,

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which ultimately activates transcription factors; and protein kinase C (PKC), which adds phosphate group to protein. Moreover, to estimate the pharmacological mechanism of sitagliptin, we assessed the anti-inflammatory effects of sitagliptin following administration before or concurrent with LPS.

Materials and Methods

Study materials and cell culture

All chemicals used in this study were of the purest grade available commercially. Sitagliptin were provided by Santa Cruz Biotechnology, Inc. (Bergheimer, Heidelberg, Germany). LPS and Phorbol 12-myristate 13-acetate (PMA; PKC activator) was purchased from Sigma-Aldrich (St. Louis, MO). The HUVECs cell line (Product Code:CC-2517), EGMTM Bullet KitTM medium, and the EGMTM Single QuotsTM kit were purchased from Lonza (Walkersville, MD). HUVECs were cultured and grown in endothelial basal medium supplemented with 10 mL fetal bovine serum (FBS), 2 mL bovine brain extracts, 0.5 mL human epidermal growth factor, 0.5 mL hydrocortisone, 50 mg/mL gentamicin, 50 μ g/mL amphotericin-B, and 0.5 mL ascorbic acid, according to the supplier's instructions (EGMTM Bullet KitTM medium and EGMTM Single QuotsTM Kit). HUVECs were maintained in a humidified atmosphere of 50 mL/L CO,, and 95 mL/L air at 37°C.

Drug treatments

All drug treatments were carried out under aseptic conditions in medium lacking FBS, bovine brain extracts, and hydrocortisone to avoid any elevation in spectrophotometric absorbance due to these substances binding to the coating antibodies. HUVECs were cultured for 24 h at densities of $1-2 \times 10^5$ cells/mL, and either pre-treated with 1, 5, 10, 50, 150, or 500 nM sitagliptin for 1 h, or left untreated. Following this, the medium was replaced and the cells were either treated with 1 µg/mL LPS together with 1, 5, 10, 50, 150, 500 nM sitagliptin; or PMA 100 ng/mL or left untreated. The concentration of LPS used (1 µg/mL) was sufficient to induce an inflammatory response in HUVECs. For measurement of IL-6 levels, 1, 5, 10, 50, 150, 500 nM sitagliptin, or PMA 100 ng/mL was used as part of the above procedure. For measurement of intranuclear NFkB p65 and p38 MAPK levels, 5 nM sitagliptin only was used.

Measurement of plasma IL-6 levels

Five hours after drug treatment, cells were harvested and the extracted medium was used to measure IL-6 levels. IL-6 levels were measured using a human IL-6 ELISA kit (Bender Medsystems, Vienna, Austria). An anti-human IL-6 coating antibody was adsorbed to the microplate wells. The human IL-6 present in the sample or standard solutions then bound to the antibodies adsorbed to the microwells. Thereafter, a biotin-conjugated anti-human IL-6 antibody, streptavidin-horse radish peroxidase (HRP), amplification reagent I (Biotinyl-Tyramide), amplification reagent II (Streptavidin-HRP), and substrate solution reactive with HRP were added in sequence. A colored product was formed in proportion to the amount of human IL-6 present in the sample or standard. The reaction was terminated by the addition of acid, and the absorbance was measured at 450 nm using a plate reader. (Spectramax, CA)

Measurement of intranuclear NFkB levels

Five hours after drug treatment, cells were harvested and the nuclei were extracted using the Nuclear/Cytosol Fractionation

Kit (BioVision Inc. Milpitas, CA). For quantitative determination of intranuclear p65 levels, the NF κ B/p65 ActivElisaTM Kit (Novus Biologicals, Littleton, CO) was used. This kit uses a sandwich ELISA protocol. The p65 antibody-coated plate captures free p65, and the amount of bound p65 is detected by adding a second biotin-conjugated p65 antibody followed by Streptavidin-HRP and colorimetric detection in an ELISA plate reader at 450 nm. (Spectramax, CA)

Detection of p38 MAPK phosphorylation in the cytosolic fractions

To evaluate the role of MAPK in LPS-induced inflammation in HUVECs, we examined the phosphorylation of p38 MAPK using the Cell-Based p38 MAPK (Thr¹⁸⁰/Tyr¹⁸²) ELISA Kit (RayBiotech, Inc. Norcross, CA). Thirty minutes after drug treatment, cells were fixed, blocked, and then incubated for 2 h at room temperature with antiphospho-p38 (Thr180/Tyr182) and anti-p38 antibodies. After incubation, cells were washed with wash buffer before being incubated again for 1 h at 37°C with HRP-conjugated anti-mouse IgG. Finally, the cells were washed with wash buffer and 3, 3', 5, 5'-tetramethylbenzidine substrate solution was added, resulting in the development of color in proportion to the amount of phosphorylated p38 MAPK. The reaction was stopped by the addition of Stop Solution from the kit, resulting in a change of color from blue to yellow. The intensity of the color was measured at 450 nm. (Spectramax, CA) The p38 MAPK phosphorylation was expressed as late of the absorbance of anti-phospho-p38 antibody divided by the absorbance of anti-p38 antibody.

