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Effects of growth hormone-releasing hormone agonistic analog MR-409 on insulin-secreting cells under cyclopiazonic acid-induced endoplasmic reticulum stress

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ABSTRACT

The endoplasmic reticulum (ER) stress is one of the mechanisms related to decreased insulin secretion and beta cell death, contributing to the progress of type 2 diabetes mellitus (T2D). Thus, investigating agents that can influence this process would help prevent the development of T2D. Recently, the growth-hormone-releasing hormone (GHRH) action has been demonstrated in INS-1E cells, in which it increases cell proliferation and insulin secretion. As the effects of GHRH and its agonists have not been fully elucidated in the beta cell, we proposed to investigate them by evaluating the role of the GHRH agonist, MR-409, in cells under ER stress. Our results show that the agonist was unable to ameliorate or prevent ER stress. However, cells exposed to the agonist showed less oxidative stress and greater survival even under ER stress. The mechanisms by which GHRH agonist, MR-409, leads to these outcomes require further investigation.

1. Introduction

Insulin is a polypeptide hormone responsible for maintaining blood glucose in the postprandial state. This hormone is secreted by pancreatic beta cells which, when stimulated by different factors, increase insulin transcription and translation. The newly synthesized peptides are redirected to the endoplasmic reticulum (ER) to be properly folded and prepared for secretion. Due to this function, ER is an organelle that requires very fine control of its homeostasis, because an imbalance in its lumen leads, in long-term, to a decrease in insulin secretion and cell death (Schuit et al., 1988; Liuet al., 2018). When the homeostasis of the ER is disturbed leading to the accumulation of misfolded proteins, the Unfolded Protein Response (UPR) is activated in order to develop adaptations to promote the correct folding of proteins. This response is mediated by three canonical transducers present in the ER membrane: inositol-requiring 1 α (IRE1α), PKR-like ER kinase (PERK) and activating transcription factor 6 (ATF6). However, in chronic conditions, this response triggers cell death through ER stress.

One of the pathways responsible for inducing apoptosis is the PERK/ATF4/CHOP signaling pathway. When PERK is activated, it phosphorylates its target protein eukaryotic initiation factor 2 alpha (eIF2α), an important component of translation machinery. This phosphorylation reduces the overall translation rate (Marciniak and Ron, 2006). Paradoxically, this global decrease in protein synthesis favors the translation of activating transcription factor 4 (ATF4) (Somers et al., 2013). ATF4 induces the transcription of antioxidant response genes, preventing the

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excessive formation of reactive oxygen species (ROS) and the increase in the influx of Ca^{2+} into the mitochondria (Görlach et al., 2015). However, ATF4 also induces the transcription of DNA damage-inducible transcript 3 protein (also known as CHOP) which regulates the expression of many genes, including the expression of pro-apoptotic genes (Hardinget al., 2000; Kaufman, 1999).

In this way, prolonged ER stress impairs insulin synthesis and causes pancreatic beta cell apoptosis, and is one of the mechanisms related to the progressive decline of islet function, contributing to the development of T2D (Eizirik et al., 2008). Therefore, the search for molecules that can promote beta cell survival is of great interest.

Only recently, GHRH agonists, of the MR series, have been explored in this context. Evidence demonstrates that in INS-1E beta cells, GHRH agonists are able to increase the activity of pathways linked to proliferation and survival, along with increased proliferation in pancreatic islets from rats (Zhanget al., 2015). However, the possible effect of GHRH agonists of MR class on ER stress in beta cells have not been yet determined. Thus, we used a beta cell line (INS-1E) exposed to the reversible ER stress inducer cyclopiazonic acid (CPA) and treated with the MR-409 agonist, aiming to explore this question.

Here, we observed that the agonist was able to promote the survival of cells under ER stress. This survival may be due to the modulation of proteins of the Bcl-2 family, reducing oxidative stress in CPA-treated beta cells. This research provides new insights into the role of GHRH agonist in cells under ER stress and adds new ideas to its antioxidant action.

2. Material and methods

2.1. Culture of INS-1E cells

Rat insulinoma INS-1E cells were kindly provided by Prof. Dr. Decio Eizirik (ULB, Brussels, Belgium), and cultured at 37 °C in a humidified atmosphere containing 5% CO_2 in RPMI-1640 medium with 11 mM glucose and supplemented with 100U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 10 mM HEPES, 50 μM 2-mercaptoethanol and 5% fetal bovine serum, or 0,1% fetal bovine serum for the treatments.

