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Effect of Vildagliptin, Compared to Sitagliptin, on the Onset of Hyperglycemia-Induced Metabolic Memory in Human Umbilical Vein Endothelial Cells

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Abstract

Background: Metabolic memory, the long-term effect of poor glycemic control in the initial stages of diabetes, leads to vascular complications that negatively affect patients' outcome. As oxidative stress plays a major role in metabolic memory onset, the use of drugs with antioxidant properties may be clinically beneficial.

Objectives: To test the effects of two dipeptidyl peptidase-4 inhibitors, vildagliptin and sitagliptin, on hyperglycemia-induced oxidative stress and apoptosis in Human Umbilical Vein Endothelial Cells (HUVECs).

Methods: HUVECs were treated with 5 nM vildagliptin or sitagliptin for 1 h, after 21 days of culture under conditions of continuous normal or high glucose (NG and HG, respectively), Oscillating Glucose (OG) or HG/OG memory (HM and OM, respectively). The effects of the two drugs on the following markers of oxidative stress were tested by different techniques: Reactive Oxygen Species (ROS), 8-hydroxy-deoxy-guanosine, 3-nitrotyrosine, thioredoxin-interacting protein (TXNIP) mRNA and PKC-β protein. Moreover, the levels of BCL-2 (anti-apoptotic) and BAX (pro-apoptotic) transcripts and of caspase-3 protein were assayed.

Results: In HUVECs, vildagliptin was able to significantly counteract the oxidative stress triggered by OG, HG and memory conditions, as measured by the levels of ROS, DNA and protein damage markers, TXNIP and PKC-β. Also, a significant increase of BCL-2 and decrease of BAX mRNA levels was observed upon OG and HG. Sitagliptin exerted a less evident effect. No significant effect on caspase-3 levels was detected by either drug.

Conclusions: Our findings point toward antioxidant and antiapoptotic properties of vildagliptin in HUVECs exposed to HG, OG and metabolic memory conditions, whereas the effects of sitagliptin were less prominent. If these results on the vascular protective effects of vildagliptin will be confirmed, its use may be implemented in the setting of diabetes to prevent the onset of metabolic memory.

Keywords: Apoptosis; Endothelial cells; Hyperglycemia; Metabolic memory; Oxydative stress; Sitagliptin; Vildagliptin

Introduction

Type 2 Diabetes (T2D) is a syndrome characterized by hyperglycemia, which plays a major role in the pathogenesis of T2D-associated complications like cardiovascular disease and nephropathy. Hyperglycemia induces oxidative stress [1-5], and increased levels of Reactive Oxygen Species (ROS) exert a detrimental effect on cellular functions by damaging lipid membranes, enzymes and nucleic acids, and by triggering apoptosis [6]. In addition to continuous hyperglycemia, post-prandial oscillations of glucose levels, like those experienced daily by diabetic patients, have been suggested as an independent risk factor for both microvascular and macrovascular complications [7-9]. This concept is consistent with the finding that intermittent High Glucose (HG) levels lead to increased damage to endothelial cells [10-13], mesangial cells [14] and tubule-interstitial cells *in vitro* [15]. Of note, it has been demonstrated that oscillating glucose levels result in increased levels of markers of ROS and vascular stress in both cultured endothelial cells and diabetic patients [3,4,10-13,16]. It has also been shown that following a hyperglycemic stress, the levels of basement membrane proteins such as collagen and fibronectin, which are markers of high glucose stress, remain elevated several days after glucose levels have normalized [16,17]. This phenomenon, defined as "metabolic memory", has been linked to poor glycemic control in the early phases of diabetes, despite a tight glycemic control achieved during the course of the disease [18-20]. Several preclinical and clinical studies have demonstrated the existence of a metabolic memory in endothelial cells [19]. Moreover, it has been shown that normalization of the extracellular glucose level does not switch off the intracellular pro-oxidant environment, suggesting a role for ROS in the onset of cellular memory [16]. Of great interest is the observation that glucose fluctuations also produce a memory in Human Umbilical Vein Endothelial Cells (HUVECs), likely through the generation of oxidative stress [5]. Therefore, from a clinical point of view, agents able to counteract the phenomenon of metabolic memory in endothelial cells may help preventing vascular complications that negatively affect the outcome of diabetic patients.

