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Isabel Maria Hipólito Reis Dias Rodrigues

Effects of liraglutide and exenatide, two GLP-1 agonists, in vascular wall cells

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DATA DE CONCLUSÃO

DESIGNAÇÃO DA ÁREA DO PROJECTO

BIOQUÍMICA

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EFFECTS OF LIRAGLUTIDE AND EXENATIDE, TWO GLP-1 AGONISTS, IN VASCULAR WALL CELLS

ORIENTADOR

DOUTORA RAQUEL SOARES

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Para o Avô, pelo exemplo.

Effects of liraglutide and exenatide, two GLP-1 receptor agonists, in vascular wall cells

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Abstract

Endothelial dysfunction underlies the pathogenesis of some type 2 diabetes mellitus cardiovascular complications, which are the major cause of morbidity and mortality in diabetic patients. Knowing that glucagon-like peptide-1 (GLP-1), a brain-gut insulinotropic peptide, plays an important role in the dynamic of these disorder, pharmacologic GLP-1 receptor agonists were developed, such as liraglutide and exenatide, for its management. The present study was undertaken to investigate the effects of these two anti-diabetic drugs on angiogenic processes, using human microvascular endothelial cells (HMECs) cultures in low (5.5mM) and high (20mM) glucose concentrations, mimicking a normal or hyperglycemic environment, respectively. No cell cytotoxicity (MTS assay) of these drugs was found upon incubation of HMECs cultures at both glucose concentrations. Cellular proliferation (BrdU assay) was increased by GLP-1 receptor agonists, reaching statistical significance for exenatide. Despite migration (wound healing assay) and apoptosis (TUNEL assay) were not statistical significant, differences were found between glucose concentrations. In addition, no difference was observed in GLP-1 receptor expression. For better understanding of this finding, further studies of the underlying downstream pathways must be carried out. In conclusion, the present study indicates that exenatide and liraglutide may exert important physiological roles in endothelial cells particularly in hyperglycemic concentrations.

Key words: Type 2 diabetes mellitus (T2DM); glucagon-like peptide receptor (GLP-1R); vascular endothelial cells; angiogenesis.

Introduction

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disease characterized in the established state by a chronic hyperglycemic state accompanied by insufficient insulin production and resistance to insulin action in body tissues (Drucker et al., 2010). Nowadays, T2DM has become increasingly important when considered its exponentially increased prevalence and its associated mortality and morbidity (Cho, 2013). Due to these facts, T2DM deserves the intensive investigation that has been done.

Diabetes is responsible for inducing neovascular mechanisms and impairing vascular regulation, resulting in several micro and macrovascular complications (Erdogdu et al., 2013; Goyal et al., 2010). Therefore, cardiovascular disease is the major cause of morbidity and mortality in diabetic patients (Kang et al., 2013; Gaspari et al., 2013).

Glucagon-like peptide-1 (GLP-1) is a brain-gut insulinotropic peptide, produced by L cells of the large intestine that plays an important role in the dynamic of metabolic disease, and regulation of homeostasis by influencing obesity (Pi-Sunyer et al., 2015), inducing glucose-dependent insulin secretion, while suppressing glucagon secretion (Aaboe et al., 2008; Kim and Egan, 2008). In addition, GLP-1 inhibits gastric motility, contributing to satiety (Krasner et al., 2014; Hirata et al., 2013). Thus, the GLP-1 actions have been an interesting field of study and a promising target for therapeutic purposes of diabetes.

It is known that endothelial cells express glucagon-like peptide receptor (GLP-1R) and evidence supports GLP-1 as an important player in endothelial function (Aronis et al., 2013; Ding and Zhang, 2012; Xie et al., 2011). Moreover, it was recently reported that endothelial dysfunction is ameliorated by acute administration of GLP-1 in type 2 diabetic patients with coronary heart disease (Nystrom et al., 2004). Regardless of these actions, GLP-1 cannot be used as a medical therapy agent due to its short half-life (Hu et al., 2013). Two minutes after its secretion, GLP-1 is rapidly degraded by dipeptidyl peptidase-4 (DPP4) to generate one metabolite that is

truncated in the NH_2-terminal incapable of connecting with GLP-1R (Aaboe et al., 2008; Hirata et al., 2013; Erdogdu et al., 2010).