2.2. Experimental groups and treatments

For the CPA concentration curve, INS-1E cells were treated with cyclopiazonic acid (CPA) (Sigma C1530-5 mg) in concentrations of 6.25 μM; 12.5 μM and 25 μM for 12 h. The treatment groups were: control, which did not receive treatment, only means of maintenance; dimethyl sulfoxide (DMSO) and CPA.

GHRH agonist MR-409 was dissolved in DMSO to obtain a concentration of 10 mM. Before use, stock solution was diluted to 2 μM in the incubation medium. The final concentration of DMSO never exceeded 0,1% in medium.

For the other experiments, cells were treated with different concentrations of CPA, 6.25 μM, 12.5 μM or 25 μM, for 12 h, with or without addition of 0.5 μM, 1 μM or 2 μM of the agonist MR-409. The treatments were carried out in triplicates and the cells were incubated with two serum concentrations: medium containing 5% serum, volume used for the INS-1E cell maintenance medium; and medium containing 0.1% serum, volume recommended for use with the MR-409 agonist.

For the evaluation of CREB phosphorylation, INS-1E cells were treated with 2 μM of MR-409, with 5 mM Caffeine (positive control) or kept without treatment, for 0, 15 and 30 min.

2.3. Assessment of beta cell apoptosis

Apoptotic cells were evaluated using DNA binding dyes Hoechst 33342 (Thermo Fisher Scientific) and propidium iodide (PI) (Sigma-Aldrich). Cells were treated with the MR-409 agonist and CPA. After treatments, 100 μl of medium was removed from the wells. Then, 100 μl of solution containing 1 mg/ml of Hoechst and 1 mg/ml of PI was added to each well and incubated for 15 min. After incubation, in order to dilute the solution, 100 μl of the solution was removed from the well and another 100 μl of medium was added. The reading and analysis of the plate for the percentage of apoptotic cells was performed by High-Content Imaging System (ImageXpress Micro Confocal, Molecular Devices), using the Live and Dead module of the MetaXpress software (Molecular Devices). To identify the populations of living and dead cells, masks were applied for the DAPI wavelengths (excitation at 350 nm and emission at 470 nm - Hoescht) and Texas Red (excitation at 496 nm and emission at 615 nm - Propidium Iodide).

2.4. Western blotting analysis

Samples were lysed using Laemmli buffer, fractioned in SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). After blocking (5% bovine albumin) the proteins of interest were detected with specific antibody (Anti-GRP78 (Bip), Abcam, ab21685; Anti-DDIT3 (CHOP), Abcam, ab11419; Anti-eIF2S1 (phospho S51), Abcam, ab32157; Anti-Bcl-xL, Cell Signaling, 54H6; Anti-Bax, Cell Signaling, 27725; Anti-GAPDH, (Sigma, G9545); SOD2 (13533, Abcam); anti-Creb (Cell Signaling 48H2) and anti-p-Creb (ser 133) (Cell Signaling 87G3), followed by incubation with the appropriate secondary antibody (1:10,000; Invitrogen, Scotts Valley, CA) containing peroxidase (HRP). Chemiluminescence (SuperSignal West Femto Maximum Sensitivity Substrate; Thermo Fisher Scientific, Carlsbad, CA) was detected by a digital imaging system (Amersham Imager 600; GE Healthcare). Optical density was quantified using ImageJ software (https://imagej.nih.gov/ij/download.html).

2.5. Real-time PCR

The samples were collected with 500 μl of Trizol (Invitrogen; Life Technologies, Carlsbad, CA) and total RNA extracted according to the manufacturer's instructions. Complementary DNA strand (cDNA) was synthesized from 1 μg RNA in 20 μl, using the High Capacity kit (Applied Biosystems), according to the manufacturer. Real-time polymerase chain reaction (PCR) was performed using Fast SYBR® Green PCR Master Mix, in a 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA). The relative expression of mRNAs was determined after normalization with HPRT, using the 2-ΔΔCt method. Primer sequences used for real-time PCR assays were as follows: *XBP1* primer sequence 5′-3 Forward: GCAGCAAGTGGTGGATTTG; Reverse: GAGTTTTCTCCCGTAAAAGCTGA. *CHOP* primer sequence 5′-3 Forward: CTGGAAGCCTG GTATGAGGAT; Reverse: CAGGGTCAAGAGTAGTGAAGGT. *Bip* primer sequence 5′-3 Forward: ACTTGGGGACCACCTATTCCT; Reverse: ATCGCCAATCAGACGCTCC. *ATF4* primer sequence 5′-3 Forward: GTTGGTCAGTGCCTCAGACA; Reverse: CATTCGAAACAGAGCATCGA. *SERCA2b* primer sequence 5′-3 Forward: TTTGTGGCCCGAAACTACCT; Reverse: GGCATAATGAGCAGCACAAAGGG. *HPRT* primer sequence 5′-3 Forward: GGTTAAGCAGTACAGCCCCA; Reverse: TCCAACACTTCGA GAGGTCC.