In diabetes, hyperglycemia also impairs the protective action exerted by Glucagon-Like Peptide-1 (GLP-1) on endothelial function [21-22],

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which can be effectively restored by the use of Dipeptidyl Peptidase-4 (DPP-4) inhibitors [23]. Among these, vildagliptin and sitagliptin have demonstrated antioxidant effects in both pre-clinical and clinical studies, significantly lowering oxidative stress-related markers [24-30]. Moreover, a recent report has shown that treatment of HUVECs with the DDP-4 inhibitor teneligliptin improved the oxidative stress and apoptotic phenotype induced by exposure to continuous HG [31]. Notably, this agent could also overcome the detrimental effects triggered by HG-induced metabolic memory conditions.

As data on the effects of vildagliptin and sitagliptin on metabolic memory in endothelial cells are currently lacking, we tested whether these inhibitors could counteract, in HUVECs, hyperglycemia-induced oxidative stress damage and apoptosis and switch-off the metabolic memory caused by exposure to either oscillating or continuous HG levels.

Materials and Methods

Cell culture and experimental design

HUVECs were purchased from Lonza (Lonza Bioresearch LBS, Basel, Switzerland) and cultured with EGM™-2 Bulletkit™ supplemented with human epidermal growth factor (hEGF), hydrocortisone, human recombinant fibroblast growth factor-beta (hFGF-b), heparin, 2% fetal bovine serum (FBS), and gentamicin/amphotericin-B (GA), at 37°C in a humidified atmosphere with 5% CO_2.

Cells were used at passage 4 and were plated in duplicate at 2×10^5 in 6-well plates for total RNA and protein extraction, and at 1×10^4 in 96-well plate for ROS measurement. After seeding, they were allowed to attach overnight and, after 24 h, were exposed to one of the following glucose conditions:

1. Continuous normal glucose (NG) (5 mM) for 21 days.
2. Oscillating glucose (OG) (5-25 mM) for 21 days.
3. Continuous high glucose (HG) (25 mM) for 21 days.
4. Cellular oscillating glucose memory (OM): OG (5-25 mM) for 14 days, followed by NG (5 mM) for 7 days.
5. Cellular High glucose memory (HM): continuous HG (25 mM) for 14 days, followed by NG (5 mM) for 7 days.

After 21 days, vildagliptin (5 nM) or sitagliptin (5 nM) were added to the culture media for 1 h.

Measurement of ROS and of oxidative stress markers

The fluorescent probe 2′,7′-Dichlorofluorescein diacetate (H_2DCFDA) was used to measure the intracellular generation of ROS. 1×10^4 HUVECs were grown in clear flat bottom treated 96-well plates during 21 days. At the end of this period, cells were treated with the above-mentioned drugs, and after removing the medium, they were stained with 20 μM H_2DCFDA for 30 min at 37°C. The fluorescence intensity of H_2DCFDA was measured kinetically at an excitation and emission wavelength of 485 nm and 530 nm, respectively, for H_2DCFDA using a fluorescent microplate reader (Synergy HT, BioTek Instruments, Inc., Winooski, Vermont, USA).

The content of 8-hydroxy-deoxy-guanosine (8-OH-dG, StressMarq biosciences, Victoria, Canada), and 3-nitrotyrosine (3-NY) was measured by ELISA (Abcam, Cambridge, USA), following the manufacturer's instructions.

RNA isolation and qRT-PCR

Total RNA was isolated from HUVECs using the total RNA isolation kit (NorgenBiotek Corp, Thorold, Ontario, Canada) following the manufacturer's instructions. The first-strand cDNA was prepared using 1-2 μg of total RNA, the Superscript III RT kit and the random hexamer primers (Invitrogen, Carlsbad, CA, USA) in a total volume of 25 μl according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed on a QuantStudio 6 flex (Applied biosystem) detection system using the SybrGreen reagents (Takara Bio Company, Clontech, Mountain View, CA, USA) and TaqMan® Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA). Primers used are listed in Table 1.

