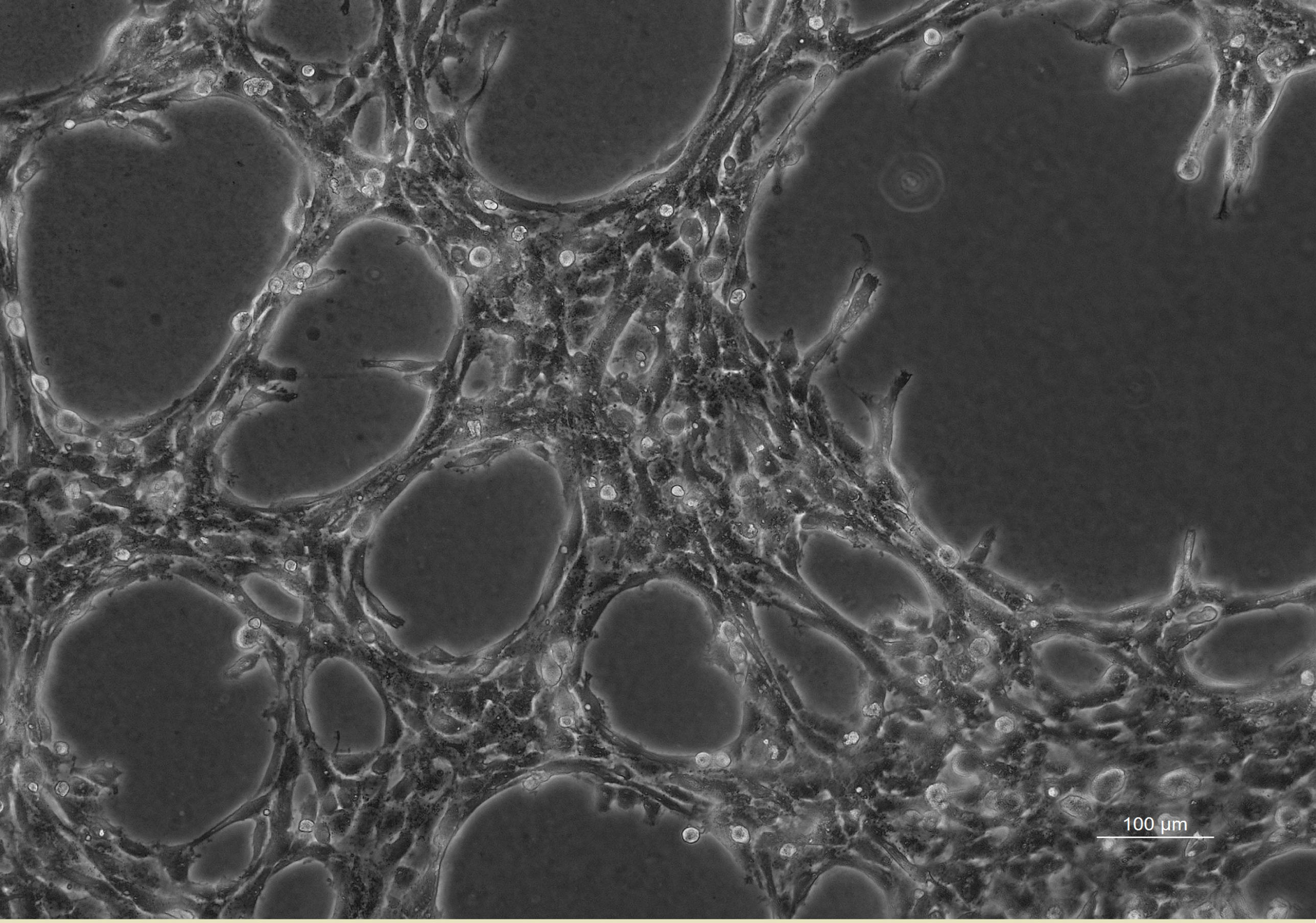

Daniela Alexandra Dinis Costa

Endothelial cell's response to proteostatic dysregulation: pursuing the protective action of ghrelin

Dissertação de Mestrado em Biologia Celular e Molecular,
orientada pela Doutora Cláudia Maria Fragão Pereira e pelo Professor Doutor Carlos Manuel Marques Palmeira,
apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

Julho 2016

· U C ·

UNIVERSIDADE DE COIMBRA

Endothelial cell's response to proteostatic dysregulation: pursuing the protective action of ghrelin

Resposta das células endoteliais à alteração das proteóstase: em busca do papel protetor da grelina

Dissertação apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra para prestação de provas de Mestrado em Biologia Celular e Molecular.

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Coimbra, Julho 2016

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Cover note

Cover contains a picture obtained during the development of this Master's thesis, specifically from *in vitro* angiogenesis assay (Rat Brain Endothelial Cells, RBE4 – electron microscopy 10x).

“The greatest pleasure in life is doing what people say you cannot do.”

Walter Bagehot

Agradecimentos

Quero começar por agradecer à Doutora Cláudia Pereira, minha orientadora científica, por me ter proporcionado as condições necessárias para desenvolver este meu trabalho aqui exposto. Foi incansável desde o início ao fim. Nada disto teria acontecido se não fosse por si. Espero ter recompensado de alguma forma toda a preocupação, apoio, dedicação e sobretudo paciência. Obrigada principalmente por ter acreditado em mim quando tudo parecia perdido! Foi um enorme prazer tê-la conhecido e trabalhado ao seu lado! Mil obrigadas e um enorme beijinho. Agradeço também ao Doutor Henrique Girão, meu co-orientador científico, por prontamente me ter ajudado em tudo que precisei, pelos recursos que disponibilizou, pela sua boa disposição logo pelo cafezito da manhã. Não podia deixar de agradecer pela preocupação face às minhas longas caminhadas entre o CNC e o IBILI. De coração, o meu obrigada. Ao Doutor Carlos Palmeira, co-orientador pelo meu departamento, um enternecedor obrigada por ter aceite o papel de representante interino. Já desde Toxicidade e Doença que tenho um enorme apreço por si!

Outro grande contributo para este trabalho foi o apoio diário que tive da Ana Plácido, da Rita Carvalho e da Teresa. Sem vocês, certamente que este trabalho não daria estes frutos. Muito obrigada, meninas! Um carinho especial para a Rosa. Passámos bons momentos juntas. Obrigada por nunca me teres negado nenhuma resposta às mil perguntas que te fiz, por estares sempre disposta a ajudar e pela constante preocupação. Significou muito! Fica aqui prometido que ainda te vou ensinar a fazer a listinha que prometi dos anticorpos. Muito, muito obrigada! Ana Duarte, obrigada pelo terno apoio, pelas explicações às minhas perguntas, que mesmo não sabendo na hora, fazias questão de te informar sobre isso. Ao resto da malta do laboratório: Sónia, Raquel, Susana, Emanuel pela boa disposição permanente. Um especial obrigada ao Rui Gomes. Esta amizade já vai longa, desde os tempos de licenciatura. Foste demasiado importante nesta jornada. Aturaste as minhas dúvidas existenciais, atendeste todos os telefonemas independentemente da hora, não sei como te agradecer, campeão! Rita Sassá e Nelson, companheiros do IBILI, obrigada pelos pontuais momentos de descontração ao solzinho no Pólo III e sábios conselhos durante este tempo.

Gostaria de agradecer também ao Dr. João Ramalho e à sua equipa, por me terem concedido acesso ao microscópio para obter estas lindas imagens. Maria Inês, obrigada por me teres ensinado e gostado das "coisas giras" que as minhas células faziam. Às minhas queridas "Isabéis", Nunes, Dantas e D. Isabel pela constante ajuda e pelos sorrisos apaziguadores que me moviam nos dias mais difíceis. À Doutora Célia Aveleira, um enorme obrigada! Foi a minha salvadora em diversas situações de aperto, sempre a desenrascar no que fosse preciso para que o meu trabalho não estagnasse. Do princípio ao fim!! Obrigadão. À maltinha da sala dos fixes: Ana Teresa, Susana, Filipa Brito, mas também à Dina, Ana Cristina, Mariana por todo o companheirismo, preocupação e suporte durante estes anos. Obrigada, minhas queridas!

Ainda gostaria de agradecer aos meus BCéMicos: Ana Cascalho, Rojões, Maria Inês, Andreia, Alberto. Foram momentos incríveis que passámos juntos ao longo do Mestrado, que certamente quero que se repitam. Cada um de vós me ajudou como pôde, mesmo a quilómetros de distância. Não poderia deixar de

incluir aqui o Pedro Cunha e o Miguel! Desde a Licenciatura que me acompanham, desde aí que fazem a diferença na minha vida, não fossem vocês meus “afilhados”. Ainda mais no Mestrado, nos piores e nos melhores momentos, sim! Vocês estavam lá. E irão continuar por mais alguns aninhos, assim espero. Obrigada, um enorme obrigada!

Rutita, este agradecimento agora é para ti! Não consigo explicar quão importante foste e és, quão bem me tens apoiado, acarinhado, quantas das minhas forças para enfrentar os dias mais cabisbaixos foram de ti que partiram. Obrigada, coração. Obrigada pela tua loucura, pelas risotas, por seres a Rute! Diogo, Pedro Filipe, Fernando, Beatriz, Filipa, Luz e Fábio, mil obrigadas a dividir por vocês todos. Cada um de vós à sua maneira contribuiu para onde cheguei hoje.

Aos meus amigos do peito: Pombo, JêPê, Ruben, Tadeu, Nuno Mendes, Anita, Lhó, Gonçalo, Helena, Apóstolo, Zé Dias. Vocês são FANTÁSTICOS, vocês são os melhores. Certamente que Bioquímica não me podia ter dado melhor família. Obrigada por tudo, pelos jantares, pela companhia, preocupação, até pelas coisas mais estapafúrdias, mas a verdade é que até nisso somos os melhores! Longe ou perto, faça muito ou pouco tempo desde a última vez que nos vimos, parece que nada muda, e ainda bem. OBRIGADA, minha gente.

Tozé e Bá, vocês nunca se conheceram, mas eu já. E quero-vos agradecer por isso, por me conhecerem tão bem, por cada palavra no momento certo. Obrigada! Gostaria ainda de agradecer a mais algumas pessoas, mas este texto já vai longo. “The north remembers” and so do I. A todas essas pessoas que me ajudaram a tornar estes dois anos incríveis, que sempre do meu lado, pegaram no mau e transformaram-no em bom, e tornaram bons momentos em magníficos, faço questão de agradecer pessoalmente a cada um de vós.

Por último, mas não menos importante, um agradecimento enorme à minha família! Tiozinhos, Madrinha, este trabalho também é vosso. Fazem sempre questão que nada me falte! Ao meu avô Mário, pela imensa sabedoria que sempre me transmitiu, e aos meus avós maternos: estejam onde estiverem, tenho a certeza que me iluminaram. Obrigada, riquezas!

Maior riqueza ainda são os meus pais. Este trabalho é vosso, inteiramente vosso! Sem vocês, nada disto era de todo possível. Todo o sacrifício, todo o apoio, todo o amor! Tudo se consegue com amor. E é bem verdade! Foi do vosso imenso esforço que construi o meu, e do qual vos tento orgulhar todos os dias. É certo que a minha ausência foi prolongada, mas foi necessário para o meu futuro. Aquele do qual vocês contruíram as bases e que, a partir de agora, eu tomarei rédeas. O meu coração é vosso, assim como é esta dissertação. Admiro-vos, meus pais! Obrigada.

Assim termino estes agradecimentos, dizendo que "Se vi mais longe foi por estar de pé sobre ombros de gigantes." E foi graças a todos estes pequenos gigantes que cheguei ao fim desta caminhada. Já estou de malas e bagagem prontas!

Bem-haja a todos vós!

Table of contents

ABBREVIATIONS

$[Ca^{2+}]_{mit}$	Mitochondrial Ca^{2+} concentration
7TMs	Seven transmembrane-spanning domain
Akt	Protein kinase B
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
Apaf-1	Apoptotic protease activating factor-1
ARC	Arcuate nucleus
ATF	Activating transcription factor
ATP	Adenosine triphosphate
Bak	Pro-apoptotic Bax and Bcl-2-homologous antagonist/killer
BAP31	B-cell receptor associated protein 31
Bax	Bcl-2/Bcl-2-associated X protein
BBB	Blood Brain-Barrier
Bcl-2	B-cell lymphoma protein-2
bFGF	basic Fibroblast Growth Factor
BiP	Immunoglobulin heavy-chain binding protein
BSA	Bovine Serum Albumin
CaM	Calmodulin
CaMKII	Ca^{2+}/Calmodulin-dependent protein kinase II
CaMKK	Ca^{2+}/Calmodulin-dependent kinase kinase
cGMP	Cyclic guanosine monophosphate
CHOP	CAAT/enhancer binding protein homologous protein
CNS	Central Nervous System
CRE	cAMP-responsive element
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DTT	1,4-dithiothreitol
EC	Endothelial cell
ECF	Enhanced ChemiFluorescence
ECGF	Endothelial cell growth factor
EDTA	Ethylenediaminetetraacetic acid
eIF2α	eukaryotic translation-initiation factor 2α
eNOS	Endothelial nitric oxide synthase

EOR	ER overload response
ER	Endoplasmic Reticulum
ERAD	ER-associated protein degradation
ERK1/2	Extracellular Signal Regulated Kinases 1 and 2
ERO1α	ER oxidoreductin 1α
FBS	Fetal Bovine Serum
Fura-2/AM	Fura-2 acetoxymethyl ester
G418	Geneticin
GADD	Growth arrest and DNA damage-inducible protein
GDP	Guanosine diphosphate
GH	Growth hormone
GHR	Ghrelin
GHS-R	Growth hormone secretagogue receptor
GPCR	GTP-binding protein (G protein)-coupled receptor
GRP75	Glucose-related protein 75
GRP78	Glucose-related protein 78
GSH	reduced Glutathione
HEPES	4-(2-hydroxyethyl)-1-piperazoneethanesulfonic acid
HRP	Horseradish Peroxidase
HSPA9	Heat shock protein A 9
IgG	Immunoglobulin G
IP_3	Inositol 1,4,5-triphosphate
IP_3R	IP_3 receptor
IRE-1	Inositol requiring element-1
JNK	c-Jun NH_2-terminal kinase
LC3	microtubule-associated protein light chain 3
LTP	Long-term potentiation
MAMs	mitochondria-associated ER membranes
MAPK	mitogen-activated protein kinase
MCEC-1	Murine Cardiac Endothelial Cells
mTOR	Mammalian target of rapamycin
MTT	Thiazolyl Blue Tetrazolium Bromide
NF-κB	Nuclear factor-kappa light chain enhancer of activated B cells
NO	Nitric oxide
NOX	Nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase
NPY	Neuropeptide Y
NRF2	Nuclear factor erythroid-2

PDI	Protein disulfide isomerase
PERK	Protein kinase RNA-like ER kinase
PI_3K	Phophatidylinositol 3-kinase
PIP_2	Phosphatidylinositol 4,5-diphosphate
PKA	cAMP-dependent protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene difluoride
RBE4	Rat Brain Endothelial cell line
RIDD	Regulated IRE1-dependent decay
ROS	Reactive oxygen species
RT	Room temperature
RyR	Ryanodine receptor
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	SDS-polyacrylamide gel
SERCA	Sarco/endoplasmic Ca^{2+}-ATPase
Sig1R	Sigma 1 receptor
sXBP-1	spliced XBP-1
TBS	Tris-Buffered Saline
TEMED	N,N,N',N'-Tetramethyl-ethylenediamine
TG	Thapsigargin
TM	Tunicamycin
TNF-α	Tumor necrosis factor-α
TRAF2	TNF-α receptor associated-factor-2
UCP2	Uncoupling Protein 2
UPR	Unfolded protein response
UPS	Ubiquitin proteasome system
uXBP-1	unspliced XBP-1
VDAC	Voltage-dependent anion channel
VEGF	Vascular endothelial cell growth factor
WB	Western blot
XBP-1	X-box binding protein-1

RESUMO

RESUMO

O retículo endoplasmático (RE) é o organelo responsável pela manutenção da proteóstase da célula. A acumulação de proteínas misfolded no lúmen do RE origina stress celular, desencadeando a ativação de vias de sinalização de defesa, como a via *unfolded protein response* (UPR), para, deste modo, restaurar a homeostasia. Em consequência, ocorre a activação de *chaperones* e a eliminação de proteínas anómalas mediada pelos sistemas lisossomal e proteassomal. No entanto, quando a severidade e/ou a duração do stress aumenta, estas vias não desempenham as suas funções, conduzindo à morte celular por apoptose. Dados recentes mostram que o stress do RE está envolvido na patogénese de várias doenças, nomeadamente doenças neurodegenerativas, cardiovasculares e em patologias associadas à disfunção de células endoteliais (CEs). Na área cardiovascular, boas perspetivas se têm obtido com o uso de grelina (GHR), dado o seu efeito cardioprotetor. Recentemente evidenciámos que a ativação da UPR induzida pelo stress do RE gera stress oxidativo, perda da homeostasia do cálcio, disfunção mitocondrial e morte celular em CEs do cérebro. Portanto, a disrupção da proteóstase surge como uma potencial característica de patologias associadas à disfunção endotelial, podendo ser revertida pela hormona GHR, apesar dos mecanismos subjacentes a estas permanecerem desconhecidos.

Temos por base a caracterização comparativa do efeito da GHR na resposta de CEs cardíacas e do cérebro (RBE4/MCEC-1) face a um ambiente proteostático hostil (stress do RE crónico). Usando linhas endoteliais cardíacas e do cérebro expostas a tapsigargina e tunicamicina, dois indutores de stress comummente usados, investigámos o tipo de modulação gerado pela GHR em debilitados mecanismos de controlo de qualidade proteicos, na perda da homeostasia redox e do cálcio e também na comunicação intercelular em CEs cerebrais, em paralelo com perturbações ao nível funcional e morfológico em ambas as linhas celulares.

Evidenciámos que o efeito dos indutores de stress do RE na activação da UPR se correlacionou com as alterações funcionais das CEs, comprometendo a sobrevivência celular através da indução de mecanismos de morte celular (apoptose) pela macroautofagia. Efeito este que foi prevenido pela GHR, em parte devido ao controlo sobre a UPR, mantendo a viabilidade celular ao proteger contra o stress oxidativo, a macroautofagia e a apoptose. No que respeita a comunicação intercelular, a GHR estimula a libertação de exossomas que possivelmente encerram em si sinalizadores deletérios para as CEs, para posterior eliminação, como o exemplo dos autofagossomas. Além disso, os parâmetros morfológicos e funcionais do endotélio são rearranjados de modo a assegurar a homeostasia na presença de GHR.

Os resultados apoiam a relação entre a perda da proteóstase e a disfunção endotelial, quer no cérebro quer no coração, mas também a ação da GHR como agente protetor. Apesar desta hipótese necessitar de confirmação experimental, este efeito benéfico da GHR resulta, possivelmente, da ativação da via de sinalização da AMPK e/ou da libertação de exossomas. Assim, estes resultados sugerem que a GHR poderá ser considerada uma nova forma de intervenção na disfunção endotelial.

Palavras-chave: Células endoteliais do cérebro e cardíacas · Proteóstase · Stress do RE · Disfunção endotelial · Grelina

SUMMARY

SUMMARY

The endoplasmic reticulum (ER) is implicated in the maintenance of proteostasis. Under stress conditions triggered by the accumulation of misfolded proteins in the ER lumen, this intracellular organelle switches on a defense signaling pathway, known as the unfolded protein response (UPR), that restores homeostasis by upregulating chaperones and clearing abnormal proteins through proteasome- and lysosome-mediated degradation systems. When the severity and/or duration of the stress increase, these pathways fail to overcome the threat, ultimately leading to cell demise by apoptosis. Growing evidences support that ER stress plays a central role in the pathogenesis of various human diseases, namely neurodegenerative and cardiovascular disorders. Accordingly, ER stress was demonstrated to occur during pathological cardiac endothelial cell (EC) dysfunction, which ghrelin (GHR) ameliorated given its cardioprotective properties. Moreover, our previous findings support that ER stress-induced UPR activation leads to oxidative stress, calcium dysregulation, mitochondrial impairment and cell death in brain ECs. Therefore, disruption of the proteostasis network emerge as a novel feature involved in endothelial dysfunction associated with several disorders that can be reversed by GHR, however, the underlying mechanisms are not yet well described.