2.6. Mitosox

To measure mitochondrial superoxide production, INS-1E cells were cultured in a 96-well plate until they reached confluence, treated with CPA and/or MR-409, as already described, for 12 h. After treatment, the cells were incubated for 10 min at 37 °C with 5 μM of the Mitosox Red probe (Molecular Probes) diluted in Krebs buffer. After this time, the cells were incubated for 10 min with 1 μg/ml Hoechst 33342 for normalization and then washed three times with phosphate buffered saline (PBS). The fluorescence intensity of Mitosox Red (emission 510 nm/excitation 580 nm) and Hoechst 33342 (emission 360 nm/excitation 500 nm) was measured in a spectrophotometer.

2.7. Insulin secretion

INS-1E cells were cultured with RPMI medium in 96-well plates until reaching 70–80% confluence, and treated with 12.5 μM CPA; 12.5 μM CPA +2 μM MR-409 or vehicle for 12 h. Soon after, the culture medium was removed and replaced with 100 μL of the Krebs pre-incubation solution (115 mM NaCl, 5 mM KCl, 2.56 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM $NaHCO_3$, 15 mM HEPES and 0.3% of BSA without glucose and equilibrated with a mixture of 5% CO_2, pH 7.4), at 37 °C for 1 h. Subsequently, the solution was gently removed and 100 μL of Krebs containing 2.8 mM or 22.2 mM glucose was added over 1 h. At the end of the incubation, the supernatant was collected and stored in a freezer at −20 °C for insulin detection by ELISA kit (Crystal Chem, USA, Catalog # 90080), according to the manufacturer's guidelines.

2.8. Mitochondria Ca^{2+} measurements

To assess mitochondrial Ca^{2+}, INS1-E cells were loaded with Rhod-2 AM (13 μM, Catalog number: R1244) at 37 °C for 30 min, followed by washout and 1 h rest at room temperature for de-esterification. Afterward, the cells were washed and incubated with Krebs without glucose for 1 h at room temperature and the fluorescence reading was performed, stimulated by glucose (2.8 mM and 22.2 mM of glucose) in the Spectramax M3 plate reader, at wavelengths excitation at 542 nm and emission at 581 nm. To normalize the results, the cells were washed with PBS, fixed with 4% formaldehyde, and incubated with Hoechst (1 μg/ml) for 15 min and then read in this same equipment, at 350 nm (excitation) and 461 nm (emission) wavelengths.

2.9. Statistics

The data were submitted to the Shapiro-Wilk normality test and, then, to the parametric and nonparametric comparison tests, the one-way ANOVA test and the Student *t*-test, followed by the Bonferroni or Kruskal-Wallis post-tests. Analyses were performed using the GraphPadPrism 7.04 program. The difference between groups was considered statistically significant if $P < 0.05$.

3. Results

First, we characterized the ER stress by quantifying the protein levels of UPR proteins, including Bip, phosphorylated eIF2α (p-eIF2α) and CHOP, in cells exposed for 12 h to 6.25 μM, 12.5 μM or 25 μM cyclopiazonic acid (CPA). After CPA incubation, there was no difference in Bip protein levels (Fig. 1A). However, p-eIF2α and CHOP protein were increased in the INS-1E cells from 12.5 μM CPA (Fig. 1B and C). Thus, the concentration of 12.5 μM CPA was determined for the following experiments. To investigate the influence of fetal bovine serum (FBS) on cell death induced by CPA, we performed a live and dead experiment to assess the percentage of death of cells exposed to 12.5 μM CPA in medium containing 5% or 0,1% FBS (DMSO < 0–1%). As expected, exposure to CPA increased the death rate in both 5% or 0,1% FBS (Fig. 1D and E). In this way, no differences were observed in the percentage of death induced by CPA in different concentrations of FBS. Due to the recommendation of medium containing 0.1% FBS for the tests with the agonist MR-409 (see Supplemental Material), we adopted this concentration of FBS for our experiments. Then, we evaluated the cell death in response to CPA and different concentrations of MR-409 0.5, 1 and 2 μM (Fig. 1F–H). The results showed lower cell death in the group exposed to 2 μM MR-409, this being the concentration of agonist used in the following experiments.