Protein analysis

For western blot analysis, HUVECs were lysed in RIPA buffer (Sigma-Aldrich) with 10% protease and 1% phosphatase inhibitors (Sigma-Aldrich). After determining the content of protein by the Bradford assay buffer (Sigma-Aldrich), 50 μg of lysates were separated by electrophoresis using PAGEr gels (4-12%) (Lonza) and transferred onto a PVDF membrane (Perkin Elmer, Waltham, MA, USA). After blocking with 5% non-fat dried milk or 5% bovine serum albumin, membranes were incubated with the respective primary antibodies (1:1,000 anti-PKC-β [Santa Cruz, Santa Cruz, CA, USA], 1:500 anti-caspase-3 [Abcam, Cambridge, UK] and 1:1,000 anti-β-actin [Sigma-Aldrich, St Louis, MO, USA) overnight at 4°C. Membranes were then incubated with the appropriate secondary horseradish peroxidase-conjugated IgG antibodies (GE Healthcare Europe GmbH, Milan, Italy) at a 1:3,000 dilution for 1h at room temperature. Blots were revealed by LI-COR ECL Reagent and C-DiGit Blot scanner (LI-COR Biosciences). Protein spots were quantitated by Image Studio software (www.licor.com). β-actin served as the loading control.

Statistical analysis

All experiments were performed eight independent times. Numerical data were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using the one-way analysis of variance (ANOVA) with appropriate *post-hoc* multi-comparison tests (Tukey's), to compare the effects of vildagliptin and sitagliptin. A P value of <0.05 was considered statistically significant.

Results

Effects of DDP-4 inhibitors on oxidative stress and damage

ROS content was measured by a fluorometric assay under 5 different glucose conditions. In untreated cells, exposure to HG (oscillating [OG], continuous [HG], or mimicking cellular memory under oscillating [OM] or continuous [HM]), but not to NG, significantly increased the production of ROS, and did so at a similar extent. Compared to untreated cells, treatment with vildagliptin significantly decreased

Gene	Sequence
TXNIP	GCAGTGCAAACAGACTTCGG TCACCTGTTGGCTGGTCTTC
β-ACTIN	CAGCCATGTACGTTGCTATCCAGG AGGTCCAGACGCAGGATGGCATG
BAX	TCAGGATGCGTCCACCAAGAAG TGTGTCCACGGCGGCAATCATC
BCL-2	ATCGCCCTGTGGATGACTGAGT GCCAGGAGAAATCAAACAGAGGC

Table 1: Primers used to assess the transcript levels of the following genes by SYBR-green quantitative-Real Time-PCR, in HUVECs either untreated or treated with vildagliptin or sitagliptin upon different glucose conditions.

the content of ROS in cells grown under cellular memory conditions (OM: $p<0.05$; HM: $p<0.01$; Figure 1A). Notably, these cells displayed a significantly lower content of ROS compared to cells grown under OG and HG, respectively. No significant difference was observed upon exposure to sitagliptin under any glucose conditions.

Next, we evaluated the levels of 8-OH-dG and 3-NY, which are markers of hyperglycemia-induced DNA and protein damage. As shown in Figures 1B and 1C, in untreated cells, exposure to any condition but HM significantly increased the levels of these markers compared to NG. Treatment with vildagliptin but not sitagliptin was able to counteract both markers in cells grown under HM conditions ($p<0.05$).

Effects of DDP-4 inhibitors on TXNIP transcript levels

We then assessed the transcript levels of thioredoxin-interacting protein (TXNIP). In untreated cells, compared to NG, all HG conditions triggered a significant up-regulation of TXNIP mRNA (Figure 2). Vildagliptin-treated cells grown under OM and HM conditions displayed a significant decrease of transcript compared to untreated cells ($p<0.01$ in both cases), which was similar to that observed in cells grown under NG. Conversely, cells grown under OG and HG had higher levels than those upon NG. The effect was less marked after sitagliptin treatment.

Effects of DDP-4 inhibitors on PKC-β mRNA levels

Prolonged exposure of HUVECs to OG, HG and memory conditions induced an increase in PKC-β levels, as demonstrated by Western blot (Figure 3). Following treatment with vildagliptin and sitagliptin, the levels of this marker decreased, although differences were not statistically significant. However, under OM and HM conditions, the expression was significantly lower than under OG and HG, respectively.