The aim of this work is to characterize the role of GHR in the response of brain ECs, in comparison with cardiac ECs, against adverse proteostatic circumstances such as chronic ER stress. We exposed rat brain and murine cardiac EC lines (RBE4/MCEC-1) to thapsigargin and tunicamycin, two widely used ER stressors. Under these conditions, we investigated GHR-mediated modulation of impaired protein quality control mechanisms, calcium and redox dyshomeostasis and cell-to-cell communication in brain ECs, in parallel to morphological and functional derangement in both cardiac and brain ECs.

Altogether, UPR induction through ER stressors is mutually related with the dysregulation of calcium/redox homeostasis and functional dystrophy of ECs, which compromises cell survival through macroautophagy-mediated apoptosis. However, GHR was able to modulate UPR, partially because it prevented oxidative stress, macroautophagy and apoptosis, leading to cell survival in brain ECs. Regarding cell-to-cell-communication, GHR stimulated the release of exosomes that might carry danger signals for elimination (e.g. autophagosomes). Additionally, GHR also instigated morphological and functional rearrangements to assure its cell homeostasis. MCEC-1 cells are differentially modulated by ER stress and GHR.

Either in the brain or the heart, there is a relationship between the loss of proteostasis and endothelial dysfunction, and GHR can be a defensive agent, possibly due to the upregulation of AMPK signaling pathway and/or the release of “ER stress freeing” exosomes, a hypothesis that deserves experimental confirmation. Furthermore, these results suggest that GHR might be considered as a potential therapeutic tool in endothelial dysfunction.

Keywords: Brain and cardiac endothelial cells · Proteostasis · ER stress · Endothelial dysfunction · Ghrelin

CHAPTER 1 – Introduction

1.1 Ghrelin

1.1.1 The hunger hormone

Originally purified from rat stomach in 1999 (Kojima et al., 1999), ghrelin (GHR) is a peptide constituted by 28 amino acids, characterized by the insertion of an octanoyl group ($C_7H_{15}CO$) on the third N-terminus amino acid, a serine residue. Strikingly, solely GHR presents this post-translational modification, a unique attribute essential for its activity. Therefore, GHR can be expressed as an acylated or desacylated peptide. GHR is a highly conserved peptide between rat and human species. Both share a third amino acid modification by the fatty acid *n*-octanoic acid, but differ on the nature of two amino acids (the eleventh and twelfth) (Kojima and Kangawa, 2005). Beforehand, GHR is obtained by the proteolytic processing of preproghrelin yielding GHR itself and other peptide, obestatin (Zhu et al., 2006). Although the main form that acts on hypothalamus is the acylated one, about 80%-90% of circulating GHR is desacyl-GHR (Ferrini et al., 2009).

The GHR's acylated form is the responsible for the secretion of growth hormone (GH) from the pituitary gland through binding to the growth hormone secretagogue receptor (GHS-R), working as a heterotrimeric GTP-binding protein (G protein)-coupled receptor (GPCR) (Howard et al., 1996; Pong et al., 1996; Korbonits et al., 1999). Accordingly, this receptor, afterwards known as type 1a GHS-R (GHS-R1a), is mainly expressed in the pituitary and hypothalamus (Howard et al., 1996). Although mostly GHR's synthesis takes place in the stomach, this peptide offers an endocrine link between stomach, hypothalamus and pituitary, and consequently, a contribution for the regulation of energy homeostasis. In fact, GHR acts as a crucial orexigenic hormone, circulating in the bloodstream under fasting conditions to transmit the hunger signal from the periphery to the central nervous system (CNS). Its serum levels are reduced by re-feeding, suggesting that, besides its role on the GH release, it signals the hypothalamic arcuate nucleus (ARC), a region known to control food intake, in order to stimulate appetite (Tschöp et al., 2000).

Like GH, GHR expression is regulated as well, and GH itself appears to be involved. Other determinants of GHR's secretion are glucose, insulin, somatostatin, glucagon, thyroid hormones, melatonin and even the parasympathetic nervous system, among others (Korbonits et al., 2004; Yin et al., 2009). In particular, glucagon was shown to upregulate the expression of GHR gene in stomach-derived cell lines (Kishimoto et al., 2003; Wei et al., 2005). Consistent with the increase in systemic glucagon levels after food restriction (Seino et al., 1980), treatments with glucagon increase the GHR mRNA levels and its concentration on plasma, supporting Tschöp's hypothesis in fasting conditions (Tschöp et al., 2000; Wei et al., 2005; Katayama et al., 2007). Seemingly regulation is provided by leptin, even though controversial data have emerged. Dose-dependently leptin *in vitro* treatments or *in vivo* infusion into rat stomach inhibited GHR secretion (Kamegai et al., 2004; Zhao et al., 2008), but intraperitoneal injection of leptin provided augmented GHR mRNA levels in mice (Toshinai et al., 2001). Regarding circulating levels of GHR, no

effect was found in humans upon leptin's administration (Chan et al., 2004). Therefore, leptin might not be a major regulator of GHR expression. Additionally, GHR secretion is also a highly regulated process. Feeding is among the most important factors for the regulation of GHR secretion. Throughout the day the endogenous GHR levels oscillate according to pre- and postprandial cycles, with especially elevation before food ingestion and during the night, and a reduction immediately after a meal. So it may suggest the putative role of GHR in meal initiation (Ariyasu et al., 2001; Cummings et al., 2001; Tschöp et al., 2001b). As a note, a powerful orexigenic effect results from an increased pulse of stomach GHR delivery together with the synchronized lower leptin (anorexigenic compound) (Bagnasco et al., 2002). Neither pituitary or hypothalamic GHR expression lies behind this process (Torsello et al., 2003).

1.1.2 Tissue distribution of ghrelin

Throughout the **gastrointestinal tract**, both GHR's isoforms are secreted by distinct organs. As mentioned before, the stomach is the primary organ producing GHR (Ariyasu et al., 2001) from X/A-like cells inserted in the endocrine cell population of adult oxyntic glands, where acyl-modified GHR is stored in round and dense secretory granules (Date et al., 2000; Dornonville de la Cour et al., 2001; Yabuki et al., 2004). Herein, GHR levels increase after birth (Hayashida et al., 2002), contrary to what happens in pancreas (Chanoine and Wong, 2004). Likewise, both acyl- and desacyl-GHR exist in this organ (Date et al., 2002a). Another GHR-producing organ is the gut, where specific portions are enriched in GHR-immunoreactive cells, following a downward concentration gradient from the duodenum to the colon (Date et al., 2000; Hosoda et al., 2000; Sakata et al., 2002).

Regarding the **CNS**, GHR is expected to be produced mainly in the hypothalamic regions owing to the highly expression of GHS-R1a in the pituitary and hypothalamus (Howard et al., 1996; Guan et al., 1997). In detail, GHR has been found in the hypothalamic ARC, because it is its target and the central core for appetite regulation in hypothalamus, controlled through the neuropeptide Y(NPY) pathway (Klok et al., 2007). Nevertheless, GHR content in the brain is very low (Kojima et al., 1999; Hosoda et al., 2000). In agreement with GHS-R1a localization in the pituitary, and given the strong GH-releasing activity of GHR (Arvat et al., 2000; Takaya et al., 2000), this peptide was also found in the pituitary gland, which can influence GH secretion in an autocrine or paracrine manner (Korbonits et al., 2001a, 2001b), similar to the adipose tissue.

Despite brain, stomach and intestinal GHR represent the majority portions of whole body content in GHR, evidences showed its expression in the lungs (Volante et al., 2002), placenta (Gualillo et al., 2001), female and male gonads (Barreiro et al., 2002; Tena-Sempere et al., 2002; Caminos et al., 2003; Gaytan et al., 2003), kidneys (Mori et al., 2000), even among cells of the immune system (Hattori et al., 2001). At last, plasma engulfs a considerable amount of GHR, where the two main molecular forms of GHR (*n*-octanoyl and desacyl-GHR) flow through the bloodstream (Hosoda et al., 2000).

1.1.3 The transport across the blood brain-barrier

The blood brain-barrier (BBB), a critical component of the cerebrovasculature, functions as a "guardian" of the CNS, keeping its homeostasis and preserving neuronal function by restraining the permeability of indiscriminate substances between CNS and blood. This includes the communication CNS-gastrointestinal tract.

In order to target neuroendocrine networks within CNS, GHR must cross BBB. This is directly dependent on the singular post-translational acylation. Once again, when comparing human to mouse GHR, two of their overall 28 amino acids are replaced: exchange of arginine with lysine at position 11 and valine with alanine at position 12. The lipophilic moiety of GHR, more specifically these two substituent amino acids, are pivotal for recognition by BBB transporters. In this way, the bidirectional transport of human and mouse GHR also differs. Using saturable transportation systems, human GHR readily crosses BBB in both directions, whether mouse GHR is a plain substrate for the brain-to-blood transporter, but it is no longer recognized by inverse carrier. So its uptake is shown to a far lesser degree (Banks et al., 2002; Pan et al., 2006). Same line of evidence, mouse desacyl-GHR enters the brain by nonsaturable transmembrane diffusion and is sequestered once within CNS, most likely converted into octanoylated form of the peptide to perform its action. In conclusion, the extent and direction of GHR transport across the BBB is, therefore, influenced by at least two features of its primary structure: (1) the post-translationally added fatty acid, and (2) the amino acid sequence.

Banks and colleagues also investigated the liaison between physiological status and the rate at which GHR passes across the BBB. Their findings pointed to lower permeability to GHR through BBB during obesity and aging phenomena, meaning that body weight is inversely correlated to GHR diffusion (Banks et al., 2008). Thus, plasma concentration is low in obese people and high in lean people (Tschöp et al., 2001a; Hansen et al., 2002; Shiiya et al., 2002; Rosická et al., 2003; Gilg and Lutz, 2006). Conversely, starvation tends to enhance its transport. Moreover, changes in GHR passage across BBB affect its bioactivity on the brain, by decreasing or improving its orexigenic effects, respectively (Banks et al., 2008). However, this orexigen and the GH-inductive release actions of GHR occur via divergent pathways. Indeed, peripherally injected GHR stimulates hypothalamic neurons (Hewson and Dickson, 2000; Rüter et al., 2003), but if the mechanism depends on the indirect vagal afferent pathway, food intake is triggered (Wren et al., 2001; Date et al., 2002b), whereas the direct mechanism results in GH secretion (le Roux et al., 2005; Gilg and Lutz, 2006).

1.1.4 GHS-R1a and downstream signaling pathways

The human GHR receptor so-called GHS-R type 1a is a typical seven transmembrane-spanning domain (7TMs) GPCR (Davenport et al., 2005), while type 1b receptor is only encoded by five of the seven predicted TM domains (truncation of GHS-R1a form at the C-terminal) (Howard et al., 1996; McKee et al.,

1997). GHS-R1b biological activity is not quite well described, but evidences implicate it as a modulator of the full-length version. It appears to act as a dominant negative of full-length GHS-R1a, decreasing its cell surface expression because type 1a is retained in the endoplasmic reticulum (ER), consequence of the GHS-R1a and GHS-R1b heterodimerization. Furthermore, GHS-R1a trafficking and constitutive activity is attenuated (Leung et al., 2007; Chow et al., 2012). Recently, GHS-R1b was described as a dual allosteric effector: not only determines the efficacy of ghrelin-induced GHS-R1a-mediated signaling but also determines the ability of GHS-R1a to form oligomeric complexes with other receptors, promoting profound qualitative changes in GHR-induced signaling (Navarro et al., 2016).

GHR receptor is ubiquitously distributed in the **brain**. Its mRNA pattern of expression has been confirmed in multiple hypothalamic nuclei and in pituitary cells, consistent with its physiological function associated to energy metabolism and GH-release (Howard et al., 1996; Guan et al., 1997; Zigman et al., 2006). Of particular interest, GHS-R1a was also localized in presynaptic boutons in ARC and in the hippocampus (Cowley et al., 2003; Diano et al., 2006), implying an additional role for GHR in hippocampal-dependent processes, aside the hypothalamus region. In the **peripheral tissues**, some authors revealed a wide distribution of GHS-R1a among several organs, such as heart, lung, liver, kidney, pancreas, stomach, intestine, adipose tissue, endocrine glands and immune cells in rodents and humans (Guan et al., 1997; Hattori et al., 2001; Kojima et al., 2001; Gnanapavan et al., 2002). Summing up with previous reported locations of its endogenous ligand, these observations support the GHS-R1a functions beyond the control of GH secretion and food intake (Chen et al., 2009).

The ability to propagate the intracellular signal in the absence of agonist is commonly known as **constitutive activity** (Aloyo et al., 2010). GHS-R1a exhibits an unusual high of such activity, where a conformational change is required to mimic agonist activation but also to stabilize and ensure the receptor to remain in the active state. GHS-R1a signal transduction is mainly mediated through the $G_{q/11}$-α subunit of the G protein, leading the activation of the phospholipase C (PLC) pathway that yields an increase in inositol 1,4,5-triphosphate (IP_3) production, which corresponds approximately to 50% the maximal agonist-induced activity (Holst et al., 2003, 2004). The same authors unravelled an additional intracellular signaling via cAMP-responsive element (CRE) cascade, which involves the activity of the transcription factor CRE binding protein (CREB). It is believed that CREB regulation arises from cAMP-dependent kinases or downstream kinases from the G_q-α pathway, including Ca^{2+}/calmodulin kinase IV or protein kinase C (PKC) (Matthews et al., 1994; Singh et al., 2001). Surprisingly, constitutive activity of GHS-R1a may depend on the cellular context, because the pituitary cell line RC-4B/C.40 does not possess any of this specific activity (Falls et al., 2006).

On the other hand, the conventional conformational modification of the receptor caused by the binding of GHR, exposing the binding sites to G proteins coupled to GHS-R1a. An exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) occurs, which, in association to G protein α subunit, initiate intracellular signaling cascades by acting on several downstream effectors (Gao and Horvath, 2007; Muccioli et al., 2007). The first mechanism underlying GHR involve the regulation of **ionic currents**: $G_{q/11}$-

α subunit leads to the activation of PLC specific for phosphatidylinositol 4,5-diphosphate (PIP_2) ensuing its cleavage into IP_3 and diacylglycerol (DAG) (Howard et al., 1996; Smith et al., 1997). Primarily, IP_3 will induce a rapid and transient rise in intracellular free Ca^{2+} concentration due to its release from IP_3-responsive ER storages. Then, inactive PKC senses the high levels of Ca^{2+} and DAG becoming activated, which, in turn, blocks potassium (K^+) channels, creating depolarization. This event begins the opening of voltage-gated L-type Ca^{2+} channels, causing more sustained accumulation of cytoplasmic Ca^{2+}, and ultimately exacerbating cellular depolarization (Fig. 1.1) (Camiña, 2006).

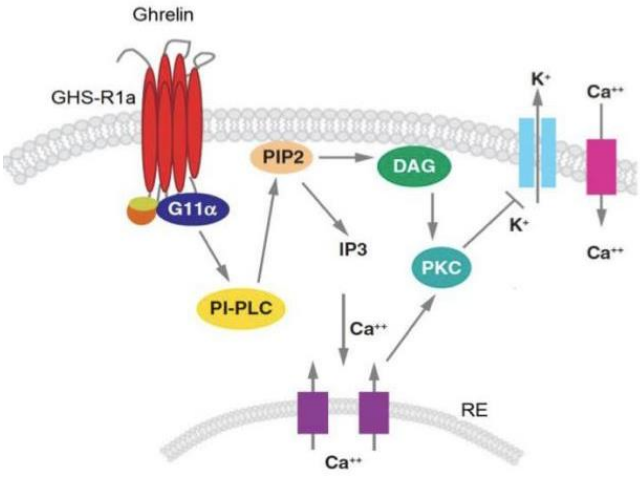


Figure 1.1 ***Prototypical signaling of GHS-R1a.***
Ligand binding activates $G_{q/11}$ protein that signals phosphatidylinositol-specific phospholipase C (PI-PLC), which hydrolyzes phosphatidylinositol 4,5-diphosphate (PIP_2), stored in the plasma membrane, generating both diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). IP_3 will induce the rapid release of Ca^{2+} stored in the endoplasmic reticulum, and the free Ca^{2+} together with DAG will activate PKC. PKC will inhibit K^+ channels, leading to a membrane depolarization and causing the opening of voltage-dependent L-type Ca^{2+} channels. Adapted from (Gao and Horvath, 2007).

Additionally, high levels of Ca^{2+} will target Ca^{2+}-binding proteins, for example calmodulin (CaM), and subsequently, via downstream events, will inactivate the mTOR protein kinase complex. This activation precedes the orexigenic signal, increasing appetite (Martins et al., 2012). GHR also activates **protein phosphorylation-based intracellular cascades** like mitogen activated protein **kinase (MAPK)** and **PI_3 kinase (PI_3K) pathways** to promote cell proliferation in various cellular systems by different G protein subunits ($G_q\alpha$ or $G_{i/o}\alpha$) (Kim et al., 2004a, 2004b; Mazzocchi et al., 2004; Camiña, 2006). Onto this matter, a different type of Ca^{2+} channels are modulated by the active cAMP-dependent protein kinase A (PKA), in particular N-type Ca^{2+} channels, promoting an influx of Ca^{2+} that boosts the intracellular concentration. This specific cascade involving $G_q\alpha$ takes place in NPY-producing neurons in the hypothalamus (Kohno et al., 2003). Still on hypothalamic cells, it was observed the enhancement of **5'-AMP-activated protein kinase (AMPK)** activity by GHR, thus responsible for energy balance mechanisms (Andersson et al., 2004; Carling, 2005; Zhang et al., 2013b). In spite of cAMP-dependent signaling cascades, **nitric oxide/cyclic guanosine monophosphate (cGMP) signaling** activation is intimately linked to GHR-stimulated GH

secretion (Rodríguez-Pacheco et al., 2005). Regarding inhibitory mechanisms, GHR is able to decrease **vascular inflammation** through the activation of the calmodulin-dependent kinase kinase (CaMKK), AMPK and endothelial nitric oxide synthase (eNOS) (Xu et al., 2008). At last, GHS-R1a is also coupled to Raf-MEK-MAPK and PI_3K/Akt/GSK3β signaling pathways, the last intimately related to suppression of apoptosis (Chung et al., 2008; Frago et al., 2011; Han et al., 2015). In line with ligand-independent GHS-R1a activity, here specific intracellular cascades are elicited according to the tissue type in which the receptor is expressed too.

1.1.5 The journey beyond adiposity

It is true that GHR exerts its main action in the hypothalamus to control appetite, but as here reviewed, active forms of GHS-R were detected outside this brain region. For example, in extrahypothalamic areas as the hippocampus, GHR plays an important role in **memory and learning mechanisms** (Carlini et al., 2002, 2004), by modulation of specific molecular intermediates for memory acquisition/consolidation (Diano et al., 2006; Carlini et al., 2010). The raise of this purpose may have an evolutionary meaning, since to maintain an energy balance, one need to locate food sources and remember those local for the sake of survival, suggesting a tight connection between feeding-associated behavior, learning capabilities and energy metabolism (Moran and Gao, 2006). However, the precise molecular mechanism of GHR action on hippocampus function is not yet fully elucidated. In the literature, it was implicated an activation of nitric oxide synthase (NOS) by endogenous GHR-GHS-R1a binding on the generation of long-term potentiation (LTP), hippocampus dendritic spine formation and behavioral outputs (Diano et al., 2006; Carlini et al., 2010; Ribeiro et al., 2014). Morphologically, those aspects of GHR's effect were shown by an increase and rearrangement of spine synapse density in CA1 regions of the hippocampus (McNay, 2007; Atcha et al., 2009). Other aspects like anxiogenesis and depression (Bali and Jaggi, 2016); rewarded behavior (Kenny, 2011; Menzies et al., 2013) and regulation of circadian cycle are described among physiological tasks underlying GHR (Nisembaum et al., 2014).

Beyond CNS, in adipocytes, both GHR isoforms also constitute an **anti-inflammatory agent** against the pro-inflammatory cytokine TNF-α-induced apoptosis and autophagy, inhibiting the activation of caspases as well as the expression of autophagic-related genes (Rodríguez et al., 2012).