To evaluate the ability of MR-409 to modulate UPR proteins, we examined the expression of Bip, p-eIF2α and CHOP proteins in cells exposed to CPA and MR-409. Exposure to MR-409 did not prevent the increase in these proteins, induced by CPA, when compared to the group exposed only to CPA (Fig. 2A–C). RT-PCR analysis showed a significant increase in the mRNA of Bip, XBP1, ATF4, CHOP and SERCA2b proteins (Fig. 2D–H) in the group exposed to CPA, and this increase remains in the group exposed CPA and MR-409, indicating that the agonist is not able to modulate UPR proteins.

We exposed cells to 2 μM MR-409 and 5 mM caffeine (Pratt et al., 2019; Connolly and Kingsbury, 2010), to determinate the phosphorylation of cAMP response element binding protein (CREB), in order to investigate the agonist's signal transduction pathway (Mayo et al., 1995). Both MR-409 and caffeine, used as a positive control, exhibited for 15 and 30 min an increase in phosphorylation of CREB (Fig. 3E). This protein has already been described as having a role in cell survival (Somers et al., 1999), being a positive regulator of the Bcl-2 gene promoter (Xiang et al., 2006). Therefore, we next evaluated proteins of the Bcl-2 family. Our results showed an increase in the Bax/Bcl-XL ratio (Fig. 3D) in the group exposed to CPA, and this increase was prevented in the group exposed to CPA and MR-409, indicating that the agonist

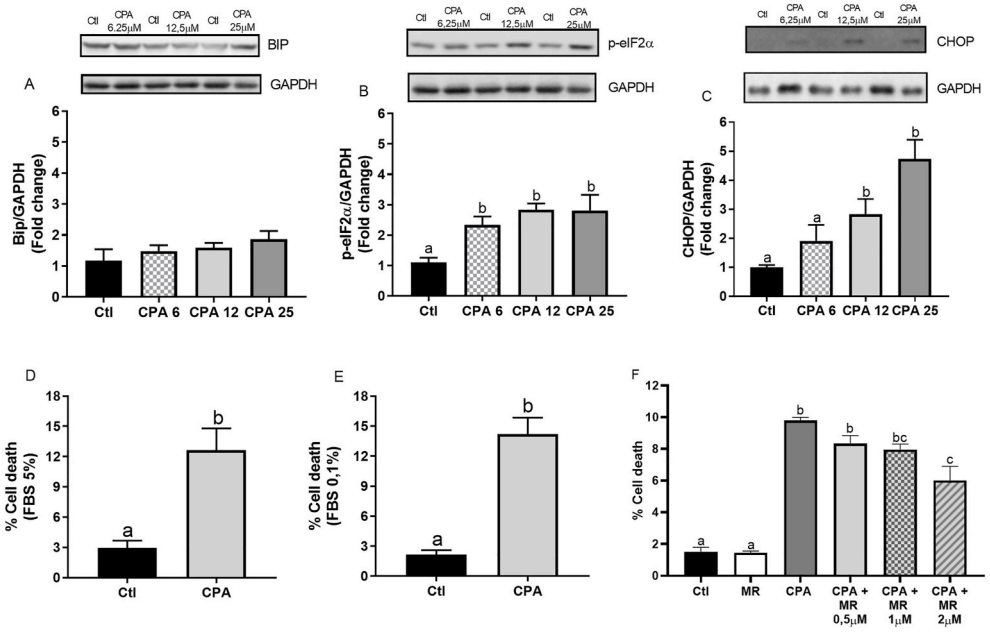

Fig. 1. Induction of ER stress by CPA and treatment with MR-409 agonist in INS-1E cells. Western blotting of UPR proteins, such as, Bip (A), p-eIF2α (B) and CHOP (C) in INS-1E cells treated with 6.25 μM, 12.5 μM or 25 μM CPA for 12 h (n = 3). Percentage of cells marked for cell death treated with medium containing 5% (D) and 0.1% (E) of FBS and 12.5 μM CPA for 12 h. Percentage of cells marked for cell death treated with medium containing 0.1% FBS, containing or not 12.5 μM CPA (n = 4), in addition to, 0.5 μM, 1 μM and 2 μM of the MR-409 agonist (F) (n = 3). The results express the mean ± SEM and were submitted to the normality test (Shapiro-Wilk) and then to ANOVA-one-way or unpaired *t*-test. Different letters (a, b or c) above the columns indicate significant difference between the groups at $p < 0.05$.