Therefore, several pharmacologic GLP-1R agonists were developed with the goal of preserving the important metabolic functions of the GLP-1 hormone (Aaboe et al., 2008), but at the same time with a longer half-life.

Liraglutide and exenatide are two novel anti-diabetic drugs included on incretin-based therapies, used in T2DM management (Meier and Nauck, 2015). These drugs act as agonists of the GLP-1R and are resistant to DPP4-mediated degradation, granting a longer half-life when compared to GLP-1 (Aaboe et al., 2008). Interestingly, they exert their actions in a glucose-dependent manner without the need for frequent dose adjustment (Kim and Egan, 2008; Meier and Nauck, 2015).

Despite the known intervention of GLP-1 in endothelial cells, it is not established whether the GLP-1R agonists are capable of positively influencing endothelial dysfunction, which accompanies the progression of T2DM and contributes to the enormous morbidity rates found in these patients.

The present study was undertaken to investigate the effects of these two anti-diabetic drugs on angiogenic processes, namely endothelial cell survival, proliferation, migration and apoptosis, using cultures of human microvascular endothelial cells in low and high glucose concentrations, mimicking a normal or hyperglycemic environment, respectively.

Materials and Methods

Cell culture experiments

Human microvascular endothelial cells (HMECs) (ATCC, Spain) were used between passage 5 and 10. HMECs were cultured in RPMI 1640 medium (Invitrogen Life Technologies, UK)

supplemented with 10% fetal bovine serum (FBS) (Invitrogen Life Technologies, UK), 1% penicillin/streptomycin (Invitrogen Life Technologies, UK), 1.176 g/L of sodium bicarbonate, 4.76 g/L of HEPES, 1 ml/L of hydrocortisone >98% (Sigma-Aldrich, Portugal), and maintained at 37° in a humidified 5% CO_2 atmosphere. Treatments were performed for 24 h in serum-free culture medium with two different concentrations of glucose (Glc): 5 mM and 20 mM, and added to cell cultures at final concentrations of 1 μM and 0.1 μM for both pharmaceutical agents. Controls were performed using identical volume of excipients.

MTS toxicity assay

HMECs were seeded (1×10^5 cells/ml) in 96-well plate, allowed to grow until 70%-90% confluence and then incubated with different concentrations of liraglutide and exanetide in 5 mM or 20 mM glucose free-serum medium for 24 h. After the incubation period, cells viability was assessed using Cell Titer 96 Aqueous ONE Solution Reagent MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxiphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] colorimetric assay (Promega, USA), according to the instructions provided by the manufacturer. Optical density was measured at 492 nm.

BrdU proliferation assay

HMECs (1×10^5 cells/ml) were cultured following standard conditions or the treatment procedures for 24 h. Cells were also incubated with 5’-bromodeoxyuridine (BrdU) solution at a final concentration 0.01 mM for 2 h. Detection was then performed at 450 nm and 690 nm using BrdU In-Situ Detection Kit (BD Biosciences Pharmingen, USA), according to the manufacturer’s instructions.

Wound healing assay

HMECs were incubated at 37°C and were allowed to grow until 90% of confluence in a 24-well plate. Then, it was made a scratch with a 200 µl pipet tip and the cells were incubated for 24 h

following the standard treatments. After incubation, cells were washed with PBS and cell migration to the damaged area was visualized and photographed under an inverted microscope at 4x objective (Nikon, UK).

Migration capacity of the HMEC cells was then evaluated by quantifying the distance between the two limits of the injured area with Image J and comparing those with controls cells migration that was considered 100%.

TUNEL assay

HMECs (6×10^4 cells/ml) were grown on glass coverslips and incubated with the different treatments and media for 24 h. TUNEL assay (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling) was performed using the In Situ Cell Death Detection Kit (Roche Diagnostics, Switzerland), according to the manufacturer's instruction. The percentage of TUNEL-stained nuclei was evaluated in relation to every DAPI-stained nuclei observed, at a 10x objective.