Effects of DDP-4 inhibitors on apoptosis-related markers' levels

The mRNA levels of the apoptosis-related markers BCL-2 (anti-apoptotic) and BAX (pro-apoptotic) were evaluated by qPCR. In untreated cells, compared to NG conditions, exposure to any of the 4 HG conditions did not alter the levels of BCL-2 mRNA (Figure 4A), while it significantly increased those of BAX (Figure 4B). Treatment with vildagliptin increased BCL-2 transcript levels, but, compared to untreated cells grown in NG, the difference was significant only upon HM ($p<0.05$, Figure 4A). No difference was observed following treatment with sitagliptin (Figure 4A). In contrast, levels of BAX mRNA decreased following treatment with both drugs, but the difference was significant only in cells grown under OM ($p<0.01$) and HM ($p<0.05$) and exposed to vildagliptin, and in those grown under OM ($p<0.01$) and exposed to sitagliptin (Figure 4B).

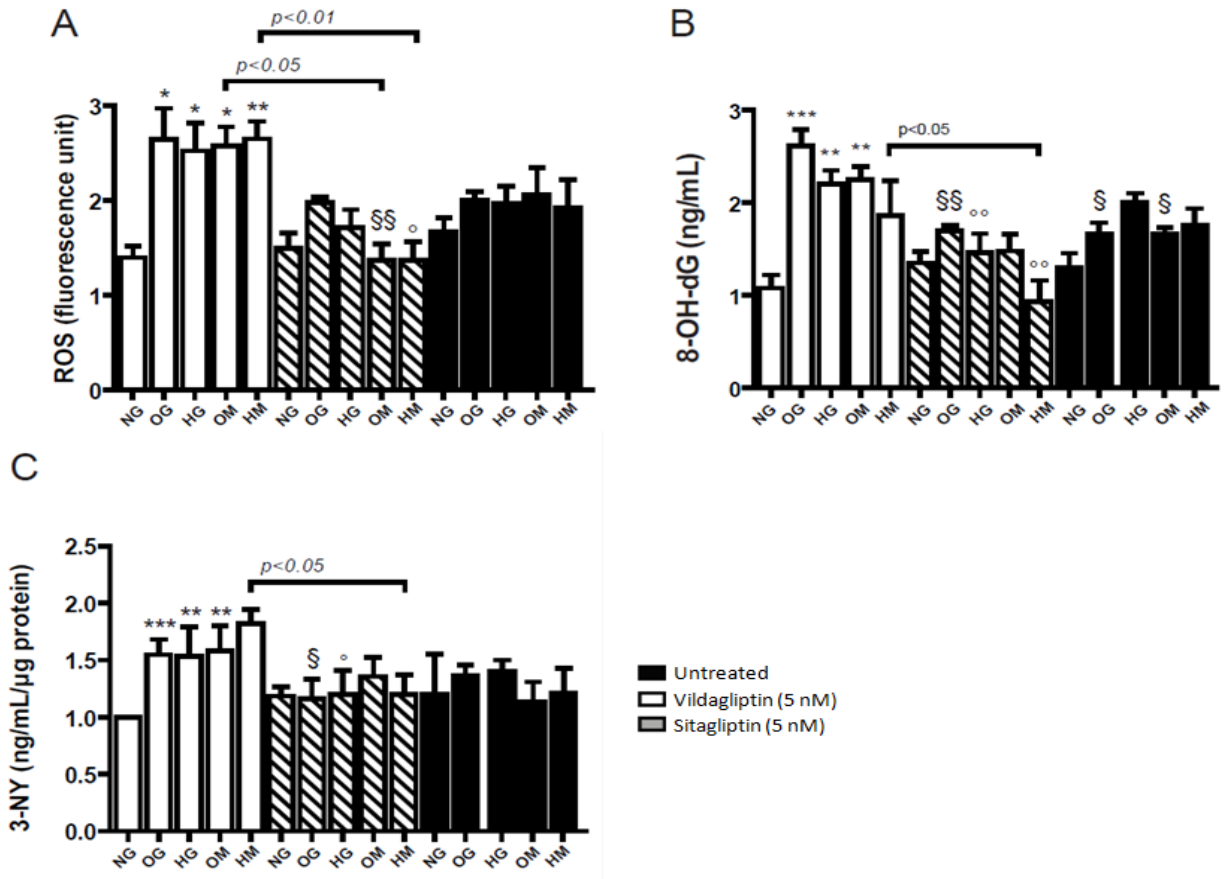

Figure 1: Oxidative stress markers' assessment. (A) Total ROS intensity measurement was performed in HUVECs after 21 days in culture under conditions of NG, OG, HG or OM-HM. Cells were treated with vildagliptin (5 nM) or sitagliptin (5 nM) for 1 h. (B) 8-OH-dG content was evaluated under the aforementioned conditions by ELISA. (C) 3-NY content was measured by ELISA and normalized to total protein content. Bars represent mean ± SEM. One-way ANOVA, followed by Tukey's *post-hoc* test was performed. Symbols represent the result of the comparison with untreated cells under NG*, OG§ and HG$^{°}$. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{§}p<0.05$, $^{§§}p<0.01$, $^{°}p<0.05$, $^{°°}p<0.01$. ROS: reactive oxygen species; NG: normal glucose; OG: oscillating high glucose; HG: continuous high glucose; OM: OG memory; HM: HG memory; 8-OH-DG: 8-hydroxy-deoxy-guanosine; 3-NY: 3-nitrotyrosine.