Being a multisystemic peptide, it is plausible to claim GHR as a link between obesity and the cardiovascular function, since the first has been considered a major risk factor for cardiovascular diseases due to elevated cardiac output (Algahim et al., 2012). The myocardium is a source of GHR (Iglesias et al., 2004), and here and in cardiomyocytes, all the components of GHR system were found, suggesting again an autocrine/paracrine role of this hormone in the heart (Beiras-Fernandez et al., 2010). In fact, some authors described a **cardioprotective effect** of GHR by decreasing blood pressure and heart rate (Lin et al., 2004; Yuan et al., 2014), corroborated by the low levels of total GHR in several heart disorders and the imbalance of both GHR isoforms in circulation during metabolic syndrome (Oner-Iyidoğan et al., 2007; Cao et al.,

2013). Thereafter, another remarkable action of GHR is its protective action, mostly credited by promoting vascular endothelial function. Among other cell types, numerous studies showed an anti-apoptotic effect of GHR in endothelial cells (Baldanzi et al., 2002; Shimada et al., 2014; Zhu et al., 2014; Li et al., 2015). According to the state of art, the signaling pathways that mediate GHR protective effect are MAPK, PI_3K, PKC, ERK1/2 and PKA (Chung et al., 2007, 2008; Xiang et al., 2011). ERK1/2 via activation of MAPK p42/44 suppresses apoptosis, in partnership with PI_3K/Akt pathway (Baldanzi et al., 2002; Lee et al., 2011). As a result, the activation of ER stress is compromised as well, since PERK cascade is hindered, and CHOP is inhibited (Chung et al., 2011; Yang et al., 2012). On the other hand, toxicity is attenuated by ERK MAPK signaling, that are thought to be involved in cell growth and survival elicited by GHR (Chung et al., 2007). PI_3K cascade, intermingled with endothelial nitric oxide release, is prompted by GHR, resulting in vasodilation. Moreover, vascular factors, as angiotensin II and endothelin-1 are reduced (Tesauro et al., 2009; Virdis et al., 2016). Apart from the abrogation of vascular superoxide species formation, GHR also inhibits other ROS generation, consequence of the increase in Bcl-2/Bax ratio, prevention of cytochrome c release, blockade of caspase 3 and higher UCP2 expression (Chung et al., 2007, 2008, 2011; Andrews et al., 2008; Liu et al., 2009; Lee et al., 2011). The higher levels of UCP2 can be beneficial to combat oxidative stress (Toda and Diano, 2014). Even in endothelial cells, inflammation is also abrogated by GHR that blocks cytokine production and nuclear factor-κB activation (NF-κB) (Li et al., 2004; Deng et al., 2015).

Recently, a GHR analog was found to protect β-pancreatic cells, *in vitro* and *in vivo*, from cytotoxicity displaying an anti-apoptotic mitochondrial pathway (Zhao et al., 2016). So, the protective effect of GHR can be attributed to the inhibition of apoptotic pathway (Chung et al., 2007), inflammatory activity (Erşahin et al., 2010) and oxidative stress (Omrani et al., 2015), and endorsing angiogenesis (Li et al., 2007; Wang et al., 2011).

1.2 Endoplasmic reticulum: structural and functional overview

The endoplasmic reticulum (ER), first reported by Porter and co-authors in 1945, is a highly organized eukaryotic organelle, strategically positioned throughout the cell by the arrangement of an interconnected network contiguous to several cellular organelles and the plasma membrane ((Porter et al., 1945; Lee et al., 1989; Baumann and Walz, 2001). It is delimited by a phospholipidic membrane, which compartmentalizes the ER luminal space, known by its oxidizing environment, from the cytosol. Being a majestic cisternae-like structure that is held together by the cytoskeleton and having a large surface area, it allows the storage of several proteins. Clearly, proteins are on the basis of ER functions. It ensures protein translation, channeled them to a sequential and highly regulated quality control mechanism, where proteins assume their correct folding/assembly, suffer post-translational modifications, such as glycosylation and disulfide bond formation, and sorting of proteins into the secretory pathway (Aridor and Balch, 1999). This is the

case of soluble proteins, despite the trafficking of integral proteins is also guarantied (English and Voeltz, 2013). Some functions are confined to distinct ER subregions. For example, rough ER is specialized for most protein synthesis, including the production and storage of glycogen and other macromolecules, mainly because of its association to ribosomes, conferring a rough aspect that makes it distinguishable from smooth ER. Later, these proteins and molecules are transported to Golgi complex for protein sorting, labeling and transport for it final destination (Baumann and Walz, 2001). Smooth ER, on the other hand, is responsible for other functions: lipids and sterol production, carbohydrates and steroids metabolism (Maxfield and Wüstner, 2002), regulation of Ca^{2+} concentration and storage (Paschen, 2001), drug detoxification and attachment of receptors to cell membrane proteins via exocytosis, as well as cell signaling (Mattson et al., 2000).

The ER lumen is the greatest reservoir of Ca^{2+} within the cell, widely used as a second messenger for fast physiological signaling via electrical or chemical stimulation (Bootman et al., 2002). Three types of Ca^{2+} modulators are distributed through the ER membrane: IP_3 (IP_3Rs) and ryanodine receptors (RyRs); and sarco/endoplasmic Ca^{2+}-ATPase (SERCA) exchanger. Both RyRs and IP_3Rs allow Ca^{2+} release from ER stores. Specifically, IP_3Rs are ligand-gated channels that open, promoting the passage of Ca^{2+} ions to cytosol, upon IP_3 binding resultant from upstream signaling cascades (Joseph and Hajnóczky, 2007; Rizzuto et al., 2009). Nonetheless, extensive release of Ca^{2+} interferes with the physiological function behind its signaling pathway, for instance, muscular contraction, exocytosis, motility, cell survival and proliferation, among other processes, which are crucial to maintain cell homeostasis (Joseph and Hajnóczky, 2007). For this reason, SERCA potentiates Ca^{2+} influx to ER, counterbalancing the efflux of Ca^{2+} from releasing channels as IP_3Rs induced by apoptotic stimuli (Verkhratsky and Petersen, 2002), and leakage via translocon involved in the entrance of newly synthesized protein into ER. SERCA promotes the Ca^{2+} refill of ER lumen, using the energy constrained in ATP molecules. ER luminal Ca^{2+}-dependent proteins, namely calnexin and calreticulin, regulate SERCA activity (Clapham, 2007; Giorgi et al., 2009; Cai et al., 2015).

1.2.1 The mitochondria-associated ER membrane (MAM): ER and mitochondrial commitment

The close contact between ER and other intracellular partners is well established. In addition to the luminal connection of ER with the nuclear envelope, organelles such as Golgi apparatus, secretory granules, lysosomes/endosomes and mitochondria belong to this dynamic membranous network (Park et al., 2008), easing the swap of ER-derived products. On this section, we will focus on the ER-mitochondria coupling in normal cellular functioning, including Ca^{2+} signaling. Nevertheless, MAMs function is also extended to lipids exchange and biosynthesis, given MAM's enrichment in metabolic enzymes, and cell survival or demise (Kornmann et al., 2009).

Most of all, Ca^{2+} mobilization is the driven force behind formation of MAMs, which implies that this tethering complex includes both ER and mitochondrial resident protein. In the last few years, some of these proteins have been identified. Structurally, it is believed the existence of a tripartite system, resulting from

the close interconnection between the ER-associated forming voltage-dependent anion channel (VDAC), unsteadily present in the outer mitochondrial membrane, the ER chaperone glucose-regulated protein 75 (GRP75) (Esposti, 2002a) that mediates VDAC's interaction with IP_3R, thus optimizing the Ca^{2+} uptake by mitochondria (Szabadkai et al., 2006), similar to what happens during apoptosis (McCommis and Baines, 2012; Raturi and Simmen, 2013). Although this mechanism still lacks clarity, VDAC acts as an ATP channel during physiological conditions, in order to provide energy for protein folding. This might be related to the unrestricted localization of VDAC in mitochondria. In fact, this MAM component was detected on the MAM-associated ER membranes in neuronal cells (Shoshan-Barmatz et al., 2004). Moreover, chaperones often play a role in MAM regarding Ca^{2+} gathering. Chaperones such as calreticulin modulate ER Ca^{2+} buffering (Michalak et al., 2009). Other ER chaperones, namely the sigma 1 receptor (Sig1R), confer stability or coordinate the sorting of signaling proteins under adverse Ca^{2+} conditions (Bernard-Marissal et al., 2015; Hayashi, 2015). The ligand-gated Sig1R, therefore, loses its physiological interaction with glucose-regulated protein of 78 kDa (GRP78) and interacts with IP_3R when ER Ca^{2+} stores are depleted, promoting cell survival during ER stress (Hayashi and Su, 2007). A slight perturbation of Ca^{2+} homeostasis at the MAM leads to the exacerbation of Sig1R shield mechanism via modulation of Ca^{2+} uptake by mitochondria (Tsai et al., 2009; Nguyen et al., 2015).

Other MAM regulators are mitofusin 2 (controls Ca^{2+} signaling) (Singaravelu et al., 2011), B-cell receptor associated protein 31 (BAP31) (Breckenridge et al., 2003), phosphofurin acidic cluster sorting protein 2 (Simmen et al., 2005) and the family of long-chain fatty-acid CoA ligases (Smith et al., 2013), not here discussed.

1.2.2 ER-mitochondria calcium flux

The regulation of intracellular Ca^{2+} homeostasis is a very complex mechanism that is vital for cell survival. Regarding this regulation, ER-mitochondria crosstalk is of utmost importance. The entry-exit cycles from ER are regulated by three receptors: IP_3R, RyRs and SERCA. The last is involved in the re-uptake of Ca^{2+} to ER stores. Given the transport against the concentration gradient, SERCA Ca^{2+}pumps depend on energy in the form of ATP for its functioning (Kaufman and Malhotra, 2014). In addition, ER-resident chaperones promote ATP hydrolysis during binding and release from other proteins. As a result, the ATP pool runs down and cells sense this energy deprivation signal, thereby expelling Ca^{2+} from ER to mitochondria (Kaufman and Malhotra, 2014). Importantly, Krebs cycle intermediaries are activated, either directly (α-ketoglutarate and isocitrate dehydrogenases) or indirectly (pyruvate dehydrogenase) in response to Ca^{2+} loading. Furthermore, the rate of ATP production increases, as well, as respiration (Gunter et al., 2004; Decuypere et al., 2011b). On the other hand, mitochondria possesses a Ca^{2+} uniporter, envisaging its transport into mitochondrial matrix driven by the negative membrane potential, caused by the respiratory chain activation or the ATP hydrolysis (Rizzuto and Pozzan, 2006). Then, Ca^{2+} can be pumped to cytosol

by diffusion via Na^+ or H^+/Ca^{2+} antiporters. These are the mechanism involved in physiological Ca^{2+} mobilization.

1.3 Endoplasmic reticulum stress: from the adaptive mechanism to the deathly end

The protein quality control system is a necessary requirement for normal ER activity, bringing about a balance between Ca^{2+} homeostasis and the proper protein folding. Thereafter, to ensure a correct protein assembly, ER lumen presents stringent environment, where chaperones and protein-modifying enzymes specialized in protein folding exist in excessive amounts under oxidant conditions (Sevier et al., 2007; Merksamer and Papa, 2010). Furthermore, healthy cells employ a selective export system of correctly folded proteins or rid of misfolded ones via retrotranslocation and subsequent proteasomal degradation in the cytosol – ER-associated protein degradation (ERAD) (Bonifacino and Weissman, 1998; Travers et al., 2000; Vembar and Brodsky, 2008; Kim et al., 2015). Once the previous balance is affected, for instance by pathological stimuli, ER performance is impaired, culminating primarily in the accumulation of misfolded or even unfolded proteins in consonance with deregulated ER Ca^{2+} homeostasis (Cnop et al., 2010). At this point ER stress is settled.

To overcome the threat, cells will readjust ER performance by activating self-protective mechanisms and preserve cell survival (Xu et al., 2005). The signaling pathway here activated is the well-known unfolded protein response (UPR) that conveys the reduction of overall translation allied to the stimulation of chaperones transcription and synthesis, rendering a tougher protein folding capacity in the ER (Harding et al., 1999; Kaufman, 2002; Walter and Ron, 2011; Gardner et al., 2013). Some authors hypothesized an additional pathway, despite identical functions: the ER overload response (EOR) (Chevet et al., 2001). Thus, EOR is distinct from UPR owing to the abnormal accumulation of normal and misfolded proteins trigger the transcriptional activation of the nuclear factor-kB (NF-κB) (Pahl and Baeuerle, 1997), a mediator of stress, cytokines or free radicals' stimuli (Gilmore, 2006). In particular, NF-κB activation can be prompted by widely used ER stress inducers, either acting on the N-glycosylation or protein transport ER-Golgi complex, i.e. tunicamycin or brefeldin A, respectively. Thus, this transcription factor integrates a signaling transduction pathway between the ER and the nucleus (Pahl and Baeuerle, 1995), requiring the release of Ca^{2+}, followed by the production of reactive oxygen species (ROS) during ER overload (Pahl and Baeuerle, 1996). Together, both adaptive mechanisms render cells with a quick response to the build-up in defective proteins within ER, either supporting the folding machinery or target them for degradation systems.

UPR is an extremely conserved process by which ER manages to restore cellular homeostasis and proteostasis, i.e. the balance between ER protein loading and folding (Wang et al., 1996; Trusina et al., 2008; Plácido et al., 2014). According to changes in cellular protein folding requirements, the ability of ER to fold proteins, as the dynamic organelle it is, is adjusted through UPR (Ron and Walter, 2007a). It is fair

then to subdivide UPR activation during ER stressful conditions into three stages: adaptation, alarm and cell death (Plácido et al., 2014). Therefore, some physiological features underlying the three pillars of UPR are:

a. Adaptive stage: summing up some previous addressed points, UPR lead to reestablishment of ER homeostasis in coordination with its environmental change via upregulation of ERAD pathway and genes encoding protein folding effectors (Xu et al., 2005). This supplies ER with an extra folding capacity, hopefully envisaging a major protein efflux. Alongside, *de novo* protein synthesis is attenuated, decreasing protein influx into ER (Fig. 1.2).

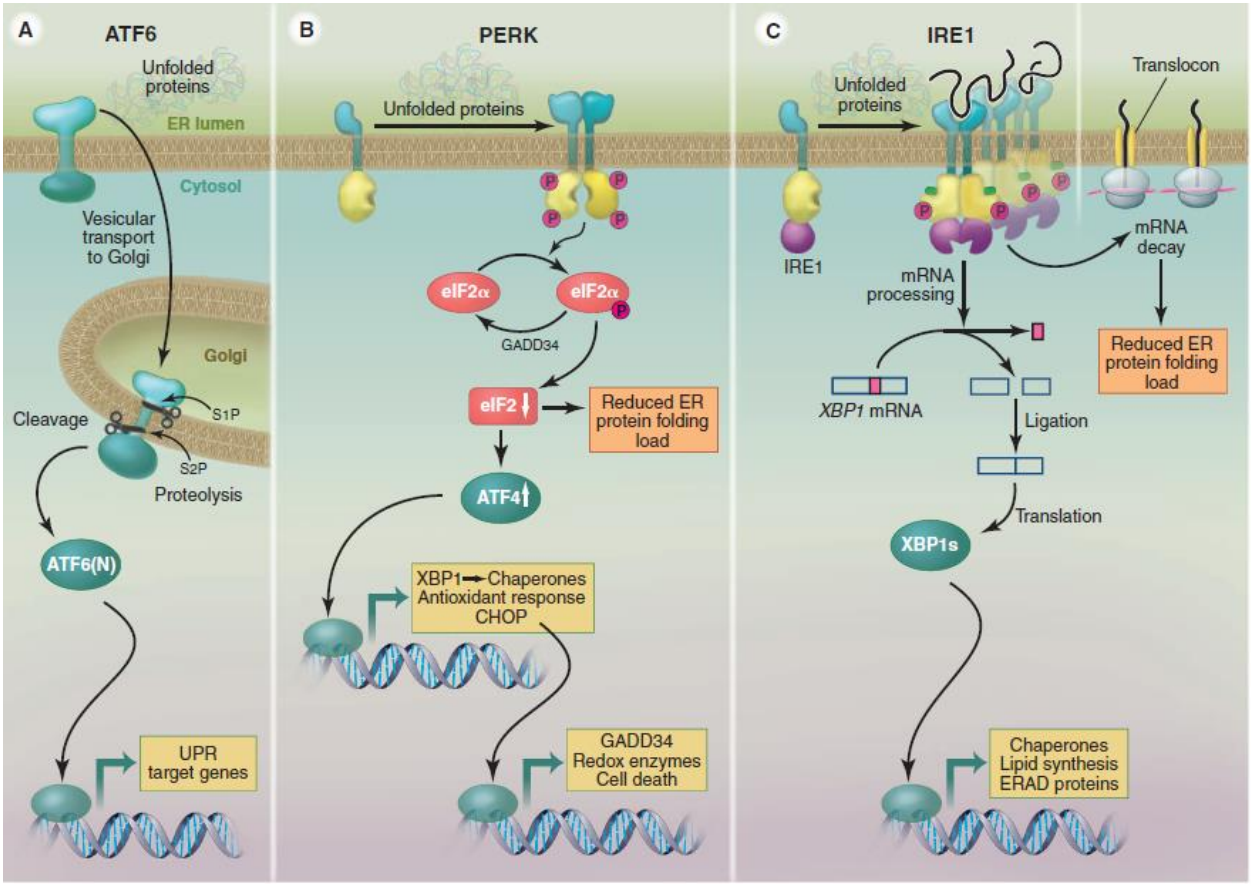


Figure 1.2. ***The three main components of the unfolded protein response.***
Upon GRP78/BiP detachment from the 3 transducers of ER stress, induced by the accumulation of misfolded or unfolded proteins in ER lumen, **(A)** ATF6, **(B)** PERK, and **(C)** IRE-1 become activated. Then, PERK phosphorylates eIF2α to attenuate protein translation and phosphorylates NRF-2 to upregulate an antioxidant response. Cleaved ATF6 translocates to the nucleus and leads to induction of molecular chaperones such as GRP78 or GRP94 to increase ER folding capacity. IRE-1 activation leads to XBP-1 splicing, transcriptional activation of chaperones and stimulation of protein degradation. Adapted from (Walter and Ron, 2011).

b. Alarm stage: after the primary failure of UPR to compensate ER stress, activation of NF-κB occurs to induce host defence genes as the p53 tumour suppressor (Plácido et al., 2014).

c. Apoptosis: at last, UPR resulting from outgrowing ER stress initiates a cell death programed process, usually via apoptosis (Fig. 1.3) (Plácido et al., 2014).

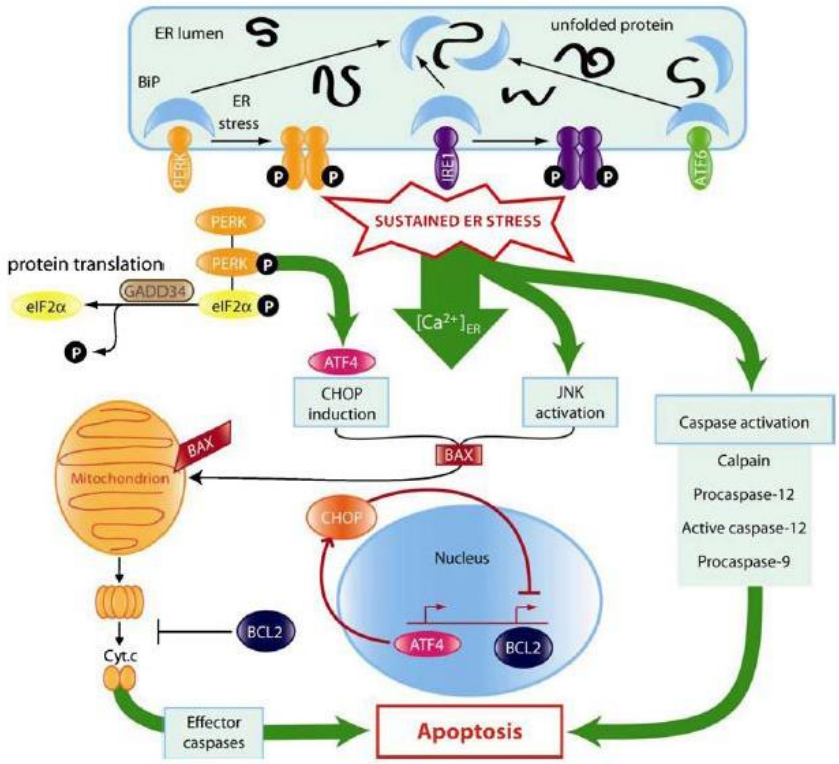


Figure 1.3. ***Sustained ER stress leads to pro-apoptotic signaling.***
Prolonged UPR activation leads to ER Ca^{2+} release and to cell death signaling. Activated IRE-1 acting on downstream factors activates JNK and caspases. ATF4-dependent transcription, induced by activated PERK, leads to augmentation of the pro-apoptotic transcription factor CHOP. CHOP inhibits Bcl-2 leading to Ca^{2+} release; higher Ca^{2+} levels sensitize mitochondria to other insults inducing cell death. Bcl-2 exerts an anti-apoptotic function in the ER. CHOP and JNK also promote the translocation of Bax to the mitochondria where it facilitates the release of cytochrome c required for caspase activation. ER specific caspases, such as the caspase-12, are thought to directly induce cell death through activation of caspases 9 and 3. Adapted from (Naidoo, 2009).