Measurement of PKC activity levels in the cytosolic fractions

We examined the activity of PKC using the PKC kinase activity Kit (Enzo Life Sciences, Inc. Farmingdale, NY). The substrate to be phosphorylated by PKC, was precoated to the wells of providing a microplate. The PKC present in the sample to be assayed are added to the appropriate wells, followed by the addition of ATP to initiate the reaction. And a phosphospecific substrate antibody was added to the wells, which was binded specifically to the phosphorylated peptide substrate. Thereafter, a phosphospecific antibody was bound by a peroxidase conjugated secondary antibody. The assay is developed with tetramethylbenzidine substrate (TMB) and a color was developed in proportion to PKC phosphotransferase activity. The color development reaction was stopped with acid stop solution and the intensity of the color was measured in a microplate reader at 450nm using a plate reader. (Spectramax, CA)

Statistical analysis

JMP statistical software (version 10; SAS Institute, Cary, NC, USA) was used for all statistical analyses. Results are represented as means \pm standard errors of means (SEM). Effects of various treatments were compared with untreated control cells using one-way analysis of variance (ANOVA) and Bonferroni post-hoc analysis. A one-sided *P* value <0.05 was considered significant.

Results

Effect of sitagliptin pretreatment and after treatment on LPS-stimulated IL-6 production

We performed preliminary experiments to determine the most effective extraction time to detect IL-6. 1, 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

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h after treatment. Accordingly, IL-6 levels were measured 5 h after simultaneous treatment with LPS and the different compounds. LPS alone induced significant IL-6 production $(10.72 \pm 1.74 \text{ pg/mL})$ compared with untreated control cells. Pretreatment of cells with sitagliptin of all different concentrations significantly reduced LPS-stimulated IL-6 production $(1 \text{ nM}: 5.92 \pm 0.38 \text{ pg/mL}, 5 \text{ nM}: 7.11 \pm 0.98 \text{ pg/mL}, 10 \text{ nM}: 6.47 \pm 0.48 \text{ pg/mL}, 50 \text{ nM}: 4.33 \pm 0.23 \text{ pg/mL}, 150 \text{ nM}: 4.50 \pm 0.29 \text{ pg/mL}, 500 \text{ nM}: 5.05 \pm 0.66 \text{ pg/mL}). After treatment of cells with sitagliptin did not inhibit LPS-stimulated IL-6 production at any tested concentration (Figure 1).$

Percentage control of IL-6 production by sitagliptin pretreatment

There was no significant difference in percentage control of IL-6 production between treated cells and untreated control cells at any sitagliptin concentration (Figure 2).

Effects of sitagliptin on nuclear translocation of NFκB/p65 in LPS-stimulated HUVECs

We performed preliminary experiments to determine the most effective extraction time to detect intranuclear NF κ B p65. Compared with untreated control cells, intranuclear p65 levels were 0.87 ± 0.18, 1.66 ± 0.59, and 0.50 ± 0.17 times higher in LPS-stimulated cells 1, 5, and 24 h after treatment, respectively. As the highest increase was observed 5 h post-treatment, we measured the nuclear translocation of p65 5 h after simultaneous treatment with LPS and sitagliptin. There was no significant difference in intranuclear p65 levels between cells treated with 5 nM sitagliptin (87.74 ± 29.27 ng/mg protein) and untreated control cells (114.40 ± 16.58 ng/mg protein). LPS alone significantly increased intranuclear p65 levels (190.32 ± 66.98 ng/mg protein) compared with untreated control cells. Pretreatment of cells with 5 nM sitagliptin

significantly reduced LPS-stimulated intranuclear p65 levels (94.93 \pm 13.17 ng/mg protein) compared with untreated control cells (Figure 3).