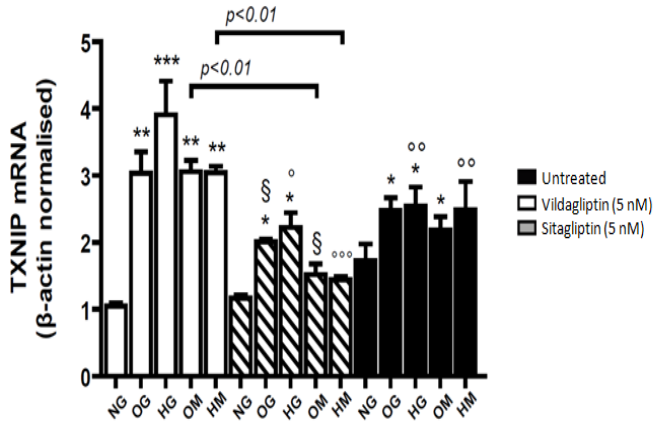

Figure 2: Expression of the oxidative stress marker TXNIP mRNA. Total cellular RNA was isolated from HUVECs after 21 days in culture under NG, OG, HG or OM-HM conditions. Cells were treated with vildagliptin (5 nM) or sitagliptin (5 nM) for 1 h. The levels of mRNA encoding for TXNIP were assessed by qRT-PCR and normalized on β-actin. The experiment was performed three times. Bars represent mean ± SEM. One-way ANOVA, followed by Tukey's *post-hoc* test was performed, where * vs. NG, § vs. OG, and ° vs. HG. *p<0.05, **p<0.01, ***p<0.001, §p<0.05, °p<0.05, °°p<0.01, °°°p<0.001. TXNIP: Thioredoxin-interacting protein; NG: Normal glucose; OG: Oscillating high glucose; HG: Continuous high glucose; OM: OG memory; HM: HG memory.

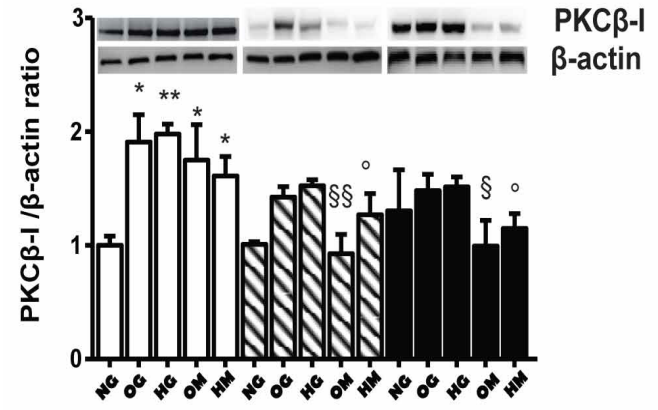

Figure 3: PKC-β expression. Total whole cell lysates were prepared from HUVECs grown under different glucose conditions per 21 days and then treated with vildagliptin (5 nM) or sitagliptin (5 nM) for 1 h. 50 µg of lysates were resolved on SDS-PAGE and incubated with an anti-PKC-β antibody. The levels of protein were normalized on β-actin. The experiment was performed three times. Bars represent mean ± SEM. One-way ANOVA, followed by Tukey's *post-hoc* test was performed, where * vs. NG, § vs. OG, and ° vs. HG. *p<0.05, **p<0.01, §p<0.05, §§p<0.01, °p<0.05. NG: Normal glucose; OG: Oscillating high glucose; HG: Continuous high glucose; OM: OG memory; HM: HG memory.