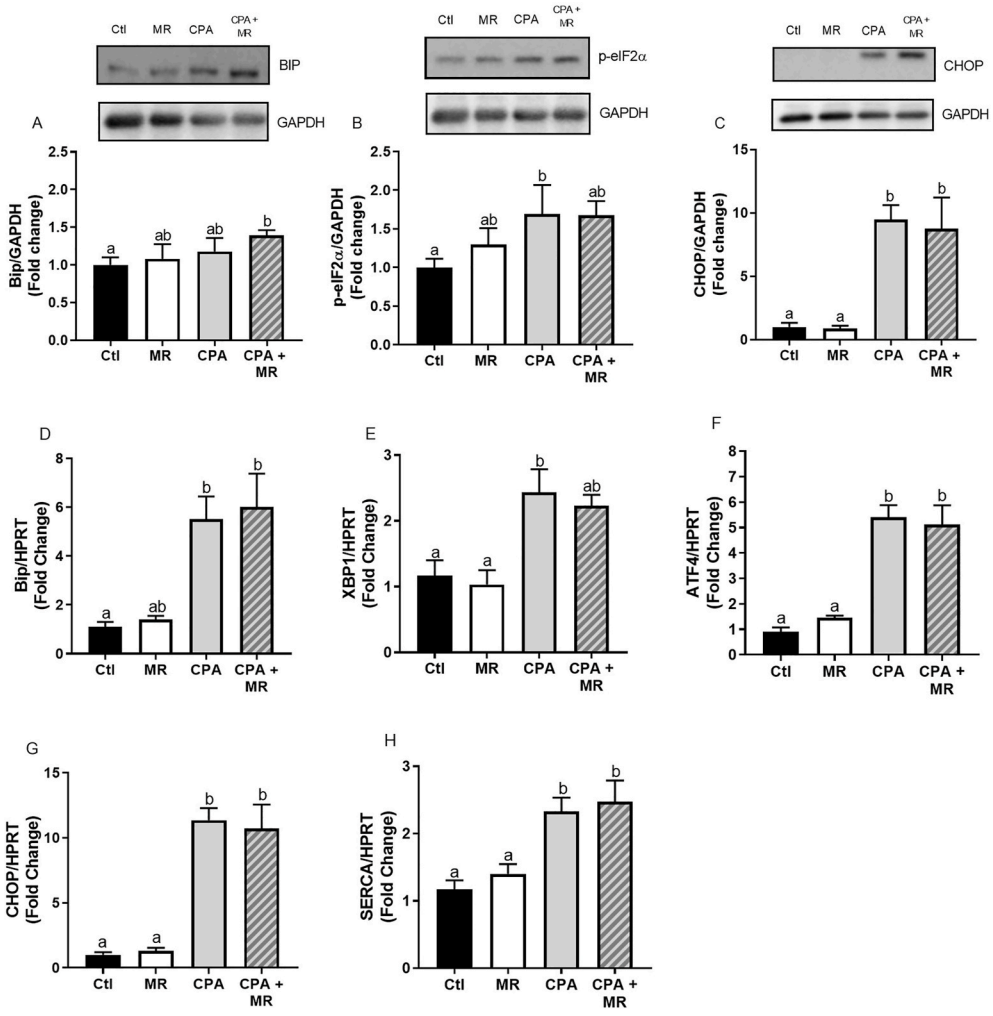

Fig. 2. Effect of agonist MR-409 on UPR proteins expression in INS-1E cells with CPA-induced ER stress. Western blotting of UPR proteins, such as, Bip (A), p-eIF2α (B) and CHOP (C) in INS-1E cells treated with 2 μM MR-409, 12.5 μM CPA, or 12.5 μM CPA and 2 μM MR-409, for 12 h (n = 3–4). Real-time PCR assay of Bip (D), XBP1 (E), ATF4 (F), CHOP (G) and SERCA (H) mRNA levels in INS-1E cells treated with 2 μM MR-409, 12.5 μM CPA, or 12.5 μM CPA and 2 μM MR-409, for 12 h (n = 3–5). The results express the mean ± SEM and were submitted to the normality test (Shapiro-Wilk) and then to ANOVA-one-way. Different letters (a, b or c) above the columns indicate significant difference between the groups at $p < 0.05$.