But, what are the main effectors, i. e. the signaling pathways, behind UPR?

Under unstressed conditions, the ER-resident chaperone GRP78, also called binding immunoglobulin protein (BiP), is tightly bounded to the luminal portion of ER resident transmembrane markers: inositol requiring element-1 (IRE-1), protein kinase RNA-like ER kinase (PERK) and activating transcription factor 6 (ATF6), precluding its signaling action (Bertolotti et al., 2000; Naidoo, 2009). GRP78 is considered a valuable ER stress marker (Liu et al., 2010), apart from its importance in protein quality control in ER. It binds to newly synthesized proteins to avoid its aggregation before being properly folded and prevent, in unsuccessful cases, from being transported to Golgi complex and downstream pathways (Kohno et al., 1993).

During short exposures to ER stress, UPR, in an attempt to defend cells from ER stress, upregulates and detaches GRP78 from the three main components of the UPR (again IRE-1, PERK and ATF6), because the available GRP78 are not sufficient for the unfolded/misfolded proteins demand; while promoting the ER stress sensors activation, GRP78 itself associates to the exposed hydrophobic portions of the abnormal proteins (Naidoo, 2009; Yoshida, 2009; Placido et al., 2015). Upon ER transducers activation,

phosphorylation and dimerization of IRE-1 and PERK is allowed, as well as the translocation of ATF6 to the Golgi apparatus. After dimerization, IRE1α is able to process mRNA encoding for X-box binding protein-1 (XBP-1) via a selective mRNA splicing mechanism yielding a more stable and active form, the spliced form, sXBP-1 (Chen and Brandizzi, 2013; Chen et al., 2014). After translocation to the nucleus, it induces the expression of chaperones and other proteins involved in protein folding and/or degradation (e.g. ERAD mediators) (Travers et al., 2000; Lee et al., 2003), and at last, upregulates ER/Golgi biogenesis (Cox et al., 1993; Calfon et al., 2002). The unspliced form, however, represses that gene expression. Nevertheless, IRE1α also degrades other mRNAs encoding proteins of the secretory pathway (regulated IRE1-dependent decay – RIDD) avoiding its translation towards the relief of ER stress (Hollien et al., 2009; Maurel et al., 2014). If the mechanism fails, IRE1α stops the mRNA splicing of XBP-1 (Placido et al., 2015). Secondly, after homodimerization and autophosphorylation of PERK, PERK itself phosphorylates the α-subunit of eukaryotic translation-initiation factor 2α (eIF2α), which results in the attenuation of mRNA translation, and at the same time, protein load into ER. Lastly, ATF6, the third ER stress sensor localized within Golgi apparatus, is cleaved by Site 1 and Site 2 proteases releasing a cytosolic domain. This cleaved ATF6 fragment migrates to the nucleus, where prompts the expression of several molecular chaperones, for instance GRP78 or protein disulfide isomerase (PDI) – reinforcing the folding machinery (Zhang and Kaufman, 2006); amongst others, ATF6 regulates quite interesting genes, such as XBP-1 (synergetic action against ER stress) (Yoshida et al., 2001), intermediaries of ERAD (Adachi et al., 2008), autophagy and organelle biogenesis (Haze et al., 1999; Yoshida et al., 2001; Schröder, 2006; Ron and Walter, 2007b).

Next, the intermediate phase, considered the alarm cell signaling pathway, is intimately linked to the activation of the immune system, namely the downstream effectors of the innate immunity (Xu et al., 2005). Briefly, IRE1α action possesses a dual character. To begin, phosphorylated IRE1α recruits the tumor necrosis factor receptor associated-factor-2 (TRAF2) to the ER membrane, an E3 ligase thereby ubiquitinating proteins via non-canonical lysine 63 and/or activating protein kinases responsible for inflammatory mechanisms (Habelhah et al., 2004). Recently, TRAF2 have been stated to initiate ER stress-induced inflammatory responses via NF-κB signaling pathway (Keestra-Gounder et al., 2016). Some of these inflammatory mediators bring up the action of c-Jun N-terminal kinase (JNK), commonly associated to upstream modulation of cell death (Urano et al., 2000), in collaboration with MAPK (Xu et al., 2005). Then, RIDD mediates mRNAs degradation, but contradicts its purpose, mitigating ER chaperone translation, ultimately setting up apoptotic events by repression of pro-apoptotic pre-miRNAs and reinforcing ER stress (Iwawaki et al., 2009; Chen and Brandizzi, 2013). To what concerns PERK ER stress sensor, it activates nuclear factor erythroid-2 (NRF2) (Harding et al., 2000a, 2003; Hotamisligil, 2010), and again NF-κB transcription factor, rendering the cell a control over redox metabolism and inflammation, respectively (Cullinan and Diehl, 2004a; Xu et al., 2005; Hetz, 2012). Besides IRE1α and PERK modulation, NF-κB is believed to be also controlled by ATF6 owing to the activation of protein kinase B (Akt). However, ATF6 implications in the alarm phase requires more insight (Nakajima et al., 2011; Hetz, 2012; Rao et al., 2014).

Using the previous mechanisms, cells are usually able to manage ER stress and recover normal functioning. But, if after all the adaptive attempts to undo ER stress burden, it was not enough, a state of prolonged or severe ER stress is inflicted. As a consequence, ER stress-induced apoptotic cell signaling is triggered (Kim et al., 2008), consenting the elimination of irreversibly damaged cells. Two major signaling cascades are involved: JNK and caspase pathways. Some transcription factors and Bcl-2 family proteins and modulators also display a role on this matter (Plácido et al., 2015).

Either IRE1α and ATF6 are ER stressors responsible for the caspases cascade activation, using different intermediates (JNK and C/EBP homologous protein – CHOP, respectively) (Schapansky et al., 2007; Pino et al., 2009; Merksamer and Papa, 2010). Thus, the activation of the pro-apoptotic ER cysteine protease caspase-12 functions as an apoptotic platform in cells experiencing ER stress (Hitomi et al., 2004; Cheung et al., 2006; Kim et al., 2009b). Although considered as one of the central mechanisms leading to apoptotic cell death, the mechanism is not yet clarified, and supposedly depends on the stimulus and cell type where it is triggered (Szegezdi et al., 2003). At the time, it is known that caspase-12 activation under ER stress conditions is the result from the conversion of the ER membrane-localized pro-caspase-12 into its cytosolic active form (Morishima et al., 2002). This way, pro-caspase-12 is overtly unavailable for interaction with IRE1α via TRAF2 adaptor (Yoneda et al., 2001). This proteolytic cleavage might be orchestrated by caspase-3/7 or calpain-2 (Xu et al., 2005; Martinez et al., 2010). Some findings report that, once translocated to the cytosol, caspase-12 activates the apoptosis' effector caspase-3, consistent with other reports from caspase-4 (human homologue of caspase-12) that acts in ER stress by enhancing caspase-3 activity too (Yoneda et al., 2001; Szegezdi et al., 2003; Kim et al., 2009b; Plácido et al., 2015). Caspase-12 shares 57% homology to human caspase-4, perhaps supporting the enrollment in apoptosis of both in ER stress (Fischer et al., 2002). Besides, it was described a partial reduction in cell death caused by thapsigargin (TG)-induced ER stress upon caspase-4 knockdown (Nakagawa et al., 2000; Szegezdi et al., 2003). On the contrary, some evidences discarded caspase-4 or -12 from the apoptotic mechanism, stating that only caspase-9 is activated once ER stress takes place in cells (Obeng and Boise, 2005). Furthermore, caspase-12 can also be modulated by other protein quality control mechanisms, as the ubiquitin proteasome system (UPS) (Song et al., 2008). Another caspase activator is BAP31 (first cited as MAMs regulator). It is an ER membrane protein that upon proteolytic cleavage by caspase-8 is active and propagates the apoptotic signal between ER and mitochondrial. This interplay is suited by the ER Ca^{2+} to mitochondria transient transfer-induced BAP31, where its accumulation triggers cytochrome c release and the activation of caspase cascade (Nguyen et al., 2000; Breckenridge et al., 2002, 2003; Plácido et al., 2015). Overlooking IRE1α downstream pathway, we also have to mention the correlation of JNK and apoptosis. Once IRE1-TRF2 complex triggers JNK phosphorylation, activated JNK promotes the translocation of Bax to mitochondria, releasing pro-apoptotic factors crucial for caspase cascade activation (Naidoo, 2009), e.g. pro-apoptotic protein Bim, now activated by JNK, and ultimately, it inhibits the anti-apoptotic Bcl-2 (Yamamoto et al., 1999; Lei and Davis, 2003; Putcha et al., 2003).

The transcription factor CHOP, also known as growth arrest and DNA damage 153 gene (GADD153), is regulated by ATF6, as aforementioned. At the same time, CHOP transcription is dependent on ATF4, upregulated via downstream effectors of PERK activation (Naidoo, 2009; Jiang et al., 2016), and XBP-1 (Harding et al., 2000b; Scheuner et al., 2001; Oyadomari and Mori, 2004). Additionally to CHOP activation, an antioxidant response is triggered via upregulation of ER oxidoreductin (ERO1α) (Harding et al., 2000a, 2003; Hotamisligil, 2010), leading to an unbalanced redox homeostasis. Thus, ER lumen becomes more oxidized due to excessive production of oxidant species, depletion of the antioxidant glutathione (GSH), and plethora release of Ca^{2+} from ER stores via IP_3R. This Ca^{2+} mobilization is a consequence of the downregulation of anti-apoptotic proteins (e.g. Bcl-2) mediated by CHOP activation (Oyadomari and Mori, 2004; Schröder and Kaufman, 2005) balanced with the upregulation of pro-apoptotic Bax and Bcl-2-homologous antagonist/killer (Bak) (Yamamoto et al., 1999; Nishitoh et al., 2002; Lei and Davis, 2003; Putcha et al., 2003). The roles of Bcl-2 family member in Ca^{2+} dyshomeostasis will be clarified below.

In parallel with UPR, and in order to combat the burden of misfolded proteins, macroautophagy system is activated as a last effort to relief cellular stress and to avoid the occurrence of apoptotic mechanism. By that, ER portions are targeted to the endocytic-lysosomal pathway (Kincaid and Cooper, 2007; Heath-Engel et al., 2008). The main events of macroautophagy are the autophagosome formation and maturation, substantially maintained by LC3II, after its conversion from LC3I (Lipatova and Segev, 2015). To sustain autophagic flux under ER stress, LC3II transcription is upregulated by eIF2α, as well as other autophagic genes (Kouroku et al., 2007). Alternatively, ATF4 promotes autophagic cell death, despite the stimulation of LC3 conversion (Milani et al., 2009; Hart et al., 2012; Moon et al., 2016). Thus, PERK appears to be the most promising branch of UPR in what concerns autophagy, but attention must be paid regarding its cytoprotective/cytotoxic role during ER stress. Nevertheless, cytoprotective action is usually predominant during ER stress conditions. Thus, the intensity of ER stress may be a deciding factor over the contradictory PERK pathway (Khaminets et al., 2015). In this context, ATF6 is the least characterized ER stress sensor. It downregulates Akt/mTOR pathway, decreasing both activities, and inducing autophagy (Yamazaki et al., 2009). However, recoverable ER stress activates Akt and MAPK cascades, leading to cell survival. Under this conditions, it is plausible that autophagy is negatively regulated (Hu et al., 2004). Regarding AMPK-dependent autophagy, AMPK is upregulated by ATF4 and by the depletion of ER Ca^{2+} stores that binds to CaMKK, an AMPK activator (Han et al., 2013). Lastly, XBP1 could suppress autophagy by clearing the aggregated protein through the UPS (Arsham and Neufeld, 2009). Overall, ER stress serves dual roles by favoring mechanisms of both autophagy induction and inhibition. During physiological ER stress, autophagy serves as an adaptive stress response that helps to sustain cell survival, whereas pathological ER stress can lead to the inhibition of autophagy (Khaminets et al., 2015).

Altogether, ER might deal with different kinds of stress upon activation of processes like UPR (Yoneda et al., 2001; Yoshida, 2009), EOR (Pahl and Baeuerle, 1997), ERAD (Travers et al., 2000) or even macroautophagy (Høyer-Hansen and Jäättelä, 2007) with a simple purpose: to regenerate ER, and subsequently, cellular homeostasis, potentiating survival. Under excessive ER stress conditions, all the

surveillance cascades fail and cell death is activated in order to eliminate damaged cells. This could contribute to several disorders, including cancer, diabetes, cardiovascular and neurodegenerative pathologies (Kim et al., 2008).

1.3.1 Endoplasmic reticulum stress side-effects: disturbance of ER Ca^{2+} and mitochondria homeostasis

During this chapter was denoted the essential importance of Ca^{2+} ion, given its impact in nearly every aspect of cellular life (Clapham, 2007). Within the ER Ca^{2+} is often associated to Ca^{2+}-binding proteins (Koch, 1990; Meldolesi and Pozzan, 1998; Michalak et al., 2002). Thereafter, loss of Ca^{2+} balance leads to the activation of Ca^{2+}-dependent pathological mechanisms, which end up in cell malfunctioning. Moreover, ER "crime partner", the mitochondrium, comes into play. In detail, ER stress disrupts ER Ca^{2+} homeostasis, which, in turn, is taken up by mitochondria that consequently releases pro-apoptotic factors, namely cytochrome c, and ultimately programmed cell death is triggered (Chami et al., 2008; Deniaud et al., 2008; Costa et al., 2010; González et al., 2010). Other common hallmarks of ER stress-induced apoptosis are Bax/Bak translocation and oligomerization in the outer mitochondrial membrane, loss of mitochondrial membrane potential and caspase-9 activation (Arduino et al., 2009). Besides its role in the control of apoptotic pathway during ER stress, members of Bcl-2 class (Bcl-2 and Bax) regulate ER Ca^{2+} homeostasis, since they are primarily integrated onto ER membrane (Szegezdi et al., 2009), but also modulate Ca^{2+} content in mitochondria (Foyouzi-Youssefi et al., 2000). Overexpressed Bax/Bak pro-apoptotic proteins favor sustained Ca^{2+} transmission from ER to mitochondria, causing cell death (Nutt et al., 2002; Scorrano et al., 2003). Apoptosis might also result from simultaneous Bax- and Bak-mediated modulation of IP_3R activity, hampering its interaction with Bcl-xL, and amplifying Ca^{2+} ions ER release (Chen et al., 2004; Oakes et al., 2005; Kaufman and Malhotra, 2014).

Of special importance, IP_3R additional modulation can result from: phosphorylation by Akt (desensitizes cells to the apoptotic stimuli, because the flux of ER-to-mitochondria Ca^{2+} is lessened) (Szado et al., 2008) and binding to released cytochrome c (pumps Ca^{2+} from ER) (Boehning et al., 2003). Binding of cytochrome c-IP_3R has a positive feedback, since ER Ca^{2+} efflux instigates the extrusion of more cytochrome c molecules from mitochondria, given rise to a stronger death signal (Boehning et al., 2004).Not-least important, this ER receptor belongs to the tripartite system inside MAMs, where Ca^{2+} signal is propagated under the control of VDAC (Weisthal et al., 2014), but also by the opening of permeability transition pore induced by IP_3, whereby cytochrome c escapes from mitochondria (Pan et al., 2013).

Moreover, large amounts of Ca^{2+} released by IP_3R and/or RyRs can, then, trespass through permeability transition pore to mitochondria (Szalai et al., 1999), activate calpains ensuing the cleavage of several pro-apoptotic mediators (Bax and Bid) (Wood et al., 1998; Chen et al., 2001; Huang and Wang, 2001), disrupt membrane biology due to interaction with phospholipids (Esposti, 2002b; McMillin and Dowhan, 2002; Xu et al., 2005), mediate mitochondria fission (Giacomotto et al., 2013), and worsen oxidative stress by

activation of NOX (Orrenius et al., 2003; Xu et al., 2005). Oxidative stress can also trigger apoptosis, ensuring the importance of the dynamic between ER and mitochondria (Csordás and Hajnóczky, 2009; Fonseca et al., 2014a). ROS can promote the accumulation of misfolded proteins within the ER (He et al., 2008; Yan et al., 2008), hampering the activity of certain chaperones, maybe because many ER proteins responsible for Ca^{2+} balance are susceptible to oxidants, thus leading to the perturbation of ER Ca^{2+} (Huang et al., 2004; Nuss et al., 2008; Fonseca et al., 2014a). To sum up, there must be an equilibrium between Ca^{2+} flux between ER and mitochondria, otherwise life and cell death will be questioned.

1.3.2 Link between the unfolded protein response and mitochondrial dysfunction

Mitochondria is a central, and yet, liable organelle involved in cell metabolism under physiological conditions. Given the close cooperation between ER and mitochondria, it is fair to conclude that mitochondria is quite susceptible to ER stress (Bravo et al., 2011, 2012). At first, this organelle functions as a deposit of resources from ER, to promote its relief from stressful conditions. During the UPR adaptive stage, mitochondrial Ca^{2+} content, as a second messenger, take some action in metabolic pathways, specifically activates dehydrogenases found in the Krebs cycle, via allosteric regulation (Cullinan and Diehl, 2004b; Plácido et al., 2014). As a consequence, the electron overflow across respiratory chain increases ATP synthesis. However, when ER stress is too severe, mitochondria is overwhelmed by the uptake of Ca^{2+}, which slows down aerobic respiration, as well as ATP levels, disruption of mitochondrial structure by fragmentation, insertion in the mitochondrial membrane of the permeability transition pore, and subsequent apoptosis (Fig. 1.4). Other evidences imply the impairment of mitochondrial biogenesis and transcription of subunits of the electron transport chain (Cullinan and Diehl, 2004b; Plácido et al., 2014). A causal effect may be due to a defective PERK pathway. Once this cascade is deregulated, both mitochondrial DNA and the activity of respiratory chain are compromised. Therefore, a defective respiratory chain leads to ROS generation (Cullinan and Diehl, 2004b; Plácido et al., 2014).

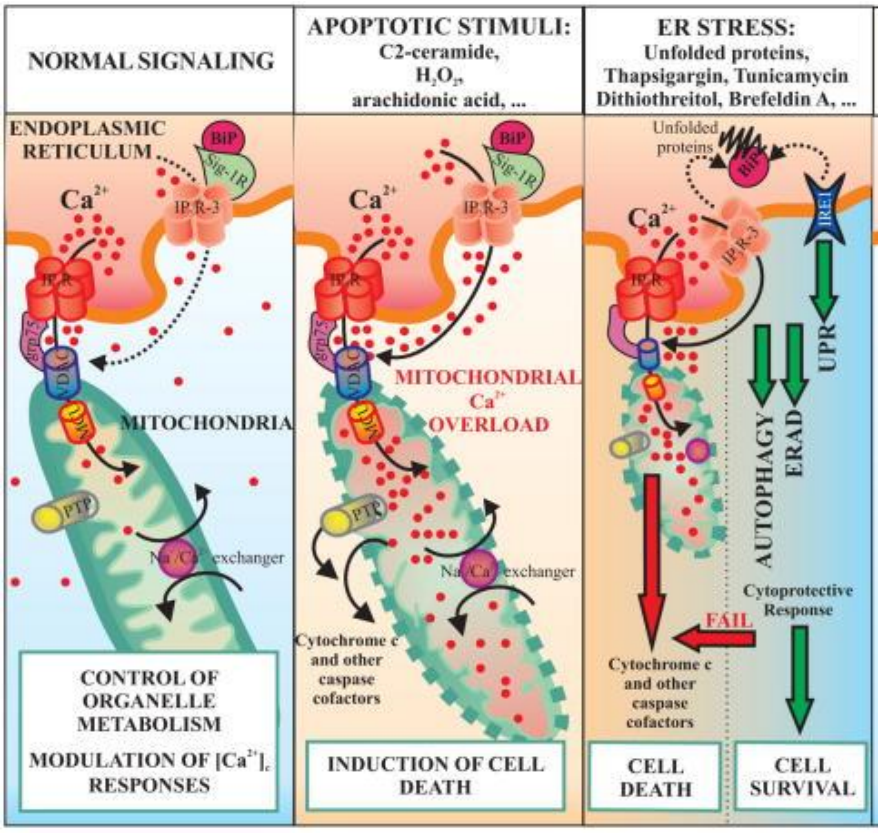


Figure 1.4. ***Calcium modulation of ER-mitochondria crosstalk before and upon chronic ER stress.***
ER-mitochondria cross-talk modulates Ca^{2+}-mediated cellular responses to different patho-physiological stimuli: in normal conditions Ca^{2+} released from the ER to mitochondria triggers a boost in cellular metabolism and ATP production. However,

mitochondrial Ca^{2+} overload induced by apoptotic stimuli or ER stressors sensitizes mitochondria to release caspases cofactors. Adapted from (Rizzuto et al., 2009).