Finally, we assessed the levels of caspase-3, a marker of apoptotic cell death. In untreated cells, a significant increase was observed only under HG vs NG. Treatment with vildagliptin led to a significant decrease only in cells exposed to HM ($p<0.05$), whereas no effect of sitagliptin was observed.

Discussion and Conclusion

Compelling evidence has demonstrated that metabolic memory is caused by poorly controlled hyperglycemia in the early stages of diabetes, and leads to vascular complications regardless of tight glucose control attained during the course of disease. Occurrence of this phenomenon has been reported in endothelial cells, in which it is driven by different mechanisms, such as oxidative stress and epigenetic changes [19]. DDP-4 inhibitors are anti-diabetic agents with vascular protective effects [32]. Yet, little is known about their impact on HG-induced stress and metabolic memory in endothelial cells. To the best of our knowledge, this is the first study addressing the effects of vildagliptin and sitagliptin on the levels of different markers of oxidative stress and apoptosis in HUVECs grown under conditions of both oscillating and continuous HG and HG-induced metabolic memory. The levels of several well established markers of oxidative stress were measured: ROS, which are important mediators of hyperglycemia-induced tissue damage [33] 8-OH-dG, a marker of oxidative DNA damage and of macrovascular complications in T2D patients [34], 3-NY a marker of ROS-induced protein damage [16], TXNIP, whose levels increase upon ROS production and hyperglycemia [35] and PKC-β [36], a mediator of hyperglycemia-induced vascular effects. Moreover, we selected BCL-2, BAX and caspase-3 as markers of apoptosis.

In untreated cells, compared to NG conditions, exposure to HG increased the levels of oxidative stress markers and of the apoptotic markers BAX and caspase-3, while leaving the levels of BCL-2 unchanged. In general, the effects observed upon oscillating or continuous HG were similar, with slight differences in few cases. Overall, under conditions of OM and HM, the changes persisted and were similar to those observed under OG and HG, despite the 7 days of growth in the presence of NG levels, confirming the occurrence of the metabolic memory phenomenon induced by both OG and HG. Overall, treatment with vildagliptin could revert these changes and the effect was more evident than upon sitagliptin, suggesting anti-oxidant and anti-apoptotic properties that can, at least in part, counteract the effects observed under HG-induced metabolic memory [5,16,37].

In a recent report, we assessed the antioxidant properties of another DDP-4 inhibitor, teneligliptin, in comparison with sitagliptin, on HUVECs grown under conditions of continuous NG, HG and HM [31]. Similarly to our results, teneligliptin was able to ameliorate the oxidative stress and apoptotic phenotype of HUVECs induced by continuous hyperglycemia, and to counteract the changes observed upon HM. Notably, the effects were dose-dependent and were more pronounced than upon sitagliptin [31].

The present study has some limitations. Indeed, all measurements of oxidative stress marker levels upon vildagliptin and sitagliptin were performed at only one time point (i.e., after 1 h of treatment), and thus no information is provided on the possible changes in ROS levels in HUVECs upon chronic administration of the drugs. Moreover, we showed that vildagliptin could ameliorate the oxidative status of HUVECs exposed to hyperglycemic conditions, but we did not investigate its capability to induce antioxidant enzymes, as this was beyond the focus of the present work. Therefore, additional studies are needed to define the protein expression profile for different markers.

Moreover, improvement on cellular redox state assessment is necessary to well-characterize the antioxidant capacity of vildagliptin. If our findings on the protective effects of vildagliptin on endothelial cells will be confirmed *in vitro* and *in vivo*, its use may be implemented as part of the therapeutic approach in diabetic patients, to prevent the onset of metabolic memory and, consequently, of vascular complications.

In conclusion, this study provides evidence supporting the protective role of vildagliptin in counteracting HG-induced oxidative stress and apoptosis in endothelial cells, and the metabolic memory induced by both oscillating and continuous HG conditions.