was able to modulate this higher ratio. Due to this result and because the Bcl-2 protein family is involved in oxidative stress (Susnow et al., 2009), we assessed the formation of mitochondrial superoxide. We observerd an increase in the production of mitochondrial reactive oxygen species (ROS) (Fig. 3B) in the group exposed to CPA and this increase was prevented in the group exposed to CPA and the agonist. We also evaluated glucose-stimulated mitochondrial calcium at two concentrations 2.8 mM and 22.2 mM (Fig. 3A). The results showed a decrease in mitochondrial calcium, at the two glucose concentrations, in the groups treated with CPA. This reduction was prevented in the group exposed to CPA and MR-409. This decrease found in mitochondrial calcium may indicate the source of mitochondrial ROS production (Cárdenaset al., 2016). Next, we investigated the modulation in the protein superoxide dismutase 2 (Sod2), which plays an important role in the defense system against mitochondrial superoxide radicals (Zelko et al., 2002). The result shows that there was no significant variation in the modulation of this protein between groups (Fig. 3C), which indicates that the antioxidant action of the agonist in INS-1E cells probably does not happen via modulation of mitochondrial antioxidant system. Despite this, MR-409 did not alter glucose-stimulated insulin secretion (Fig. 4).

4. Discussion

ER stress is a process triggered by different stimuli and in beta cells it can lead to decreased insulin secretion and apoptosis, contributing to the onset of diabetes (Eizirik and Cnop, 2010). In this way, it has been shown that the MR-409 agonist is able to increase cell proliferation of INS-1E cells, as well as insulin secretion, two important conditions in the prevention of diabetes (Ludwiget al., 2010). Here, we extend these findings, showing the effects of the MR-409 agonist on insulin-secreting cells under ER stress. In our study, cells exposed to CPA showed an increase in protein content (Bip, p-eIF2α and CHOP) and mRNA (Bip, XBP1, ATF4, CHOP and SERCA2b) of UPR proteins, indicating the installation of ER stress. However, the exposure of cells to CPA and MR-409 did not prevent the increase in both protein and gene levels, suggesting that the agonist was unable to act preventing or mitigating the installation of ER stress in such conditions.

Interestingly, cells exposed to 12.5 μM of CPA together with 2 μM of the agonist, showed a lower percentage of cell death, although there was no decrease in UPR proteins. Due to ability of ER stress to increase ROS production in the mitochondria (Cao and Kaufman, 2014), we evaluated the mitochondrial oxidative stress of cells that received the agonist. This group had less oxidative stress than the group that was exposed only to CPA. When evaluating mitochondrial calcium as a possible source of ROS production, we found, in contrast to currently held views (Brookes et al., 2004), that groups exposed to CPA showed a decrease in glucose-stimulated calcium, at low and high concentrations, while the groups exposed to CPA together with MR-409 show a significant partial recover in the calcium levels in this organelle. These results indicate the possibility that the agonist acts in other pathways that lead to cell survival and that have no direct relationship with ER stress (Cao and Kaufman, 2014).

Highlighting the antioxidant action that the agonist demonstrated in the mitochondria, and the greater survival of cells that received the