From the three UPR modulators, PERK is the one that seemingly regulates mitochondria, at the morphological and the functional level. In fact, PERK interacts with mitofusin 2, and both mitochondria and mitofusin 2 might be correlated (Muñoz et al., 2013). Findings pointed out the reduction of ER-mitochondria contact in the absence of PERK, whether its overexpression restores these contact sites, and consequently the stabilization of MAM (Bender et al., 2010; Verfaillie et al., 2012). Thus, PERK might reside at MAM complex and exerts a structural action, regardless its canonical pathway via eIF2α (Zhang et al., 1998; Rainbolt et al., 2014). PERK also protects mitochondria from ROS during ER stress, stabilizing mitochondrial proteostasis by upregulation of quality control proteases (Bota and Davies, 2002; Bender et al., 2010) and chaperones (e.g. heat shock protein A 9 – HSPA9) (Chacinska et al., 2009). Moreover, PERK indirectly abrogates damaged mitochondrial proteins, products of oxidation mechanisms (Hori et al., 2002; Ngo and Davies, 2009; Bender et al., 2010), and via HSPA9, it accomplishes protein refolding and importation of newly synthesized protein into mitochondrial matrix (Iosefson et al., 2012; Rainbolt et al., 2014). So PERK can be a therapeutic target (Li et al., 2016).

From the previous mechanism recently emerge a novel pathway, called the mitochondrial UPR, as a transcriptional response to the accumulation of unfolded protein in the mitochondria (Lin and Haynes, 2016). Although the existing information about this subject is sparse, mitochondrial UPR underlies metabolic adaptation and chromatin reorganization by cells (Tian et al., 2016), possibly through ATF5-mediated UPR, proved to maintain mitochondrial activity during mitochondrial stress and promote organelle recovery (Fiorese et al., 2016).

1.4 Endothelial dysfunction – the novel therapeutic holy grail

Endothelial cells (ECs) are spread throughout the whole body systems. Vascular ECs have the ability to regulate vascular tone and coagulation, control the exchange of fluids and nutrients between blood and tissues, participation in immune and inflammatory responses, and control blood flow, as well as, vascular cell growth (Pober et al., 2009; Lenna et al., 2014). Among several molecules, ECs secrete growth factors such as vascular endothelial growth factor (VEGF), which promotes survival, migration, vasculogenesis and angiogenesis (Milkiewicz et al., 2006), coagulation and inflammation effectors, proteases and vasoconstrictor/dilator mediators that influences nearby cells (Grammas et al., 2011).Specifically, the cerebrovasculature is composed by a monolayer of brain ECs, part of BBB constitution. ECs accomplishes the barrier function of BBB, restricting paracellular transport, because neighbouring ECs are connected via tight junctions, or transcellular transport (Jia et al., 2013; Aman et al., 2016). On the surroundings, pericytes and astrocytic foot processes lie closest to ECs to maintain the integrity of BBB, being an extra safety

mechanism for the interaction of blood and brain interstice. This way, the permeability of BBB is conditioned by ECs alongside to pericytes and glial cells, that together with the basement membrane, confers biochemical support and form the neurovascular unit (Cardoso et al., 2010). The endothelium in the brain is highly specialized and the EC carries a large amount of mitochondria (Grammas et al., 2011; Fonseca et al., 2014b). Regarding cardiac endothelium, recent studies have elucidated the critical role of ECs in the support of cardiomyocytes survival and myocardial contraction. After stress stimulus, cardiomyocytes affect ECs, promoting angiogenesis (Kuruvilla and Kartha, 2003; Wan and Rodrigues, 2016). Comparing to vascular contraction, angiogenesis is also a balanced mechanism between pro- and anti-angiogenic factors from different signaling pathways, and it is controlled by a number of growth factors (Carmeliet and Jain, 2011).

The principal cause of endothelial dysfunction is cell injury, most often result from the endothelium-dependent vasodilation through a decrease in eNOS activity (Pober et al., 2009). In parallel, reduced anticoagulant properties, increased adhesion molecules expression, chemokine and cytokine release and ROS production by the endothelium are also part of this dysfunction (Kuruvilla and Kartha, 2003; Ferreiro et al., 2012). The last few years, several pathologies have been associated to the impairment of endothelium, including stroke, neurodegenerative disorders, hyperhomocysteinemia, hyperlipidemia, hyperglycemia and chronic inflammation, that induce endothelial dysfunction promoting an amplified ER stress response (Paschen and Frandsen, 2001; Zipser et al., 2007; Cimellaro et al., 2016). As referred above, some reports attributes ECs dysfunction to the inefficient response over vasodilator/constrictor molecules balance (Esper et al., 2006). However, a lot of causes are yet unravelled, but it is certain that stress, either oxidative, ER or metabolic, is among its foundation (Pober et al., 2009).

Although the oxidative stress mechanism extends to other types of ECs (Pober et al., 2009; Lenna et al., 2014), in the brain, the oxidative burden has been broadly studied. This might be explained by the fact that brain oxygen consumption and the amount of mitochondria in these areas are indicators of a presumable formation of ROS. As a consequence, NO signaling is hindered, automatically endothelial-induced vasodilation tilts over vasoconstriction (Dawson and Snyder, 1994; Shulman et al., 2004; Grammas et al., 2011). NO is an important cell messenger, clearly impacting vascular relaxation, but due to its nature, it is also a source of reactive nitrogen species (RNS). The attenuation of NO levels is caused by the formation of peroxynitrite (major mediator of endothelial dysfunction) from an excess of ROS, that indirectly oxidizes eNOS. On the other hand, the presence of mitochondria plus the neglected leakage of electrons from the respiratory chain underlies ROS formation that begins with the assembly of superoxide anion by reduction of oxygen or also obtained from electron transfer chain, in small amounts. Due to its reactivity, superoxide is converted to hydrogen peroxide (H_2O_2), stabilizing it to a certain point. Either cytosolic and mitochondrial superoxide dismutases play this role (Fridovich, 1998; Zhang et al., 1998). Next, the enzymes catalase and glutathione peroxidase reacts with H_2O_2 obtaining water and diatomic oxygen, or the hydroxyl radical via Fenton reactions (Kowaltowski et al., 2009). In addition, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) provides ROS to ECs' environment, whether its cofactor NADPH is the major

substrate for vascular enzymes, resulting in cell proliferation and survival, apoptosis or inflammation (Griendling et al., 2000). Taken up of xanthine oxidase from plasma could also result in ROS generation (Cai and Harrison, 2000). ROS also target DNA molecules, breaking it and activating the DNA repair enzyme poly-ADP-ribose-polymerase (PARP), and endothelial enzymes such as cyclooxygenase (COX - promotes vascular constriction, and recently COX-2 was discovered as the main source of intravascular ROS generation) (Virdis and Taddei, 2016), mediates lipid peroxidation and protein nitration via peroxynitrite, and ultimately might triggers apoptotic cell death (Aliev et al., 2014). To face these threats, ECs antioxidant defences are reinforced, that's the reason why high levels of GSH, glutathione reductase and peroxidase and catalase are present in ECs (Tayarani et al., 1987; Fridovich, 1998; Freeman and Keller, 2012; Larson et al., 2012).

1.4.1 Endoplasmic reticulum and cell death in endothelial dysfunction

Injuries or cell demise, consequences of unbearable inflammation, excitotoxicity, ischemia, are in the origin of diverse pathologies. According to the ability of ECs to antagonize adverse conditions, and given the wide range of cell signaling pathways, multiple mechanisms of cell death can occur, for example, necrosis, caspase-independent apoptosis or ER-mediated apoptosis, previously revised (Chan, 2001; Rizzo and Leaver, 2010; O'Carroll et al., 2015). Some studies implicate p38-MAPK, ERK and JNK cascades in apoptosis, more specifically acting as pro-apoptotic factors, by phosphorylation of Bcl-2 family members, activating transcription factors with pro-apoptotic character and modulating cell death receptors (Yamamoto et al., 1999; Tournier et al., 2000; Deng et al., 2003). At the same time, these kinases also regulate ROS-dependent cell death via JNK and p38-MAPK activation (Deng et al., 2003; Hsu et al., 2007; Correia et al., 2012; Plácido et al., 2015). During ER stress, p38-MAPK downstream effectors are enhanced (Galán et al., 2014).

ER stress is a causal mechanism for ROS production in ECs, since during stressful conditions NOX is upregulated, namely NOX4 isoform (Pedruzzi et al., 2004; Radermacher et al., 2013). Moreover, due to the close cooperation between ER and mitochondria, ER stress is a source of ROS via mitochondrial electron transfer system. In fact, a study showed that the blockage of ER-mitochondria crosstalk induces endothelial dysfunction (Sutendra et al., 2011). As referred previously, MAMs are well-defined structures that mediate Ca^{2+} fluxes, including Ca^{2+} transport to mitochondria or cytosol. Hence, Ca^{2+} is also a causal mechanism that leads to ROS production in mitochondria, and an impairment in its trafficking leads to ECs dysfunction (Liu and Hajnóczky, 2009; Kluge et al., 2013), perhaps consequence of ROS formation and vasodilation, as highlighted in a coronary artery disease study (Bubolz et al., 2012). Also, under ER oxidizing conditions ERO1α localizes into MAM, whether the reduction in ER environment promotes its detachment from there, regardless ER stress-dependent mechanisms (Gilady et al., 2010). However, ERO1α mediates ER stress responses, where it devises Ca^{2+} efflux from ER to mitochondria, increasing ROS and, likelihood apoptosis

(Seervi et al., 2013). So, it is clear the influence of ER-mitochondria partnership in ECs dysfunction, still more evidences are needed.

Although moderated oxidative stress is required in the physiological context, ECs dysfunction may be triggered by its deregulation (Higa and Chevet, 2012). Regarding ROS production, another consequence from ER stress results from the attempt to overcome this injury by UPR (Cimellaro et al., 2016). Herein, the chaperone PDI from adaptive stage and ERO1α resultant from the alarming phase are the major players (Santos et al., 2009; Kim et al., 2012; Radermacher et al., 2013). During protein quality control, there is the formation of disulfide bonds, secured by PDI and ERO1α, where ROS are the byproduct of the transfer of electrons from the protein thiol group to molecular oxygen. Besides, cells have to deal with ROS accumulation given GSH depletion (Tu et al., 2000; Tu and Weissman, 2002; Xiao et al., 2004; Kim et al., 2009a).

As already revised, ER Ca^{2+} leakage to cytosol can occur during UPR, in this case, upon the high ER loading of unfolded proteins that leads to ROS generation. Again, we cannot discard the crucial role of mitochondria, which provides energetic substrates to cells. Prolonged UPR activation leads to ATP depletion. By increasing ATP synthesis via oxidative phosphorylation, demanded for folding/refolding in ER lumen, mitochondria forms more ROS as a side-effect (Malhotra and Kaufman, 2007; Kaufman and Malhotra, 2014). Altogether, ROS might drive endothelial dysfunction, independently from its source. Nonetheless, ER stress has been associated to ECs, oxidative stress and vascular disorders. Thus, if therapeutic approaches towards ER stress are developed, maybe ECs dysfunction might be targeted and minimized.

CHAPTER 2 – Objectives

Endothelial dysfunction pops up as a potential contributor in the pathogenesis of neurodegenerative and cardiovascular disorders, worldwide spread and representing the majors causes of death and economic burden. ER stress is further entitled as the main causative factor in several of these sets of diseases, probably due to the vascular failure component. Considerable progress has been made in recent years towards better understanding ER stress causes and consequences, for as much as putative stressors-modifying agents. Moreover, the orexigenic hormone GHR becomes a suitable approach, given the earlier findings about how remarkably GHR ameliorated ER stress, mostly its mediated apoptotic pathway, and oxidation in different cellular models. Thus, GHR might be an effective and protective tool against ER stress-induced endothelial dysfunction.

Therefore, this study was designed to investigate whether GHR attenuates ER stress in cardiac and brain ECs, focusing on molecular targets that underly the ER stress-induced loss of endothelial proteostasis. Specifically, it was brought to question: (1) the morphological and functional consequences of ER stress, (2) the Ca^{2+} and redox dysfunction, (3) the ER stress downstream apoptotic cascade and (4) macroautophagy and exosomes release as ER stress-induced UPR consequences. To appraise these purposes, different endothelial cell lines were used, namely cardiac (MCEC-1) and brain endothelial cells (RBE4), and thapsigargin and tunicamycin were used as ER stressors.

MCEC-1 and RBE4 endothelial cells allowed us to discriminate the role of GHR during stressful conditions in ECs angiogenic response, using different complementary approaches such as migration and tube-like formation, and the protective effect of GHR against ER stress-induced toxicity.

To further strength the potential role of GHR to restore proteostasis, mitochondrial Ca^{2+} and ROS dyshomeostasis in parallel to ER-mitochondria crosstalk were accessed in RBE4 cells. Additionally, to clarify the recovery from apoptotic cell death pathway by GHR, putting the focus on ER stress-induced caspase cascade, the different stages of ER UPR signaling were studied, giving emphasis to the chaperone GRP78 and two downstream effectors of the PERK and IRE1α signal transduction. To gain additional insights into protein quality control in the cytosol, macroautophagy induction was monitored in RBE4 cells challenged by GHR under ER stress conditions. Finally, GHR implication on the cell-to-cell communication, accomplished by the release of exosomes, was ultimately investigated as a novel strategy to eliminate unfolded/misfolded proteins from the overloaded ER. Lastly, all these aims were tested during moderate and acute states of ER dysfunction, in an attempt to establish a relationship between the severity of ER stress and the protective action of GHR.

From this Master's work, we expect to gather more intel from the "friendly" GHR's means of action to fight ER stress and subsequently endothelial dysfunction.

CHAPTER 3 – Materials and Methods

3.1 Materials

Table 3.1.1. Detailed description of the materials used in this study and the suppliers.

Antibodies (Ab)	
Goat alkaline phosphatase-linked IgG anti-rabbit secondary Ab	Amersham Pharmacia Biotech (Buckinghamshire, UK)
Goat alkaline phosphatase-linked IgG + IgM anti-mouse secondary Ab	Amersham Pharmacia Biotech (Buckinghamshire, UK)
Goat polyclonal anti-sigma 1 receptor (Sig1R)	Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA)
Mouse monoclonal IgG1 anti-β-tubulin	Sigma Chemical Co. (St. Louis, MO, USA)
Mouse monoclonal IgG2a anti-CHOP	Cell Signaling Technology, Inc. (Danvers, MA, USA)
Mouse monoclonal IgG2a anti-GRP78	BD Biosciences (Heiderlberg, Germany)
Mouse monoclonal IgG2b,k anti-ERO1A/ERO1L	LifeSpan BioSciences, Inc. (Seattle, WA, USA)
Rabbit alkaline phosphatase-conjugated IgG anti-goat secondary Ab	Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA)
Rabbit monoclonal IgG anti-eIF2α	Cell Signaling Technology, Inc. (Danvers, MA, USA)
Rabbit monoclonal IgG anti-phospho-eIF2α	Merck KGaA (Darmstadt, Germany)
Rabbit polyclonal anti-caspase-12	Cell Signaling Technology, Inc. (Danvers, MA, USA)
Rabbit polyclonal anti-LC3B	Cell Signaling Technology, Inc. (Danvers, MA, USA)
Rabbit polyclonal IgG anti-VDAC	Abcam plc (Cambridge, UK)
Rabbit polyclonal IgG anti-XBP-1	Abcam plc (Cambridge, UK)
Cellular lines	
Murine Cardiac Endothelial Cells (MCEC-1)	A thoughtful gift from Dr. Henrique Girão (Faculty of Medicine, Coimbra, Portugal)
Rat Brain Endothelial cell line (RBE4)	Gently provided by Dr. Paula Moreira (Faculty of Medicine, Coimbra, Portugal)

Cell culture reagents

RBE4:

Collagen	Trypsin-EDTA solution Roche Applied Science (Mannheim, Germany)
F-10 Nut Mix with GlutaMAX-1	GIBCO BRL, Life Technologies (Paisley, UK)
Fetal Bovine Serum (FBS)	Invitrogen Life Science (Paisley, UK)
Geneticin (G418)	GIBCO BRL, Life Technologies (Paisley, UK)
MEM Alpha Medium with GlutaMAX-1	GIBCO BRL, Life Technologies (Paisley, UK)
Recombinant human basic Fibroblast Growth Factor (bFGF)	Sigma Chemical Co. (St. Louis, MO, USA)

MCEC-1:

Dulbecco's Modified Eagle's Medium (DMEM)	Life Technologies (Carlsbad, CA, USA)
Endothelial Cell Growth Factor (ECGF)	Sigma Chemical Co. (St. Louis, MO, USA)
Fetal Bovine Serum (FBS)	Life Technologies (Carlsbad, CA, USA)
Gelatin	Sigma Chemical Co. (St. Louis, MO, USA)
GlutaMAX	Life Technologies (Carlsbad, CA, USA)
Heparin	Invitrogen (Carlsbad, CA, USA)
Penicillin/Streptomycin	Life Technologies (Carlsbad, CA, USA)

Enzyme inhibitors

Phenylmethylsulfonyl fluoride (PMSF)	Sigma Chemical Co. (St. Louis, MO, USA)
Protease inhibitor cocktail (leupeptin, pepstatin A, chymostatin and antipain)	Sigma Chemical Co. (St. Louis, MO, USA)
Thapsigargin	Sigma Chemical Co. (St. Louis, MO, USA)
Tunicamycin	Sigma Chemical Co. (St. Louis, MO, USA)

Fluorescent dyes

Amplex Red	Molecular Probes (Eugene, Oregon, USA)
Rhodamine-2 acetoxymethyl ester (Rhod-2/AM)	Molecular Probes (Eugene, Oregon, USA)

Peptides

Ghrelin (rat)	Sigma Chemical Co. (St. Louis, MO, USA

Western blotting reagents and material

30% Acrylamide/Bis solution	Bio-Rad (Hercules, CA, USA)
Ammonium Persulfate (APS)	Sigma Chemical Co. (St. Louis, MO, USA)
Bromophenol Blue	Sigma Chemical Co. (St. Louis, MO, USA)
Enhanced ChemiFluorescence (ECF) reagent	Amersham Pharmacia Biotech (Buckinghamshire, UK)
Glycine	Merck KGaA (Darmstadt, Germany)
N,N,N',N'-Tetramethyl-ethylenediamine (TEMED)	Sigma Chemical Co. (St. Louis, MO, USA)
Polyvinylidene difluoride (PVDF) membrane	Millipore (Billerica, MA, USA)
Precision Plus Protein™ All Blue Prestained Protein Standards	Bio-Rad (Hercules, CA, USA)
Tween 20	Sigma Chemical Co. (St. Louis, MO, USA)

Other chemicals

1,4-dithiothreitol (DTT)	Sigma Chemical Co.
2-Propanol	Merck KGaA
4-(2-hydroxyethyl)-1-piperazoneethanesulfonic acid (HEPES)	Sigma Chemical Co.
A23187	Sigma Chemical Co.
Acetic acid (glacial) 100%	Merck KGaA
BCA Protein Assay Reagent	Thermo Scientific, Pierce Protein Research Product
Bovine Serum Albumin (BSA)	Merck KGaA
Calcium chloride ($CaCl_2$)	Merck KGaA
Dimethyl sulfoxide (DMSO)	Sigma Chemical Co.
Glucose ($C_6H_{12}O_6$)	Sigma Chemical Co.
Growth factor reduced Matrigel	BD Bioscience
Horseradish Peroxidase (HRP)	Sigma Chemical Co.
Hydrochloric acid (HCl)	Merck KGaA
Magnesium chloride hexahydrate ($MgCl_2$)	Merck KGaA
Methanol	VWR International, LI
Paraformaldehyde	Sigma Chemical Co.
Potassium chloride (KCl)	Merck KGaA
Resazurin, sodium salt	Sigma Chemical Co.
Sodium chloride (NaCl)	Calbiochem
Sodium deoxycholate (DOC)	Sigma Chemical Co.
Sodium dodecyl sulphate (SDS)	Amre
Sodium hydroxide (NaOH)	Merck KGaA
Sodium phosphate monobasic Monohydrate (NaH_2PO_4)	Sigma Chemical Co.
Thiazolyl Blue Tetrazolium Bromide (MTT)	Sigma Chemical Co.
Tris	Calbiochem

Table 3.1.2. Information regarding the source, host species, molecular weight and dilutions of the primary and secondary antibodies used for Western blot analysis.