Western blotting assay

Proteins were isolated from HMEC lysates using RIPA (Chemicon International, USA). Protein concentrations were determined using BCA kit (Thermo Fisher Scientific, USA), and 30 µg of protein were subjected to 8% SDS-PAGE with a 5% stacking gel. After electrophoresis, proteins were blotted into a Hybond nitrocellulose membrane (Amersham, USA). Immunodetection for GLP-R1 (1:200; Santa Cruz Biotechnology, USA) and β-actin (1:3000; Abcam, UK) was accomplished with enhanced chemiluminescence (ECL kit, Bio-Rad, USA). The relative intensity of each protein blotting analysis was measured using a computerized software program (Bio-Rad, USA), and the expression of GLP-1R was normalized with β-actin bands, to compare the expression of proteins in different treatment groups.

Statistical analysis

All experiments were performed in duplicate or triplicate. The results are expressed as standard deviation (SD) ± S.E.M. Differences between the groups were evaluated for statistical significance using the one-way ANOVA method in GraphPad Prism program, and was considered significant whenever the P value was <0.05.

Results

GLP-1R agonists did not affect cell viability either in euglycemic or in hyperglycemic concentrations

In order to examine whether liraglutide or exenatide have cytotoxic effects, cell viability was evaluated in 70-90% confluence HMECs cultures after incubation with the GLP-1R agonists by MTS assay. As illustrated in Figure 1, incubation with 0.01-1 µM of each agent did not significantly affect the number of viable cells relative to excipient-treated cell cultures (controls) in any of the glucose concentrations tested, indicating that no cytotoxicity was found in HMECs after incubation with the five different concentrations of GLP-1R agonists examined. Nevertheless, cell viability was slightly reduced upon incubation with increasing concentrations of liraglutide, particularly in hyperglycemic concentrations. Given these preliminary findings, the next experiments were performed using 0.1 µM, the less cytotoxic concentration, and whenever possible 1 µM of each agent, in the two different glycemic concentrations.

Cellular proliferation was significantly augmented by both GLP-1R agonists

The effect of liraglutide and exenatide in endothelial cells proliferation was then assessed using BrdU incorporation assay. Treatment with both GLP-1R agonists led to a significant increase in cell growth in normal (Glc 5.5 mM) and in hyperglycemic (Glc 20 mM) concentrations, being the results more prominent at 0.1 µM concentration of both agents.

Nevertheless, significant differences were only seen between controls in both glycemic environments with the 0.1 µM concentration of exenatide in the respective glucose doses, and also with 1 µM concentration of this compound in normoglycemic conditions, as observed in Figure 2.

Apoptosis and migration was not significantly induced by incretin receptor agonists

For the study of apoptosis in HMECs treated with GLP-1R agonists, TUNEL assay was performed. Interestingly, in normoglycemic concentrations (Glc 5.5 mM) the number of apoptotic cells doubled the one from control for both incretin receptor agonists, although not reaching statistical significance (Figure 3A). In contrast, in hyperglycemia, apoptosis was similar for both agents studied. However, significant differences were only detected between the glucose concentrations (5.5 mM vs 20 mM) in liraglutide 0.1 µM (Figure 3A).

Angiogenesis also requires endothelial cells to migrate. Therefore, we next examined whether GLP-1 receptor agonists influenced the capacity of HMECs to migrate using a wound healing assay. Although a tendency towards an increase in migration in hyperglycemic cultures was noted, those differences did not reach statistical significance (Figure 4A). No difference was observed among the distinct concentrations of each compound.

Incretin receptor agonists affected GLP1-R expression in HMEC by Western blotting

To further investigate whether GLP-1R agonists played a role in GLP-1 signaling, the expression of GLP-1R was examined on HMEC lysates treated with 0.1 µM and 1 µM of either liraglutide or exenatide, in both glycemic concentrations. As it can be seen in Figure 5, no statistical differences were obtained regarding the expression of GLP-1R when subjected to different agents and glycemic conditions.

Discussion

Liraglutide and exenatide are two GLP-1R agonists used in medical practice for the treatment of T2DM. They are especially interesting because they have a glucose dependent action and many other additional beneficial functions for the patient. Knowing that endothelial cells express the GLP-1R as it was demonstrated by Nystrom et al (2004), the present paper aimed to study the effects of these agents in the endothelial cell behavior in two different environments, high and low glucose concentration.