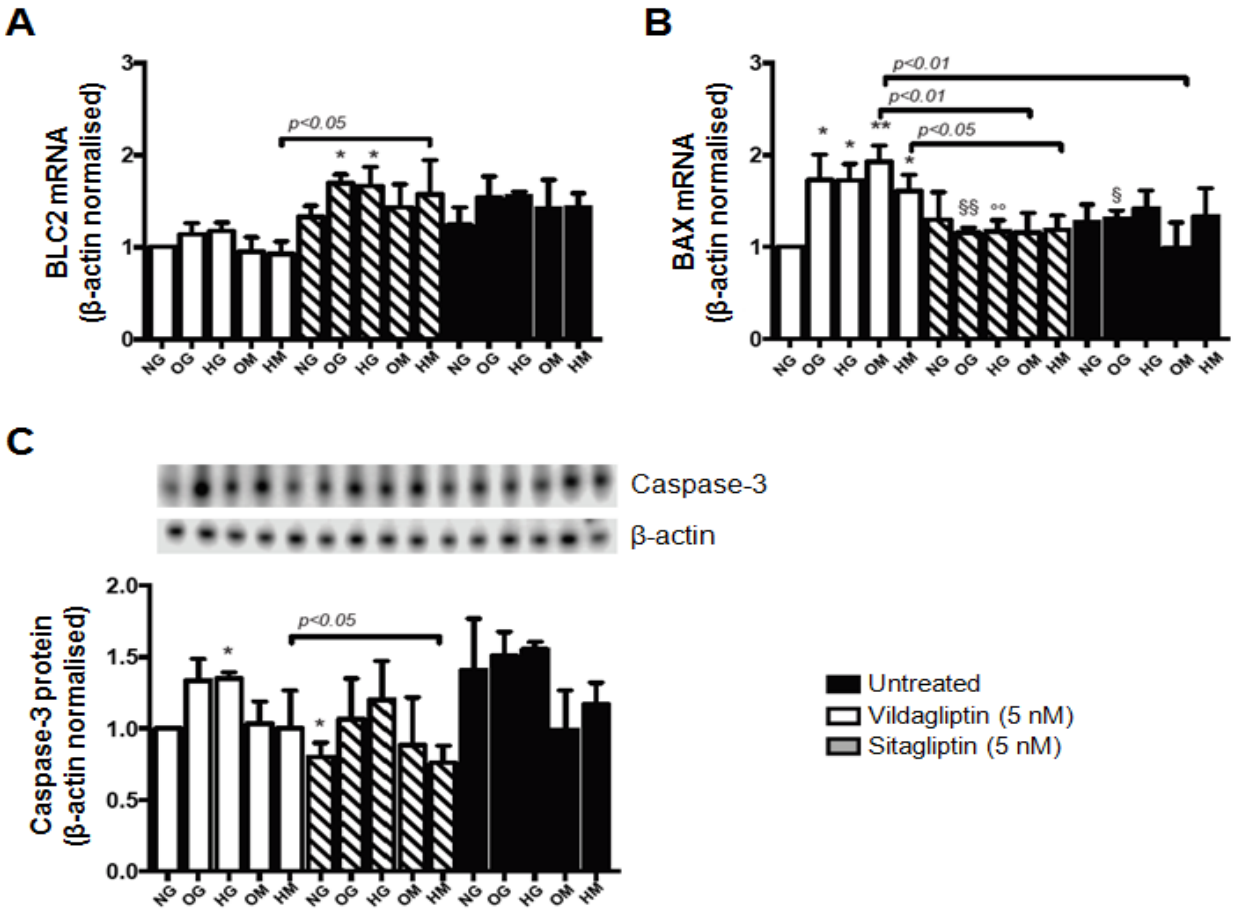

Figure 4: Regulation of apoptosis-related markers. Total RNA or proteins were extracted from HUVECs grown for 21 days under NG, OG, HG or OM-HM conditions and treated with vildagliptin (5 nM) or sitagliptin (5 nM) for 1 h. The expression of BCL2 (A) and BAX (B) mRNA was quantified by qRT-PCR and normalized on β-actin. (C) 50 µg of protein lysates were resolved on SDS-PAGE and the expression of caspase-3 was normalized on the expression of β-actin. The experiment was performed three times. Bars represent mean±SEM. One-way ANOVA, followed by Tukey's *post-hoc* test was performed, where * vs. NG, § vs. OG, and ° vs. HG. *p<0.05, §p<0.05, §§p<0.01, °p<0.05, °°p<0.01. NG: Normal glucose; OG: Oscillating high glucose; HG: Continuous high glucose; OM: OG memory; HM: HG memory.

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Authorship Contributions

LSL contributed to the acquisition, analysis and interpretation of data and manuscript writing. SG and AC contributed to analysis and interpretation of data and manuscript writing. All authors have read and approved the final manuscript.

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