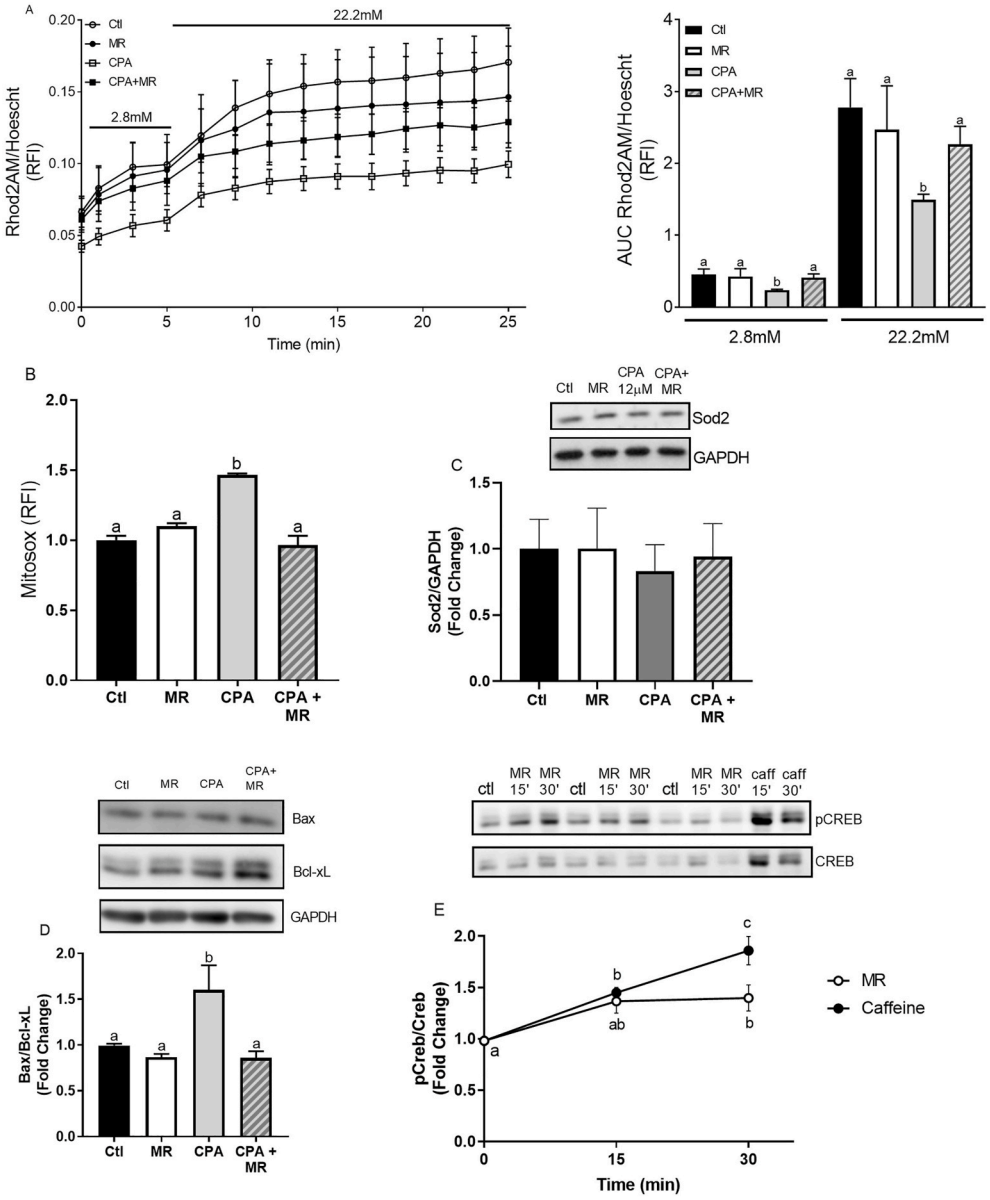

Fig. 3. Effect of agonist MR-409 on activation of Creb/Bcl pathway and mitochondrial superoxide production in INS-1E cells with CPA-induced ER stress. Mitochondrial calcium concentration curve stimulated by low and high (2.8 mM and 22.2 mM) glucose. Area under the curve of the glucose-stimulated mitochondrial calcium assay (A) (n = 5–6). Quantification of mitochondrial production of ROS (B) and Sod2 protein content (C) (n = 5) in INS-1E cells treated with 2 μM MR-409, 12.5 μM CPA, or 12.5 μM CPA and 2 μM MR-409, for 12 h (n = 3). Ratio of Bax/Bcl-XL (D) in INS-1E cells treated with 2 μM MR-409, 12.5 μM CPA, or 12.5 μM CPA and 2 μM MR-409, for 12 h (n = 4). Western blotting of Creb phosphorylation (E) in INS-1E cells treated with 2 μM of MR-409 agonist for 0, 15 and 30 min (n = 3). The results express the mean ± SEM and were submitted to the normality test (Shapiro-Wilk) and then to ANOVA-one-way. Different letters (a, b or c) above the columns indicate significant difference between the groups at $p < 0.05$.

agonist, we evaluated the ratio of Bax/Bcl-XL proteins, involved in cell survival. Our results showed an increase in the Bax/Bcl-XL ratio in cells that received the CPA, and this increase was prevented in cells that received the agonist. This result indicates that the survival of cells exposed to MR-409 may be due to the action of the agonist in the modulation of proteins in the Bcl-2 family. Other results point in the same direction. The decrease in mitochondrial calcium that was found in our experiments may be associated with cell death via pro-apoptotic proteins of the Bcl-2 family.