Herein, incubation of liraglutide and exenatide in confluent HMEC cultures did not result in significant decreased cell viability when compared with controls. These preliminary findings point to the lack of cytotoxicity of these agents within the concentrations used, and prompted to further investigate the effect of these two agents in endothelial proliferation, death and migration.

Interestingly, HMEC proliferation was increased by 0.1 μM and 1 μM liraglutide and exenatide, particularly in normoglycemic concentrations. Nevertheless, only exenatide at 0.1 μM showed a statistical significant increase in endothelial cells proliferation in both glycemic environments. Erdogdu et al (2010) and Xie et al (2011), both claimed that GLP-1 enhances endothelial cell function, namely by promoting vascular endothelial cell proliferation. Using an *in vitro* model, our findings further indicate that GLP-1R agonists likely promote cell growth, and consequently survival, in a glucose independent manner.

Inversely, cell migration and apoptosis were not affected by the incubation with the two GLP-1R agonists. The percentage of apoptotic cells was not changed by treatment with any of the GLP-1R agonists. However, a tendency to an increased number of apoptotic cells observed in normoglycemic environment was attenuated in high glucose conditions. A vascular protective role of GLP-1 has already been reported by Zhan et al (2012). These findings regarding

apoptosis, together with the lack of migration enhancement, are consistent with the ones reported for the GLP-1 endogenous hormone.

We also examined whether the presence of GLP-1R agonists affected GLP-1R expression. Unexpectedly, no difference was observed when HMEC were incubated with the agents in comparison to controls. These findings led to the assumption that GLP-1 signaling could occur in a GLP-1R independent manner. However, the lack of GLP-1R overexpression is insufficient to deny any role of this receptor in GLP-1R agonists signaling, given that GLP-1R activity can be enhanced without changing the receptor expression. For disclosing this hypothesis further studies of the underlying downstream pathways must be carried out.

Altogether, our findings showed that the GLP-1R agonists studied exhibit different effects in low and high glucose concentrations, implying a protective role against endothelial dysfunction.

Nonetheless, further studies are needed to clarify these effects, namely using *in vivo* models of diabetes, where endothelial cells are stabilized by the vessel structure comprising a basement membrane, pericytes or smooth muscle and fibroblasts, which exert paracrine effects on endothelium. Moreover, a crosstalk between cells and the extracellular matrix components, play a further role in the vascular environment, which might as well influence the effect of GLP-1R agonists in vessel structures.

In conclusion, our findings indicate that GLP-1 receptor agonists represent a promising field for the treatment of T2DM not only due to the numerous health benefits already established, such as the glucose-dependent action in glycemic levels, but also because of this endothelial protective role. Furthermore, GLP-1R agonists may ameliorate the complications arising from diabetes, which have a major impact on morbidity and mortality in diabetic patients.

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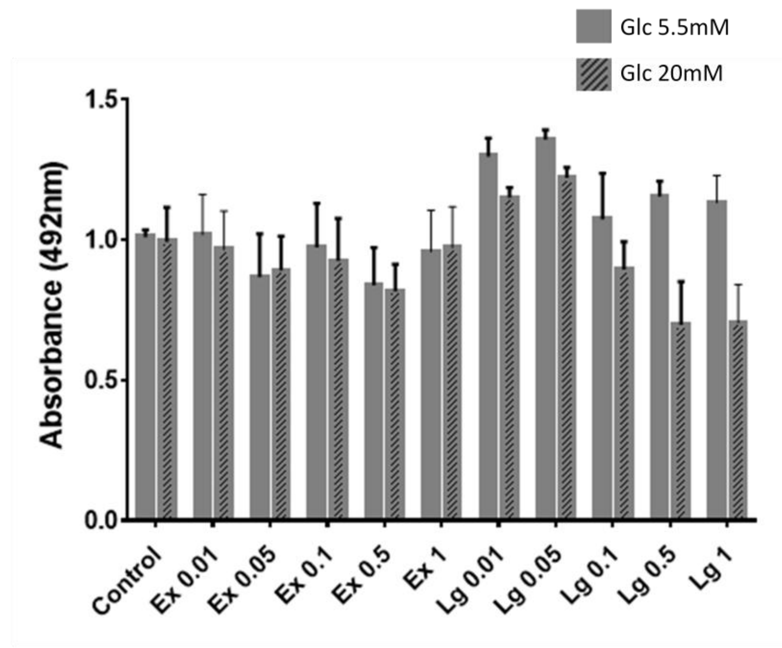


Figure 1: Cytotoxicity evaluation in confluent HMEC cultures using MTS assay. No significant cytotoxicity was found either for liraglutide or exenatide at any of the concentrations tested and at any of the glycemic concentrations of the culture media. Results are expressed in absorbance values at 492 nm.
Glc - glucose; Ex - Exenatide; Lg - Liraglutide.