Antibodies	Catalog number	Host specie	Molecular weight (kDa)	Dilution
Primary Antibodies				
Anti-β-tubulin (anti-C-terminal)	Sigma T7816	Mouse monoclonal	55	1:5000
Anti-caspase-12 (cleaved and full-length)	Cell Signaling #2202	Rabbit polyclonal	42; 55	1:1000
Anti-CHOP	Cell Signaling #2895	Mouse monoclonal	30	1:500
Anti-eIF2α	Cell Signaling #5324	Rabbit monoclonal	38	1:1000
Anti-ERO1A/ERO1L	LS-C133740	Mouse monoclonal	65	1:1000
Anti-GRP78	BD610979	Mouse monoclonal	78	1:1000
Anti-LC3B (LC3BII; LC3BI)	Cell Signaling #2775	Rabbit polyclonal	16; 18	1:1000
Anti-phospho-eIF2α (Ser51)	Milipore 04-342	Rabbit monoclonal	38	1:1000
Anti-sigma 1 receptor	sc-22948	Goat polyclonal	25	1:750
Anti-VDAC	ab34726	Rabbit polyclonal	32	1:750
Anti-XBP-1 (non-spliced and spliced)	ab37152	Rabbit polyclonal	33; 54	1:1000
Secondary Antibodies				
Anti-goat	sc-2771	Rabbit	-	1:5000
Anti-mouse	RPN5781	Goat	-	1:20 000
Anti-rabbit	RPN5784	Goat	-	1:20 000

3.2 Experimental models and treatment

3.2.1 Murine Cardiac Endothelial Cells (MCEC-1)

The cardiac endothelial cell line from murine heart microvasculature was provided by Dr. Henrique Girão (University of Coimbra, Portugal). MCEC-1 cells are conditionally immortalized, mimicking the cardiovascular endothelium, although endothelial cells (ECs) from the endocardium and coronary vessels may be displayed. Thus, it is likely to be a heterogeneous cell line. MCEC-1 cells were plated at a density of 3 x 10^5 cells/well for cell viability and wound healing assays (12-well plates). These cells were passaged approximately 10-30 times, and maintained on 75 cm^2 tissue culture flasks coated with 1% (w/v) gelatin in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) Fetal Bovine Serum (FBS), penicillin (100 U/mL), streptomycin (100 µg/mL), heparin (10 U/mL), 1% GlutaMAX and 75 mg/L Endothelial Cell Growth Factor (ECGF). Cells were initially expanded at 33 ºC in a humidified atmosphere of 95% air and 5% CO_2 (Lidington et al., 2002). During experimentation, cell cultures were switched to 37 ºC under the same atmospheric conditions.

3.2.2 Rat Brain Endothelial Cells (RBE4)

The RBE4 cell line was kindly supplied by Dr. Paula Moreira (University of Coimbra, Portugal), however it was firstly obtained on Dr. Jon Holy's laboratory (University of Minnesota, Duluth, USA). This continuous and immortalized cell line is commonly retrieved from the rat brain vasculature, being established as a model for BBB endothelium given its ability to retain in culture a non-transformed endothelial phenotype (Roux et al., 1994; Couraud et al., 2003; Roux and Couraud, 2005). Cells were plated onto 100 mm petri dishes at a density of 6.6 x 10^5 cells/dish for WB, 12-well plates at a density of 3.0 x 10^5 cells/well for the scratch assay or 24-well plates at a density of 2.0 x 10^4 cells/well for all other protocols. RBE4 ECs are routinely used up to passage 35 and cultured onto 4.15 µg/cm^2 collagen-coated 75 cm^2 tissue flasks in MEM-Alpha medium with GlutaMAX-1 and Nut Mix F-10 W/GLUTAMAX-1 (1:1 v/v), supplemented with 10% (v/v) heat inactivated FBS, 0.3 mg/mL geneticin, and 1 ng/mL bFGF (Roux et al., 1994). Cells were maintained as monolayer cultures at 37 ºC in a humidified incubator under an atmosphere containing 5% CO_2.

3.2.3 Cellular treatments

Rat ghrelin was prepared in 1% acetic acid to a final concentration of 1 mg/mL. This solution was diluted to 1:10 in sterile PBS to 30 µM and kept at -20 ºC.

Both lyophilized powder of the stress inducers thapsigargin (5 mM stock solution) and tunicamycin (2 stock solutions: 1 and 5 mg/mL) were reconstituted in DMSO or ethanol/methanol, respectively. Afterwards, thapsigargin was stored at -20 ºC, whereas tunicamycin at 4 ºC.

Prior to the seeding of cells, the suitable number of viable cells in suspension was quantified by a standard Trypan blue exclusion method using a hemocytometer chamber. When 50% confluency is achieved, both cell lines were pre-incubated for 24 hours with (1) ghrelin (0.01 and 0.1 µM) for cell viability assays; (2) ghrelin (0.1 µM) for the remaining experimental procedures. After the pre-incubation period, during 2-12 hours, cells were treated with thapsigargin (2 µM) or tunicamycin (2.5 µg/mL). This is the standard experimental design (Fig. 1.1), which was adapted for tube formation assay: all reagents are added to cultured cells without any specific incubation time. Thereafter experiments were performed to evaluate the cardio/neuroprotective effect of ghrelin against ER stress. Untreated cells were used as control.

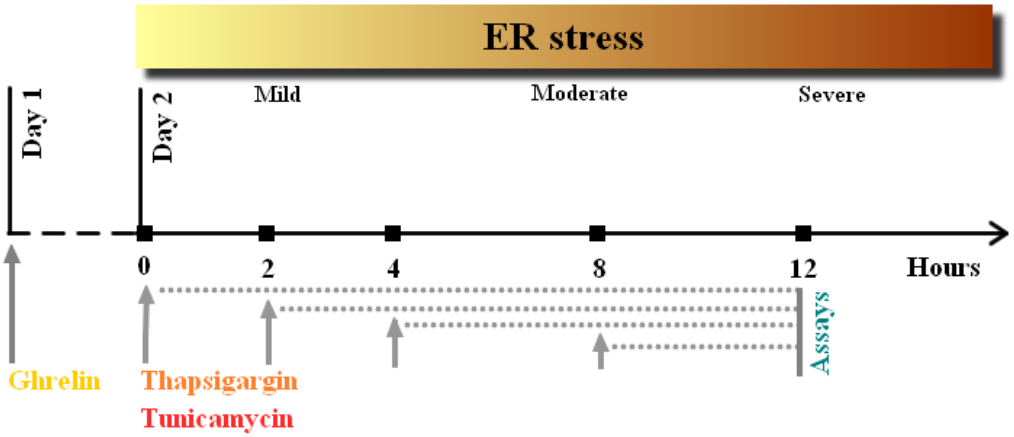


Figure 3.1 ***Experimental procedures outline.***
Standard experimental design showing the interconnection between the time course and concomitant degree of ER stress: mild (adaptive stage), moderate (alert signals stage) and severe (cell death stage).

Table 3.1.3. Summary of cell treatments performed in the different experimental protocols.

Treatments	Function	Abrev./Conc.	Incubation period
Ghrelin	Brain-gut peptide that regulates growth hormone release, food intake and cardiovascular function.	GHR (0.1 µM); a concentration of 0.01 µM was used in cell viability experiments.	24 hours (pre-incubation time)
Thapsigargin	ER stress inducer (SERCA Ca^{2+} ATPase inhibitor). Positive control for ER stress.	TG (2 µM)	2, 4, 8 or 12 hours
Tunicamycin	ER stress inducer (N-acetylglucosamine transferase inhibitor). Positive control for ER stress.	TM (2.5 µg/mL)	2, 4, 8 or 12 hours

3.3 Methods

3.3.1 Evaluation of cell viability

3.3.1.1 MTT reduction assay

To assess endothelial injury caused by the exposure to ER stressors in brain ECs, cell viability was evaluated using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay, which measures the ability of metabolic active cells to reduce MTT into formazan via cleavage of MTT's tetrazolium ring, yielding a colorimetric product that absorbs light at 570 nm (Mosmann, 1983).

Following cell treatments, RBE4 were washed with Krebs buffer [in mM: 132 NaCl, 4 KCl, 1.2 NaH_2PO_4, 1.4 $MgCl_2$, 6 glucose, 10 HEPES, and 1 $CaCl_2$ (pH 7.4)] and incubated with 0.5 mg/mL MTT for 2 hours at 37 ºC. The purple formazan crystals formed, insoluble in aqueous solution, were dissolved in an equal volume of 0.04 M HCl in isopropanol and quantified spectrophotometrically by measuring the absorbance at 570 nm using a microplate reader (SpectraMax Plus 384, Molecular Devices, CA, USA). Results were expressed as a percentage of the average absorbance determined in control cells.

3.3.1.2 Alamar Blue assay

Another cytotoxicity test was used in both endothelial cell lines: Alamar Blue or Resazurin reduction test. Alamar blue is a soluble, non-toxic fluorimetric growth indicator, designed to measure quantitatively the chemical transformation of the oxidized substrate (non-fluorescent, blue) to a reduced product (strong fluorescent, red) (Fields and Lancaster, 1993; Page et al., 1993). After the incubation period, a 10% (v/v) Alamar blue was added to the culture medium. After 2 hours' incubation at 37 ºC, the supernatant was collected and the absorbance was recorded at 570 and 600 nm using SpectraMax Plus 384 microplate reader or Biotek Synergy HT spectrophotometer (Biotek, Winooski, VT, USA). Cell viability (% control) was calculated according to the equation:

$$\frac{(A_{570} - A_{600})_{\text{treated cells}}}{(A_{570} - A_{600})_{\text{control cells}}} \times 100$$

3.3.2 Characterization of functional endothelial parameters

3.3.2.1 Scratch assay

ECs' migration was monitored by the wound healing assay, as previously described (Liang et al., 2007), in both cells lines. 24 hours after ghrelin pre-incubation, the confluent layer of cells over a 12-well plate was wounded with a sterile pipette tip to remove cells by linear scratches. Detached cells were washed away with PBS and ECs were maintained in 1 mL medium with the compounds to be tested at 37 ºC. The progress of migration was photographed using a Zeiss Axio HXP IRE 2 microscope (Carl Zeiss AG, Jena,

Germany) – 10x objective – immediately after injury and 4 and 12 hours later, near the scratched point. The wound was calculated by tracing along the border of the gap using ImageJ analysis software (National Institutes of Health, available at http://rsb.info.nih.gov/ij), and results were expressed according to the formula (Goetsch and Niesler, 2011):

$$\frac{[\text{Wound Area (0h)} - \text{Wound Area } (x\text{h})]}{\text{Wound Area (0h)}} \times 100 = \% \text{ Wound Closure}$$

3.3.2.2 Matrigel angiogenesis assay

The angiogenic potential upon stimulation of MCEC-1 and RBE4 cells was evaluated by tube formation assay. The day before experimentation, growth factor reduced Matrigel was thawed at 4 ºC overnight to become liquid. Briefly, 10 µL solidified Matrigel was filled into the lower well of the µ-Slide Angiogenesis (ibidi GmbH, Martinsried, Germany) and an extra humidity chamber was prepared with water soaked paper towels where the setup was inserted. After polymerization at 37 ºC (about 1 hour), cell suspension (25 000 cells) is applied to the upper well in a total volume of 50 µL of medium with or without test compounds (Zengel et al., 2011). Then, the network formation was observed microscopically and about 5 hours after plating the pictures were captured by phase-contrast using Zeiss Axio HXP IRE 2 microscope (Carl Zeiss AG, Jena, Germany) – 10x objective, and automatically analysed using angiogenesis' plugin for ImageJ software (NIH, Bethesda, MD) (e.g., determination of tubes, loops, cell covered area, and branching points).

3.3.3 Measurement of mitochondrial Ca^{2+} content with Rhod-2/AM

To monitor the dynamics of mitochondrial $[Ca^{2+}]$ ($[Ca^{2+}]_{mit}$), an experimental procedure that exploits the properties of the fluorescent Ca^{2+}-sensitive probe Rhod-2/AM was used, as described previously (Deniaud et al., 2008) with some minor modifications. Rhod-2/AM is a low affinity cell-permeable Ca^{2+} indicator that is visible-wavelength excitable. Due to the presence of the acetoxymethyl ester group (AM), its accumulation into mitochondria is promoted effectively, most likely via membrane potential-driven uptake. Once inside the mitochondrium, this AM group is cleaved by non-specific esterases highlighting the sequestration of the probe inside the organelle.

Control and treated RBE4 cells were washed twice in sodium medium without Na_2HPO_4 (in mM: 132 NaCl, 4 KCl, 1.4 $MgCl_2$, 6 glucose, 10 HEPES, and 1 $CaCl_2$ add posteriorly; pH 7.4) supplemented with 0.1% (w/v) fatty acid-free BSA. Then cells were loaded with Rhod-2/AM (5 µM; 1 mM in DMSO) in Krebs medium for 40 min. To assure a selective accumulation of Rhod-2/AM into mitochondria, probe loading was performed at low temperature followed by incubation at 37 ºC in the dark. Later, cells were washed and incubated for 30 min in Ca^{2+}-free Krebs buffer to allow the complete deesterification of the AM ester group. Experiments were run for 5 or 15 min, and mitochondrial maximal Ca^{2+} uptake was assessed by challenging mitochondria with the subsequent addition of 5 µM Ca^{2+} ionophore A23187 (stock: 2 mM in absolute ethanol) after a fluorescence baseline was stabilized. Then, Rhod-2/AM fluorescence was

measured in a microplate reader (SpectraMax Gemini EM fluorocytometer, Molecular Devices, CA, USA) with excitation at 552 nm and emission at 581 nm. The peak amplitude of Rhod-2/AM fluorescence was used to evaluate mitochondrial Ca^{2+} content. The fluorescence intensity is proportional to mitochondrial Ca^{2+} binding to the probe. Acquired results were normalized to control values.

3.3.4 Determination of Reactive Oxygen Species (ROS) production with Amplex Red

The accumulation of reactive oxygen species (ROS), in particular the hydrogen peroxide (H_2O_2), was evaluated using the Amplex® Red Hydrogen Peroxide/Peroxidase assay kit, as previously described (Mohanty et al., 1997). Amplex Red is a colourless, highly sensitive substrate that reacts with H_2O_2 to produce highly fluorescent Resorufin. Thereafter, once in the cell, the horseradish peroxidase catalyses H_2O_2-dependent oxidation of non-fluorescent probe Amplex Red into fluorescent Resorufin Red.

After incubation under the experimental conditions previously outlined, RBE4 cells were rinsed twice with Krebs medium and then loaded with 100 µM Amplex® Red reagent (stock: 10 mM in DMSO) and 0.2 U/mL HRP (stock: 10 U/mL in Krebs buffer) in the same buffer for 30 min at 37 ºC, protected from light. After that, the endpoint fluorescence signal, corresponding to H_2O_2 generation, was registered at 560 nm excitation and 590 nm emission, using a temperature-controlled SpectraMax Gemini EM spectrofluoremeter (Molecular Devices, CA, USA). Fluorescence values were normalized to those determined in controls.

3.3.5 Quantification of exosomes with "Nanosight" system

Cells were cultured in exosome-depleted medium, prepared accordingly to *Lässer et al* (Lässer et al., 2012). In short, RBE4 serum was depleted of exosomes by ultracentrifugation at 120 000 x *g*, for 16 hours at 4 ºC (Optima™ L-100XP Preparative Ultracentrifuge; Beckman Coulter Inc., Pasadena, CA, USA), in a 1:1 dilution to minimize serum proteins loss due to its viscosity. Exosomes derived from cultured cells were isolated from conditioned medium after culture in exosome-depleted medium during the entire workflow. Afterwards, the medium was collected and exosomes were isolated by ultracentrifugation. Harvested supernatants were subjected to differential centrifugation at 4 ºC, starting with a 10 min centrifugation at 300 x *g* (Centrifuge 5810R; Eppendorf AG, Hamburg, Germany), followed by 20 min at 16 500 x *g* (Avanti® J-26 XP High-Performance Centrifuge; Beckman Coulter Inc., Pasadena, CA, USA). To thoroughly remove cellular debris and larger particles, supernatants were filtered through a 0.22 µm filter unit (Fisher Scientific, Porto Salvo, Lisboa), followed by ultracentrifugation at 120 000 x *g* for 70 min. The resultant pellet was washed with phosphate buffered saline (PBS) (in mM: 1.5 KH_2PO_4, 155 NaCl and 2.7 $Na_2HPO_4.7H_2O$; pH 7.4) and, after a second ultracentrifugation again at 120 000 x *g* for 70 min, exosomes were ressuspended in PBS.

Afterwards, it was measured their particle size and concentration distribution upon dilution in 1 mL PBS. Thenceforth, exosomes isolated from RBE4 cells were subjected to Nanosight tracking analysis

(NTA), using a NanoSight LM 10 instrument (NanoSight Ltd, Amesbury, UK). Settings were optimised and kept constant between samples. Each video was analysed to give mean, mode, median and estimated concentration for each particle size (Dragovic et al., 2011; Sokolova et al., 2011). Data were processed using NTA 2.2 analytical software.

3.3.6 Detection of protein levels by Western Blotting (WB)

3.3.6.1 Sample preparation for WB

WB analysis was performed to evaluate changes in the levels of several proteins of interest, including macroautophagic and ER stress markers.

RBE4 cell line was first washed 2 times with PBS (pH 7.4). Then, whole cell extracts were prepared from treated or untreated cells by scrapping and resuspension in ice-cold RIPA lysis buffer containing: 150 Mm NaCl, 1% (v/v) NP-40, 0.1% (w/v) SDS, 0.5% (w/v) DOC, 50 Mm Tris-HCl (pH 7.4); supplemented with 100 µM PMSF, 2 mM DTT, and 1:1000 dilution of a protease inhibitor cocktail (1 µg/mL leupeptin, pepstatin A, chymostatin and antipain). In order to favour disruption, the cellular suspension was rapidly submitted to three frozen/defrosted cycles in liquid nitrogen, and centrifuged at 17 000 x *g* for 15 min at 4 ºC (Eppendorf 5417R; Eppendorf AG, Hamburg, Germany). The pellet was discharged and the protein concentration in the supernatant was accessed using the Bio-Rad protein dye assay reagent. Samples were denaturated at 100 ºC for 5 min in a concentrated sample buffer (6x): 100 mM Tris, 100 mM DTT, 4% (w/v) SDS, 20% (v/v) glycerol and 0.2% (w/v) bromophenol blue. Samples were then processed for WB analysis.

3.3.6.2 Western immunoblotting analysis

After denaturation, cellular extracts from each sample containing 40 µg of protein prepared as described above were separated by electrophoresis on a 15% (for LC3B and CHOP) or 12% (for all the other proteins) SDS-polyacrylamide gels (SDS-PAGE). The identification of proteins of interest was facilitated by the usage of the prestained Precision Plus Protein All Blue Standard, which was run simultaneously. Proteins were then electroblotted onto PVDF membranes, which were further blocked for 1 hour at room temperature (RT) with 5% (w/v) BSA in Tris-buffer [TBS; 150 mM NaCl, 25 mM Tris-HCl (pH 7.6)] with 0.1% (v/v) Tween 20 (TBS-T) to eliminate nonspecific binding. Membranes were next incubated overnight at 4 ºC with gentle agitation using one of the following primary antibodies prepared in TBS-T containing 5% (w/v) BSA against: CHOP (1:500 dilution), sigma 1 receptor (Sig1R), total VDAC (1:750 dilution), caspase-12, total and phosphorylated eIF2α, ERO1α, GRP78, LC3B, XBP-1 (1:1000 dilution). Mouse monoclonal anti-β-tubulin antibody (1:5000 dilution) was used as control of protein loading.