Effects of sitagliptin on p38 MAPK phosphorylation in LPSstimulated HUVECs

As with NFkB/p65, we performed preliminary experiments to determine the most effective extraction time to detect phosphorylated p38 MAPK. Phosphorylation of p38 MAPK occurs upstream of a reaction cascade that leads to transcription factor activation. Therefore, we estimated the peak in p38 MAPK phosphorylation to occur earlier than 5 h in cells stimulated by LPS. Compared with untreated control cells, phosphorylated p38 MAPK levels were 0.88 ± 0.04 , 1.20 ± 0.08 , 0.96 ± 0.08 , and 0.92 ± 0.02 times higher in LPS-stimulated cells15 min, 30 min, 1 h, and 5 h after treatment, respectively. As the increase was highest at the 30 min time point, we measured p38 MAPK phosphorylation in the cytosolic fractions 30 min after simultaneous treatment with LPS and sitagliptin. There was no significant difference in p38 MAPK phosphorylation between 5 nM sitagliptin treatment (0.57 \pm 0.07) and untreated control cells (0.50 \pm 0.02). There was significant upregulation of p38 MAPK phosphorylation in LPS-treated cells (0.60 ± 0.04) compared with untreated control cells. Pretreatment of cells with 5 nM sitagliptin significantly inhibited LPS-stimulated p38 MAPK phosphorylation (0.49 \pm 0.01) compared with cells treated with LPS alone (Figure 4).

Effect of after sitagliptin and PMA treatment on LPSstimulated IL-6 production

PMA alone induced significant IL-6 production (5.67 \pm 0.43 pg/mL) compared with untreated control cells. The treatment of cells with 5 nM sitagliptin significantly inhibited LPS-stimulated IL-6







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Figure 2: Percentage control of interleukin (IL)-6 production by pretreatment with sitagliptin. IL-6 levels were measured using a human IL-6 ELISA kit (Bender Medsystems, Vienna, Austria). The absorbance was measured at 450 nm with Spectra Max 340 pc (Molecular Devices Co., CA). IL-6 percentage control is expressed relative to untreated control cells at 1, 5, 10, 50, 150, and 500 nM sitagliptin concentrations. Values are expressed as mean ± standard error. Sita, sitagliptin.



Milpitas, CA) and NFκB/p65 ActivElisa™ Kit (Nevus Biologicals, Littleton, CO) were used for measurements. Values are expressed as mean ± standard error. *P< 0.05 vs. control; #P< 0.05 vs. LPS 1 µg/mL; Sita, sitagliptin; LPS, lipopolysaccharide.

production (3.95 ± 1.16 pg/mL). Furthermore, PMA significantly increased sitagliptin inhibited IL-6 production $(7.26 \pm 0.62 \text{ pg/mL})$

Effects of sitagliptin on PKC activity in LPS-stimulated **HUVECs**

There was no significant difference in PKC activity among all 4 groups (untreated control cells: 0.016 \pm 0.003 µg/mg protein, sitagliptin treatment: 0.015 \pm 0.003 µg/mg protein, LPS treatment: 0.012 \pm 0.001 µg/mg protein, LPS and sitagliptin treatment: 0.017 \pm 0.002 µg/mg protein) (Figure 6).

Discussion

In this study, pretreatment of HUVECs with sitagliptin significantly inhibited LPS-stimulated IL-6 production, intranuclear p65 levels, and p38 MAPK phosphorylation. This suggests that the anti-inflammatory effects of sitagliptin were induced by reduced MAPK phosphorylation and were not due to increased GLP-1 levels. This study is the first to clearly demonstrate the anti-inflammatory effects of sitagliptin via MAPK-dependent mechanisms in vitro using HUVECs. We previously investigated the anti-inflammatory



Figure 4: Effect of sitagliptin on the phosphorylation of p38 MAPK in LPS-stimulated HUVECs. We evaluated the phosphorylation of p38 MAPK using the Cell-Based p38 MAPK (Thr¹⁸⁰/Tyr¹⁸²) ELISA Kit (RayBiotech, Inc. Norcross, CA). The absorbance was measured at 450 nm. Values are expressed as mean ± standard error. **P*< 0.05 vs. control; **P*< 0.05 vs. LPS 1 µg/mL; Sita, sitagliptin; LPS, lipopolysaccharide.



effects of linagliptin, another DPP-4 inhibitor, in hemodialysis patients with diabetes [15] and suggested three possible underlying mechanisms: increased GLP-1 (including an anti-diabetic effect) [16-21], suppression of DPP-4 [13], or xanthine-related skeletal system activity [22]. However, the study of anti-inflammatory mechanisms *in vivo* is complex owing to the presence of various overlapping anti-inflammatory factors, making it difficult to analyze each factor independently. For these reasons, our study did not use either GLP-1 or its analogs, and all experiments were conducted

using HUVECs, which do not secrete GLP-1. Therefore, the antiinflammatory effects of sitagliptin observed in this study can be attributed to the independent pharmacologic actions of sitagliptin, and not to increased GLP-1. Four previous studies conducted in leucocytes have reported the anti-inflammatory effects of DPP-4 inhibitors, including sitagliptin, *in vitro* [12,13,23,24]. Dai reported that two DPP-4 inhibitors, sitagliptin and vildagliptin, suppress the expression of Nod-like receptor family pyrin domain-containing 3, toll-like receptor 4 (TLR4), and the proinflammatory cytokine IL-1β