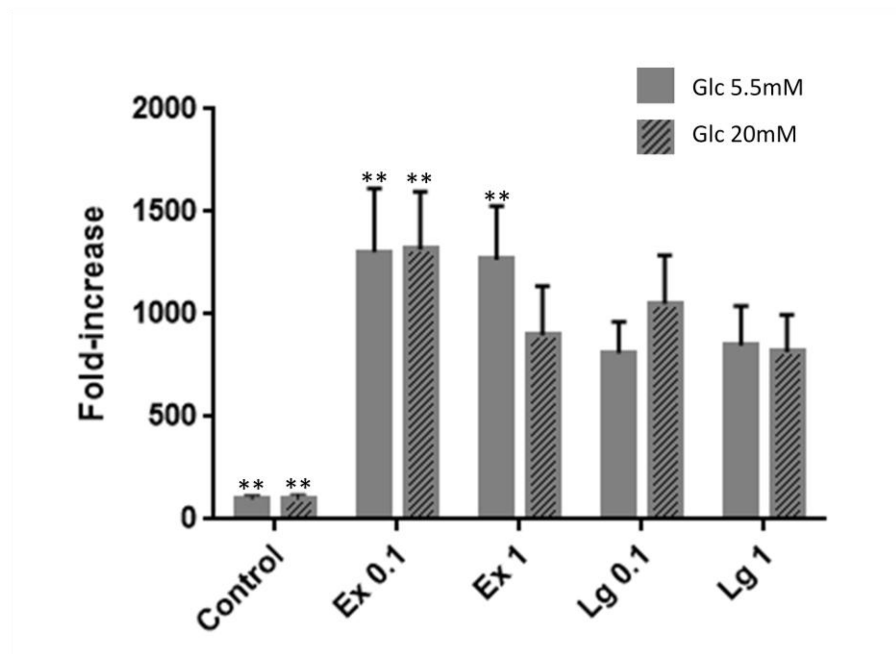


Figure 2: Cell proliferation was assessed by immunocytochemistry analyses of BrdU incorporated HMECs. Cell proliferation was only increased significantly in the two concentrations of exenatide when compared with controls. Results are expressed as percentage of the controls. ** $p < 0.05$ vs Controls.
Glc - glucose; Ex - Exenatide; Lg - Liraglutide.