After washing with TBS-T several times (10 min each), membranes were re-incubated for 1 hour with agitation at RT with alkaline phosphatase-conjugated anti-mouse, anti-rabbit or anti-goat secondary antibody (1: 20 000, 1:20 000 and 1:5000 dilution, respectively), also prepared in 5% (w/v) BSA in TBS-T. Upon another TBS-T washing, immunoreactive protein bands were detected on a Bio-Rad Versa Doc 3000 Imaging System after incubation of membranes with ECF reagent at most 5 min. Densities of protein bands were calculated using the Quantity One 1-D Analysis Software Version 4.6.5 (Bio-Rad; Hercules, CA, USA). The ratios between pro-caspase-12, caspase-12, CHOP, total and phospho-eIF2α, ERO1α, GRP78, LC3II, Sig1R, total VDAC, XBP-1 (un- and spliced) and β-tubulin were calculated and normalized to control values.

Antibody dilutions are depicted in the Table 1.1.2.

3.3.7 Statistical analysis

Data are expressed as means ± the standard error of the mean (SEM) of measurements performed in duplicate, from at least three independent experiments. Statistical significance was determined using one-way analysis of variance (ANOVA), followed by Dunnett's or Sidak's post-hoc tests for multiple comparisons and by the unpaired two-tailed Student's *t*-test in the GraphPad Prism Software (San Diego, CA, USA). The differences were considered significant for P values less than 0.05.

CHAPTER 4 – Results

4.1 Cell survival is differentially impaired upon ER stress in cardiac versus brain ECs.

Previous studies have established a causal relationship between TG- and TM-induced prolonged ER stress and cell survival in brain endothelial and cortical cells (Chung et al., 2011; Plácido et al., 2015). Moreover, GHR protective properties can rescue neuronal and cardiac cells against various insults, including ER and/or oxidative stress (Chung et al., 2011; Shimada et al., 2014). Taken together, these studies highlight the potential role of GHR on cell viability in both cardiac and brain ECs after ER stress stimulation.

Here, the differential response to GHR in RBE4 and MCEC-1 was compared, parallel to the effect of TG and TM, two classical ER stressors (Li and Lee, 1991; Rogers et al., 1995; Kuo et al., 1998). For that, we first evaluated the metabolic capacity of both cell lines using MTT and Alamar blue colorimetric assays, which correlate with cell survival. After pre-incubation of RBE4 and MCEC-1 cells with 0.01 or 0.1 µM GHR for 24 hours, cells were treated with 2 µM TG or 2.5 µg/mL TM for 2, 4, 8 or 12 hours, in order to follow the different stages of ER stress: reestablishment of homeostasis (short exposure) vs. induction of apoptosis (prolonged exposure).

Regarding RBE4 cells, both stimuli enhanced cytotoxicity in a time-dependent manner, particularly after 4 hours of exposure to the stressors, whereas GHR slightly reversed that effect on the preceding time periods (Fig. 4.1). In accordance with MTT data, results from Alamar Blue assay, which is a more sensitive approach to evaluate cytotoxicity, shown in Fig. 4.2, highlight the deleterious effect of chronic ER stress in brain ECs.

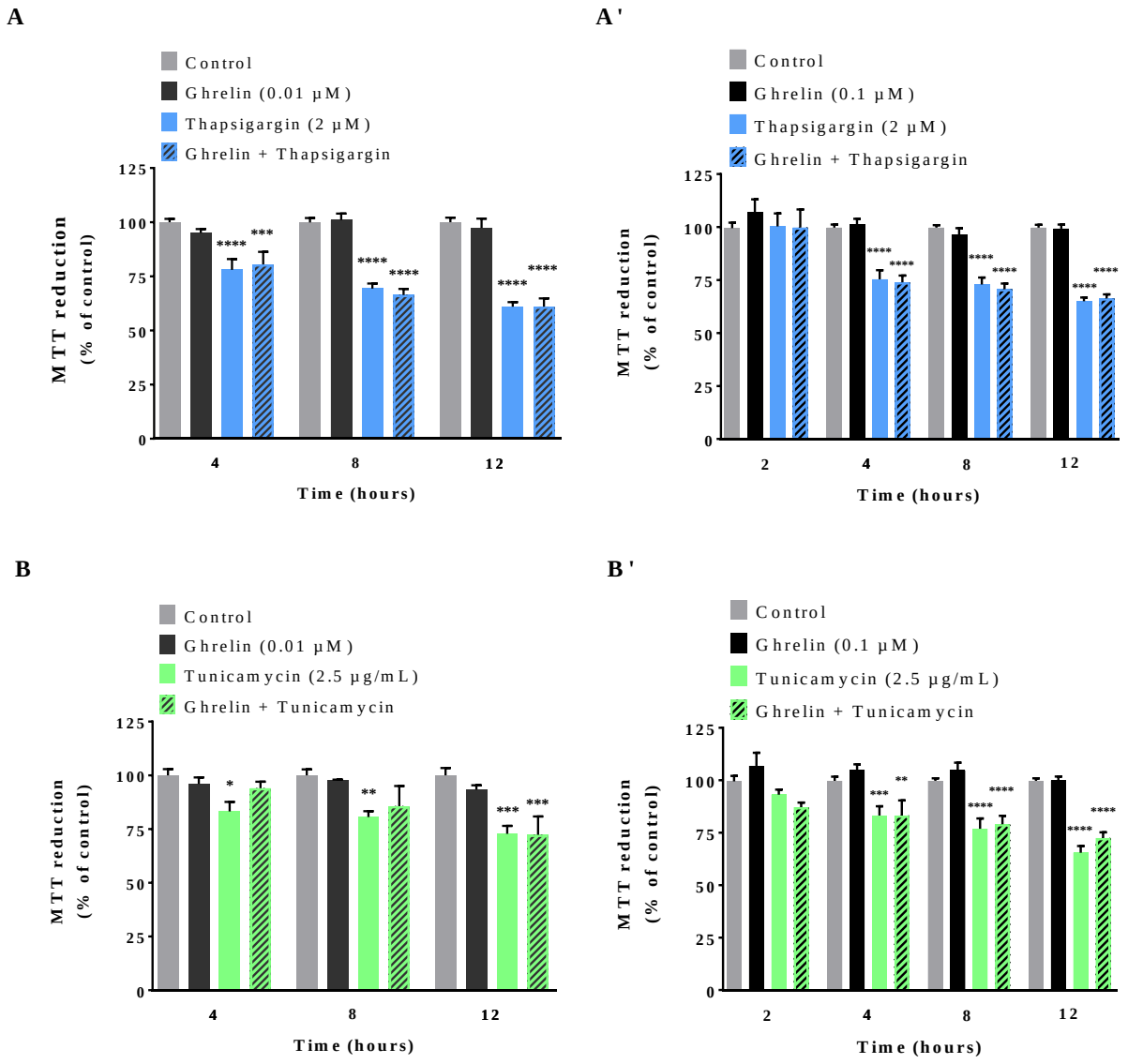


Figure 4.1 ***Role of ghrelin on ER stress-induced loss of brain ECs survival.***

RBE4 cells were incubated with ghrelin (0.01 and 0.1 µM) for 2-12 hours, in the absence or in the presence of: **(A and A')** 2 µM thapsigargin or **(B and B')** 2.5 µg/mL tunicamycin. Thapsigargin and tunicamycin treatments were used as positive control for ER stress. Cell viability was evaluated by the reduction of the tetrazolium salt MTT, as described in Methods section. Results are the means ± SEM of values corresponding at least to three independent experiments, performed in duplicate, and are expressed as the percentage (%) of the absorbance determined under control conditions (untreated cells). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ significantly different with respect to control data using the one-way ANOVA test, followed by Sidak's post hoc test.

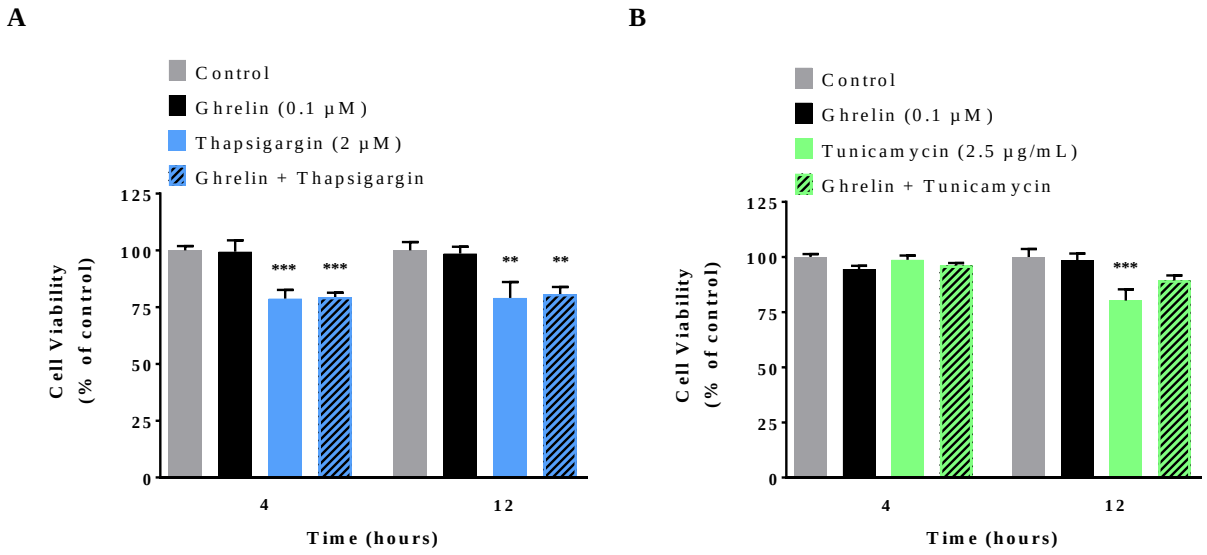


Figure 4.2 ***Effect of ghrelin on brain EC's survival upon ER stress.***

RBE4 cells were treated for 4 or 12 hours with two widely used ER stressors: **(A)** 2 µM thapsigargin or **(B)** 2.5 µg/mL tunicamycin, in the absence or presence of ghrelin (0.1 µM). Cell viability was determined by the Alamar Blue assay. Data represent the mean ± SEM of at least three independent experiments performed in duplicate and are expressed as the percentage (%) of control values (untreated cells). Statistical significance between control and treated cells was analysed using the one-way ANOVA test, followed by Sidak's post hoc test: $^{**}p < 0.01$; $^{***}p < 0.001$.

When compared to brain ECs (approximately 60 - 70 % cell survival), the cardiac MCEC-1 cell line is less susceptible to the TG effect, due to higher and broader cell viability rates (between 70 - 90%). Herein, time-dependent changes in ER stress persists, but became significant only after 12 hours (Fig 4.3 A, A'). At the concentration tested, TM was not able to affect the viability of cardiac MCEC-1 cells (Fig. 4.3 B).

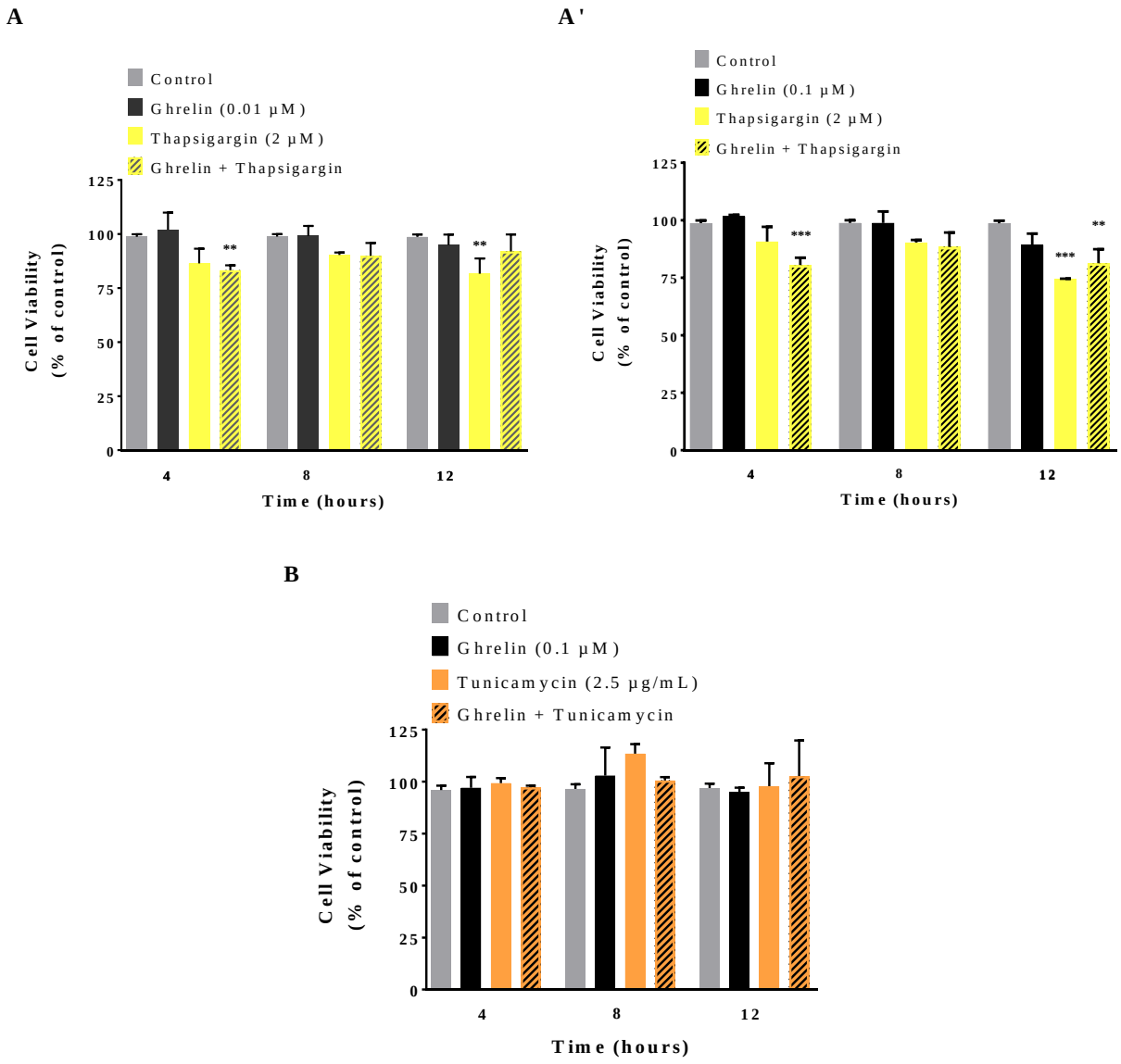


Figure 4.3 ***Ghrelin response to ER stress-induced cytotoxicity in cardiac ECs.***

MCEC-1 cells were incubated for 4-12 hours at 37°C with **(A and A')** 2 µM thapsigargin or **(B)** 2.5 µg/mL tunicamycin, in the presence or absence of increasing concentrations of ghrelin (0.01-0.1 µM). Cell viability was evaluated by monitoring the changes in cell reduction capacity by the Alamar Blue assay, as described in Methods section. The results were presented as the percentage (%) of absorbance measured in controls and express the means ± SEM of three independent experiments, performed in hexaplicate. $^{**}p < 0.01$; $^{***}p < 0.001$ significantly different from control operating the one-way ANOVA test, followed by Sidak's post hoc test.

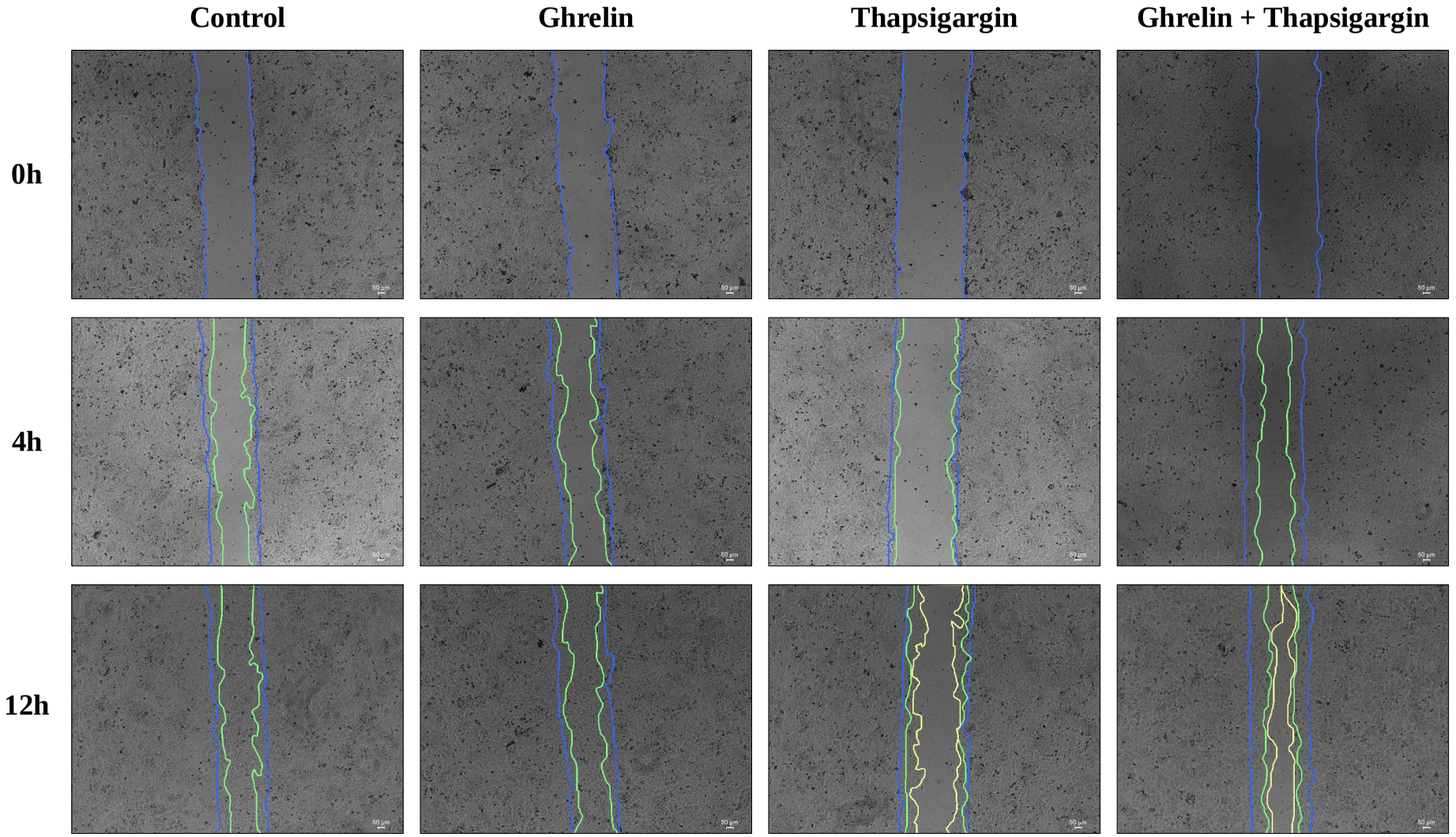
Results from ECs incubation with GHR in the absence of toxic insults demonstrate that this hormone did not affect cell viability *per se* (Fig. 4.1 - 4.3), therefore, a concentration of 0.1 µM GHR was used in subsequent studies. Taken together, these findings support the loss of cardiac and brain ECs survival during ER stress conditions, in part prevented by GHR.

4.2 Brain and cardiac functional endothelial parameters are affected by GHR and ER stressors.

After demonstrating a correlation between ER stress and the decline of ECs survival, and an attempting of GHR to avoid ER stress-induced toxicity, we next evaluated its influence on specific characteristics common to ECs, namely their invasive and angiogenic features, accomplished by scratch and endothelial tube formation assays, respectively. Angiogenesis phenomena is the arrangement into capillary tubes closely dependent on ECs migration. Evidences pinpointing ER stress and GHR implications on migration and capillary-like tube formation of ECs are controversial. To date, GHR markedly antagonized tube formation of rat brain ECs and HUVECs, besides its inhibitory action over angiogenic molecules (Baiguera et al., 2004; Conconi et al., 2004), while other authors stated its involvement on vascularization, including on cardiac ECs (Li et al., 2007; Ahluwalia et al., 2009; Wang et al., 2012a, 2012b). Conversely, GHR was reported to promote angiogenesis and to control ECs migration by inhibition of angiotensin-II, becoming an indirect angiostatic molecule (Rossi et al., 2007; Wang et al., 2011, 2012b; Chen et al., 2013). ER stress seems to modulate angiogenesis/migration according to the surrounding environment, whether it is a physiological or a pathological context one. Usually, EC migration and angiogenesis is prompted under physiological conditions. The reversed effect happens ensuing a pathological mechanism. Moreover, the magnitude of ER stress might induce different outcomes as well (Kimura et al., 2001; Pereira et al., 2010; Nakamura et al., 2013; Ying et al., 2016).