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in THP-1 macrophages [12]. Ikeda reported that sitagliptin inhibits tumor necrosis factor (TNF) a, extracellular signal-regulated kinase (ERK), c-Fos, NFkB, and the cut-like homeobox 1 expression induced by LPS and soluble DPP-4 in THP-1 cells and monocytes [13]. Ta discovered that alogliptin suppresses TLR4-mediated ERK activation and ERK-dependent matrix metalloproteinase manifestation in U937 histiocytes [23]. Ervinna indicated that anagliptin decreases LPSinduced TNFa production by inhibiting ERK phosphorylation and nuclear translocation of NFkB in THP-1 cells [24]. Two in vitro studies have described the anti-inflammatory effects of DPP-4 inhibitors in endothelial cells. Hu demonstrated that sitagliptin attenuates TNFamediated induction of NFkB and orphan nuclear receptor NUR77 mRNA expression in vascular endothelial cells [14]. Ishibashi showed that linagliptin inhibits advanced glycation end product-induced reactive oxygen species (ROS) generation in endothelial cells [25]. There have been no reports on anti-inflammatory mechanisms of sitagliptin in relation to p38 MAPK phosphorylation in HUVECs, although another in vitro study investigated proximal tubular cells (Human kidney 2 cells) in this context. This study showed that the anti-apoptotic activity of Diportin A, a DPP-4 inhibitor, ameliorates indoxyl sulfate-induced renal damage, which might be partly attributed to the ROS/p38MAPK/ERK and phosphoinositide 3kinase (PI3K)-AKT pathways [26]. These in vitro studies provide increasingly convincing evidence that almost all DPP-4 inhibitors, including sitagliptin, possess anti-inflammatory functions that are independent of GLP-1. LPS is found in the outer membrane of gram-negative bacteria, and elicits strong immune responses. Since studies have reported that 1 µg/mL LPS treatment can significantly induce inflammation in HUVECs [27-30], we used this concentration in our experiments for the same purpose. Following single administration of 12.5 mg, 25 mg, and 50 mg sitagliptin, the maximum blood concentration is 60 \pm 7 nM, 145 \pm 33 nM, and 319 \pm 83 nM in healthy volunteers [31]. Therefore, when investigating IL-6 production in LPS-stimulated cells, we decided to use sitagliptin

after treatment. Sitagliptin pretreatment significantly reduced LPSstimulated IL-6 production, but sitagliptin after treatment did not prevent LPS-stimulated IL-6 production. We proposed that these differences might be due to sitagliptin functioning PKC. Dai reported that two DPP-4 inhibitors, sitagliptin and vildagliptin, suppress the inflammatory signals through inhibition of phosphorylated PKC at 24 hr after administration of medicine [12]. Therefore, we examined PKC activity with sitagliptin. The induction of inflammation by PMA (PKC activator) was confirmed. However, PKC activity did not change by sitagliptin or LPS at 30 minutes after administration of medicine. PKC activity may be greatly affected by the measurement time. Further investigations of the inhibitory effects of sitagliptin on PKC activity are ongoing. We acknowledge certain limitations of our study. Inflammation induced by agents other than LPS was not studied. Cascade reactions upstream from p38 activation were not evaluated. Moreover, in this study, we chose to study the effect of only a single sitagliptin concentration (5 nM) on intranuclear p65 and phosphorylated p38 MAPK levels; evaluation of these effects at a range of sitagliptin concentrations would have been more robust. In our investigations of LPS-stimulated IL-6 production, 5 nM sitagliptin pre- or after treatment was the least effective concentration we tested for reducing LPS-stimulated IL-6 production. Therefore, examining the effect of 5 nM sitagliptin on intranuclear p65 levels and phosphorylated p38 MAPK levels represents the most conservative approach. Nevertheless, LPS-stimulated intranuclear p65 levels and p38 MAPK phosphorylation were inhibited significantly by 5 nM sitagliptin. Detailed anti-inflammatory mechanisms for DPP-4 inhibitors other than sitagliptin are still uncertain. Further research on the anti-inflammatory effects of DPP-4 inhibitors will enable the use of each of the nine DPP-4 inhibitors individually, depending on the patient context. We evaluated the anti-inflammatory effects of sitagliptin via MAPK-dependent mechanisms in vitro by using HUVECs. Overall, our results strengthen the evidence for the use of

concentrations of 1, 5, 10, 50, 150 and 500 nM for pretreatment and

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sitagliptin as an efficient and low cost DPP-4 inhibitor for diabetic patients with chronic inflammation.

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Declaration of Interest

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