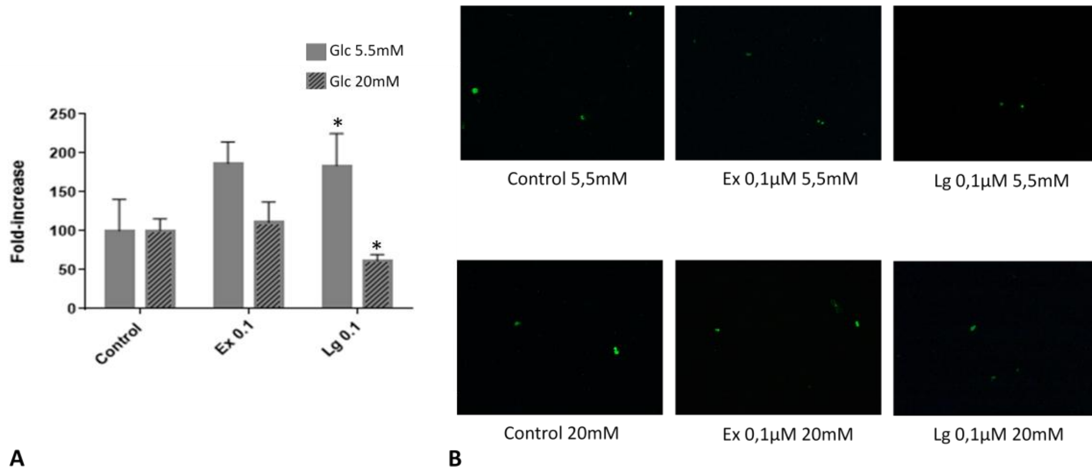


Figure 3: (A) Apoptosis was evaluated in HMECs by TUNEL assay after incubation with 0,1 μM of liraglutide or exenatide in two different glucose concentrations serum-free medium (5.5 mM vs 20 mM). The different glucose environments in liraglutide 0.1 μM were the only who affected significantly apoptosis rate, with hyperglycemia reducing 4-fold apoptosis to normoglycemia. Results are expressed as percentage of the controls.** $p < 0.05$ vs Controls. **(B)** Images of the TUNEL assay are shown. Amplification 100x.
Glc - glucose; Ex - Exenatide; Lg - Liraglutide.

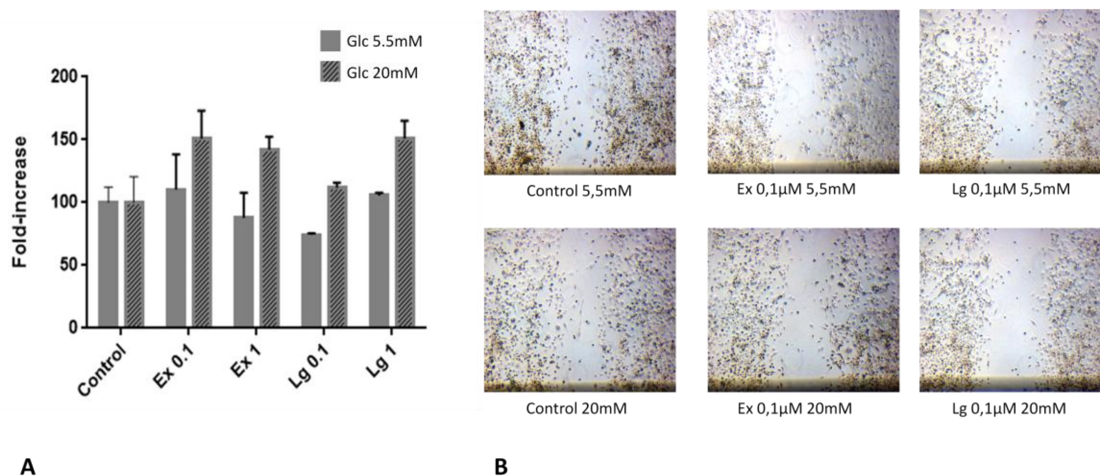
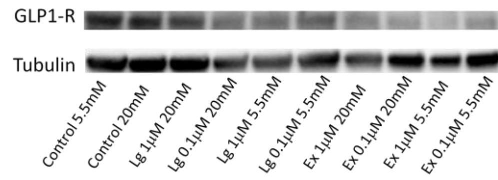
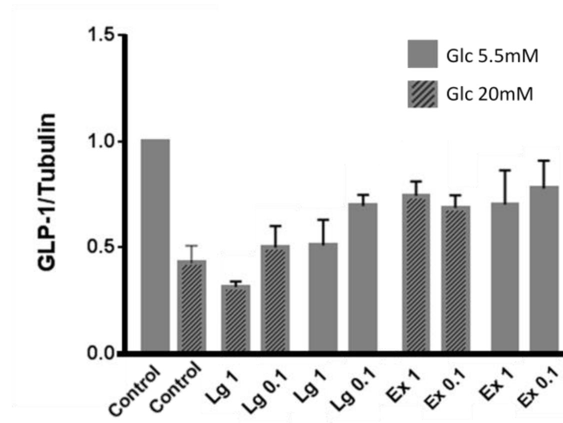


Figure 4: (A) Cell migration was examined in HMECs by wound healing assay upon incubation with 0,1 μM of liraglutide or exenatide in two different glucose concentrations serum-free medium (5.5 mM vs 20 mM). **(B)** The images show the quantitative effect of compounds in the two glucose concentrations tested. Results are expressed as percentage of the controls. Amplification 100x.
Glc - glucose; Ex - Exenatide; Lg - Liraglutide.