Calcium is necessary for the production of adenosine triphosphate (ATP), and a disturbance in its concentration leads to the activation of AMP-activated protein kinase (AMPK) (Cárdenaset al., 2016; Hinchy et al., 2018). AMPK is an enzyme that plays an important role in cellular energy homeostasis, including cell growth and apoptosis (Herzig and Shaw, 2018). A reduction in ATP production, caused by a decrease in mitochondrial calcium, and a consequent increase in adenosine diphosphate (ADP) and adenosine monophosphate (AMP), leads to the activation of AMPK, which is then able to modulate pro-apoptotic proteins of the Bcl-2 family and induce cell death in many cell types (Hinchy et al., 2018; Concannonet al., 2010; Haikalaet al., 2019; Yi et al., 2013). An increase in anti-apoptotic proteins would suppress AMPK activation via a decrease in mitochondrial ROS (Aharoni-Simonet al., 2016). The increase in CREB phosphorylation, induced by the agonist, corroborates this hypothesis, since CREB has a role in cell survival and is the positive regulator of the Bcl-2 promoter gene (Xiang et al., 2006). This pathway may explain the association between increased mitochondrial oxidative stress, caused by the lower calcium influx to this organelle, and the increase in the Bax/Bcl-XL ratio, probably due to AMPK activation. MR-409, via CREB phosphorylation, increases the transcription of anti-apoptotic proteins, reducing oxidative stress and cell death. Furthermore, there was no modulation of the mitochondrial antioxidant response, via Sod2, which also indicates that the agonist action does not take place directly in the mitochondria.

Despite increased cell survival and decreased oxidative stress, cells exposed to the agonist were unable to maintain glucose-stimulated insulin secretion at the same level as the control group. This indicates that the presence of ER stress impairs insulin secretion, probably due to the decrease in the translation of insulin mRNA and the protein folding process (Eizirik and Cnop, 2010).

In conclusion, our results indicate that the action of the agonist, in

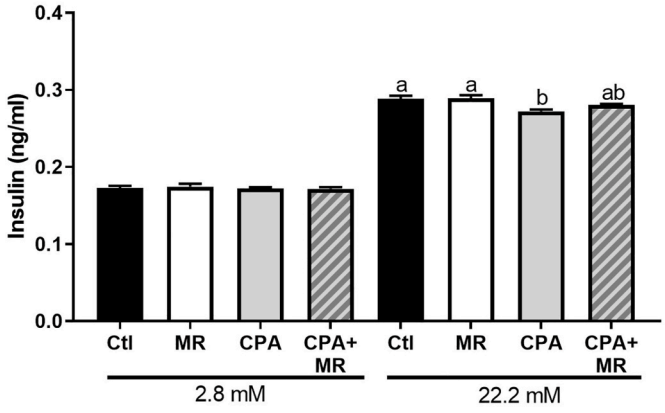

Fig. 4. Effect of agonist MR-409 on insulin secretion in INS-1E cells with CPA-induced ER stress. Glucose-stimulated insulin secretion assay of INS-1E cells treated with 2 μM MR-409, 12.5 μM CPA, or 12.5 μM CPA and 2 μM MR-409, for 12 h (n = 3–4). The cells were incubated in the presence of low (2.8 M) and high (22.2 M) glucose for 1 h and insulin was quantified in the supernatant. The results express the mean ± SEM and were submitted to the normality test (Shapiro-Wilk) and then to ANOVA-one-way. Different letters (a, b or c) above the columns indicate significant difference between the groups at $p < 0.05$.

INS-1E cells under ER stress, is due to the increase in CREB phosphorylation, which leads to the modulation of anti-apoptotic proteins. These proteins, in turn, decrease mitochondrial oxidative stress, decreasing cell death. In summary, our study demonstrates the anti-apoptotic action of the agonist MR-409 on cells under ER stress. Future studies are needed to better explain the exact antioxidative mechanism by which the agonist acts on beta cells under ER stress, promoting their survival.

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CRediT authorship contribution statement

Karina Rodrigues-dos-Santos: designed the study, conducted the experiments and acquired data, Writing – original draft, revised the manuscript. **Gabriela M. Soares:** designed the study, revised the manuscript. **Dimitrius S.P.S.F. Guimarães:** conducted the experiments and acquired data. **Thiago R. Araújo:** conducted the experiments and acquired data. **Jean F. Vettorazzi:** designed the study, conducted the experiments and acquired data. **Lucas Zangerolamo:** conducted the experiments and acquired data. **Emilio Marconato-Júnior:** conducted the experiments and acquired data. **Renzhi Cai:** provided reagents and supported the study. **Wei Sha:** provided reagents and supported the study. **Andrew V. Schally:** provided reagents and supported the study, revised the manuscript. **Antônio C. Boschero:** provided reagents and supported the study. **Helena C.L. Barbosa:** designed the study, provided reagents and supported the studyrevised the manuscript, All authors reviewed and approved the final version of the manuscript.

Declaration of competing interest

A.V.S. and R.C. are listed as co-inventors on the patents on GHRH agonists, assigned to the University of Miami and Department of Veterans Affairs. The remaining authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.mce.2021.111379.

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