A **B**

Figure 5: Expression of GLP-1R in HMEC was determined by Western blotting assay. **(A)** Representative bands obtained after immunostaining are shown. **(B)** Quantification of band intensity was obtained by densitometry analysis. Data presented as mean ± SEM of at least three independent experiments.
Glc - glucose; Ex - Exenatide; Lg - Liraglutide.

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Anexos

Normas da revista

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Figures: No more than 8 figures may be presented, approximately equivalent to 3 pages-worth total. Figures should be sized to one column width (20 picas), or two column width (40 picas), as appropriate. Image height may not exceed 40 picas. Multi-panel images should be composed as a single image with width and height limitations applying to the final, combined image. Image resolution must be at least 300 dpi for raster images (e.g., photographs, gels, stains) and 600 dpi for line-art images (e.g., charts and graphs). Image-related text and labeling must be clearly legible, a font size of 10 points or greater should be present in the final image

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No more than 8 figures may be presented, approximately equivalent to 3 pages-worth total. Figures should be sized to one column width (20 picas), or two column width (40 picas), as appropriate. Image height may not exceed 40 picas. Multi-panel images should be composed as a single image with width and height limitations applying to the final, combined image. Image resolution must be at least 300 dpi for raster images (e.g., photographs, gels, stains) and 600 dpi for line-art images (e.g., charts and graphs). Image-related text and labeling must be clearly legible, a font size of 10 points or greater should be present in the final image.

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How to handle authorship disputes: a guide for new researchers By Tim Albert and Liz Wager

This document aims to help new researchers prevent and resolve authorship problems. In particular it provides:

- suggestions for good authorship practice that should reduce the incidence of such dilemmas,
- advice on what to do when authorship problems do arise, and
- a glossary of key concepts in authorship, with some reading lists and websites for those who wish to take this further.

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The objective is to provide access to novel experimental strategies. "Benchmark" articles should be developed to enhance awareness of capabilities to resolve complexities of cell structure and function. Submission of articles that focus on development of technologies and instrumentation to enable exploration of regulatory mechanisms previously unapproachable are encouraged. Coverage of topics can include (but not limited) to:

-Cell-based and animal models for in vitro and in vivo validation of molecular components to regulatory networks that mediate cell cycle control, proliferation, phenotype, cell fate and responsiveness to physiological cues.

-Breakthroughs in imaging and experimental approaches to discriminate subtleties in common or total control contributing to resolve and complexities in biological processes that include development, lineage commitment, tissue remodeling, apoptosis, pluripotency transformation and tumorigenesis.

-Capabilities to acquire, configure and integrate data from a rapidly evolving high throughput genomic and proteomic screens.

-Bioinformatic strategies that support dimensions for biological control that link to the prevention, early detection and treatment of human disease.

Abstract: In 150 words describe capability of the method to provide novel insights into biological/biomedical control. Indicate applications for resolving fundamental regulatory mechanisms and/or clinically relevant questions. Present validations for the approach and summarize strengths as well as potential limitations.

Presentation: Begin each “Benchmark” with an introduction that provides the background for the method. Focus on the unique capabilities of the approach to address compelling biological/biomedical questions. Highlight opportunities to increase resolution and/or scope of structural as well as regulatory parameters that can be evaluated.

The method should then be presented with schematic illustrations and described with emphasis on instrumentation, resolution, experimental applications and complexities with alternative strategies. Validation should be presented and examples of data that can be anticipated are encouraged. Considerations of options to accommodate specialized requirements is informative.

“Benchmarks” should not exceed 2500–3500 words. To maximize the utility of “Benchmarks” it is important for descriptions of technology to be accessible to a broad audience.

Experimental protocols and specifications for instrumentation and reagents are valuable; but, in general, will be presented as supplemental material that is available online. “Benchmarks” articles will be invited by the Journal of Cellular Biochemistry editors to cover emerging methods and strategies. However, authors are encouraged to submit unsolicited manuscripts or letters of inquiry to the editorial office.

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· Constructing figures from different gels, fields, exposures, and experimental series is discouraged. When this is necessary the component parts of composite images should be indicated by dividing lines clearly demarcated in the figure, and described in the legend.

These recommendations are based on guidance developed at the Journal of Cell Biology and Rossner and Yamada's discussion. Cromey discusses image manipulation in " Avoiding twisted pixels: ethical guidelines for the appropriate use and manipulation of scientific digital images ."

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