Wound healing is normally used to investigate collective EC migration. To begin, the assessment of migratory phenotype of cardiac and brain ECs modulated by GHR during ER stress was conducted by the scratch assay. After a day of pre-incubation with GHR, a scratch was created onto the cell monolayer. The scratch recovery was recorded 0, 4 and 12 hours after the scratch was made in RBE4 (Fig. 4.4, 4.5 A) and MCEC-1 cells (Fig. 4.6, 4.7 A), and the percentage of closure being calculated in relation to the initial scratch width (Fig. 4.4 - 4.7 B). In brain ECs, only TG exert changes on cell motility (Fig. 4.4). At 12 hours, RBE4 cells incubated with TG migrate significantly less than controls (about 50% less) or those with pre-incubation with GHR, suggesting that GHR acts as a pro-angiogenic compound, since it partially counteracts TG effect. However, TM clearly has no effect on these cells (Fig. 4.5).

A


Control
Ghrelin
Thapsigargin
Ghrelin + Thapsigargin
0h
4h
12h

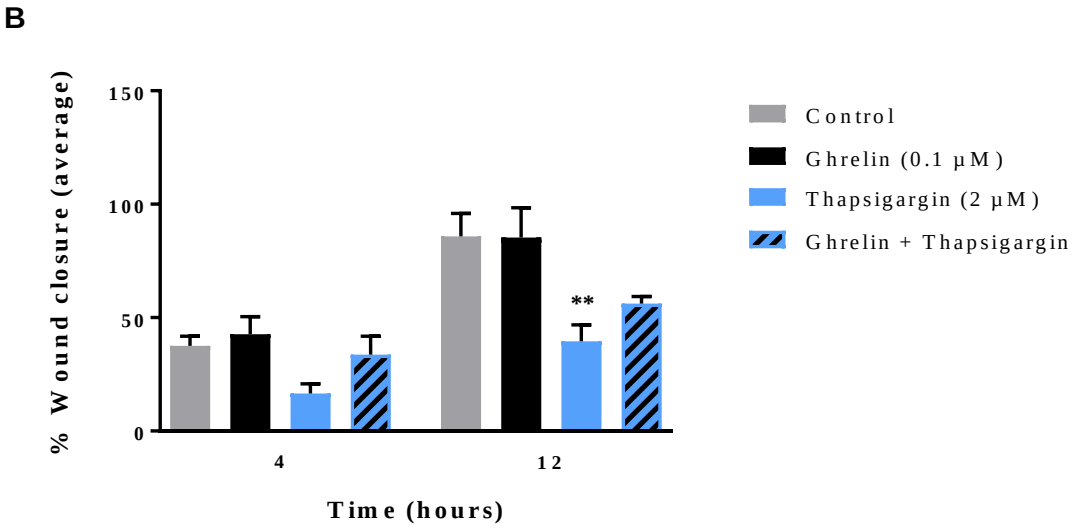
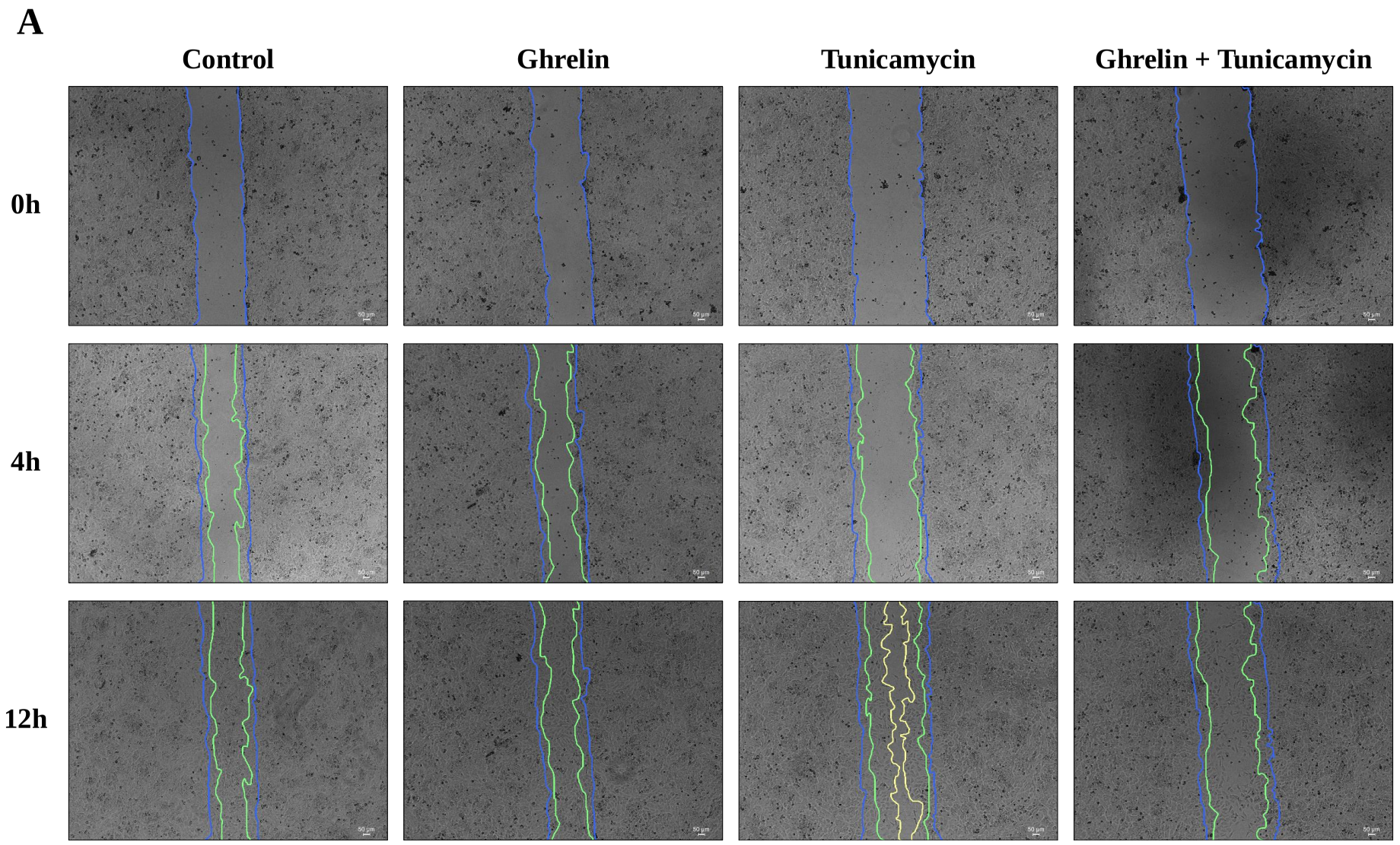


Figure 4.4 ***Impact of ghrelin on wound healing ability of brain ECs upon ER stress.***

The scratch assay on RBE4 cells was displayed by a 200 µL pipette tip upon incubation with or without ghrelin (0.1 µM) and thapsigargin (2 µM). **(A)** Phase contrast photographs of the cultures taken immediately after scratching (time 0) and at 4 and 12 hours' time points. The lines indicate the wound edge at 0h (blue), 4h (green) and 12h (yellow). All images were taken at 100x magnification. Scale bar 50 µm. **(B)** Average recovery obtained by analysing the gap size using Image J image software with respect to the initial scratch size. Results are the means ± SEM of values corresponding at least to three independent experiments, performed in duplicate. $^{**}p < 0.01$ significantly different from control, in the absence of ghrelin treatment, using the one-way ANOVA test, followed by Sidak's post hoc test.


A
Control
Ghrelin
Tunicamycin
Ghrelin + Tunicamycin
0h
4h
12h

B

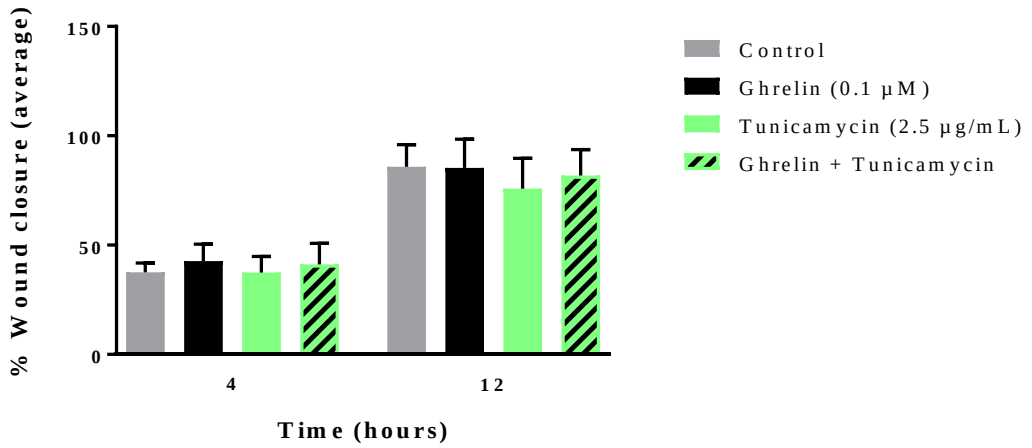
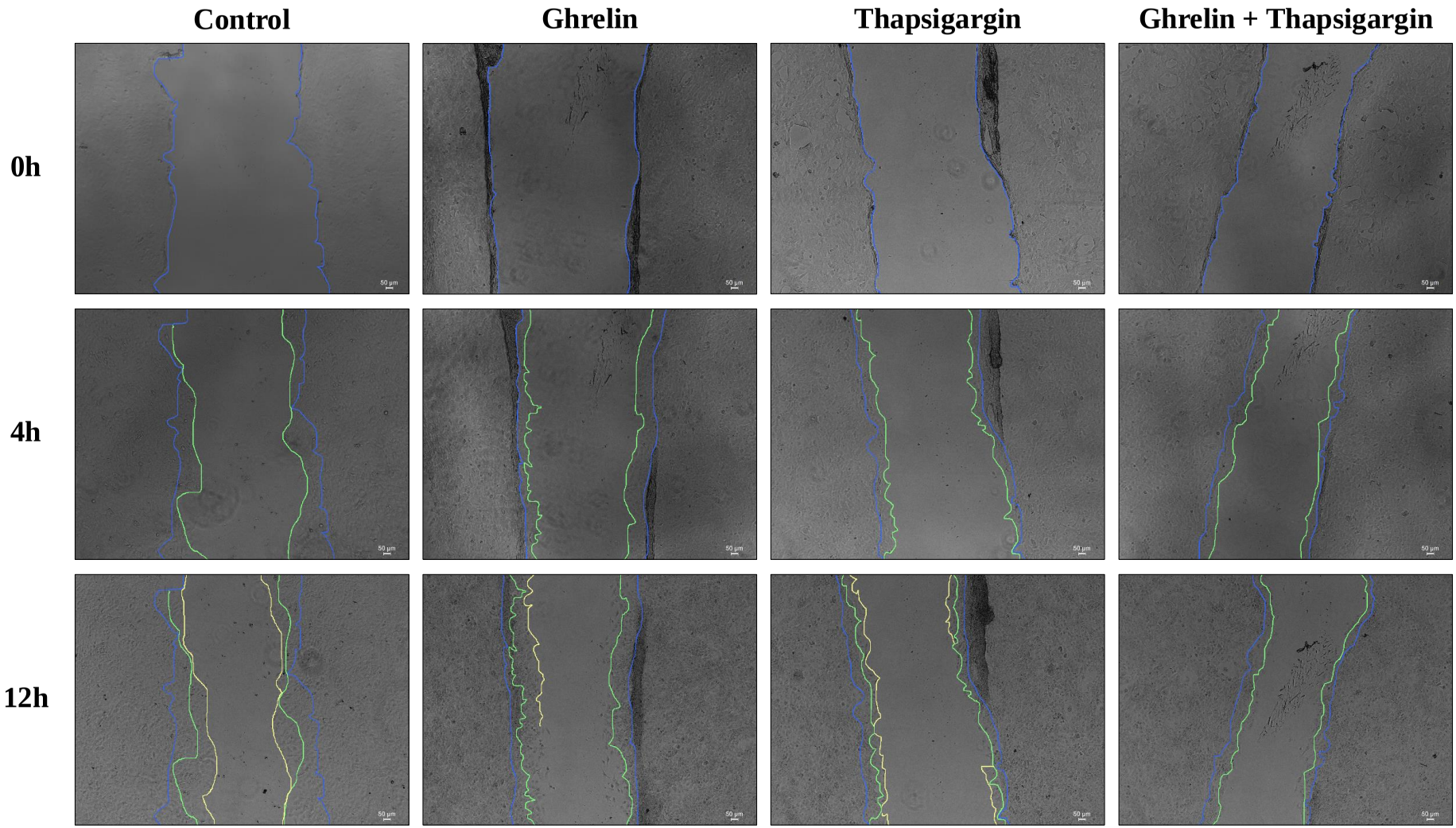


Figure 4.5 ***Role of ghrelin on migration of brain ECs under ER stress conditions.***

Migration capacity of RBE4 cells was analysed by the wound healing assay: EC's confluent monolayer was scratched after ghrelin (0.1 µM) pre-incubation and, thereafter, medium replacement with tunicamycin (2.5 µg/mL), in the presence or absence of ghrelin. Cell migration was monitored for 4-12 hours' period after the injury. **(A)** Representative phase-contrast images at time 0-12 hours after the scratch are shown, along with blue, green and yellow linings for 0-12 hours' time points, respectively. Scale bar 50 µm and 100x magnification. **(B)** Quantification was performed using Image J software onto cell-free area from each set of images. Data was expressed as the mean ± SEM percentage of recovered area of duplicate samples from four independent experiments.

By contrast, both TG and TM affect MCEC-1 capacity to recover from the scratch. Heart ECs behave similarly under the influence of the tested agents (Fig. 4.6, 4.7). However, only the migration of TG-treated MCEC-1 cells was significantly slower than that observed in control cells, while GHR pre-treatment could not attenuate migration capacity in the presence of TG (Fig. 4.6).

A


Control
Ghrelin
Thapsigargin
Ghrelin + Thapsigargin
0h
4h
12h

B

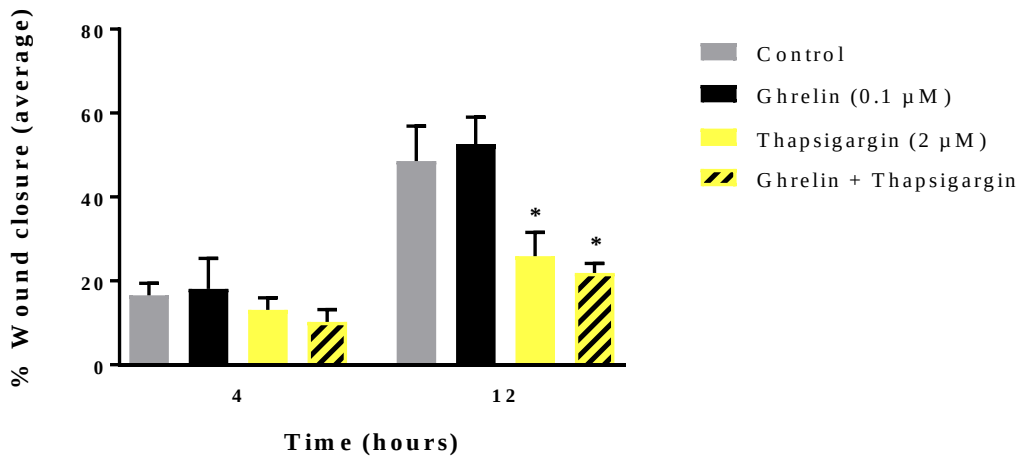
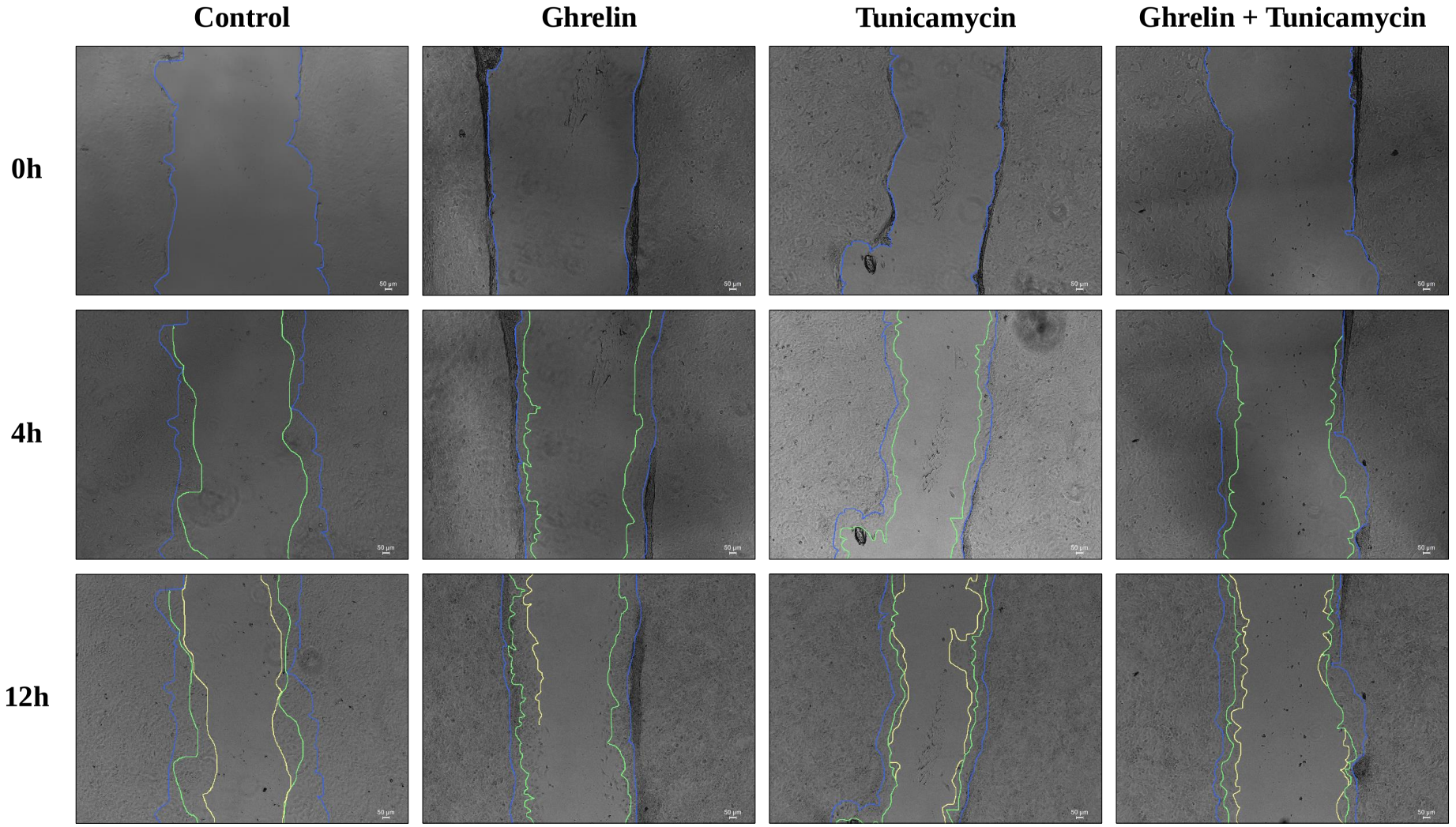


Figure 4.6 ***Influence of ghrelin on cardiac ECs' motility upon ER stress.***

MCEC-1 cells at 100% confluence were wounded with a sterile pipette tip to remove cells, before treatment with thapsigargin (2 µM) either with or without ghrelin (0.1 µM) pre-incubation. **(A)** Photographs were taken (objective magnification 10x; scale bar 50 µm) at 0, 4, and 12 hours after injury. Wound edges are also depicted in blue, green and yellow in accordance to the progressive timing points. **(B)** Wound closure was evaluated using the equation described under Methods section. For this panel, data were expressed as the mean ± SEM of three independent experiments in duplicate. *$p < 0.05$ *versus* control values using the one-way ANOVA test, followed by Sidak's post hoc test.

A


Control
Ghrelin
Tunicamycin
Ghrelin + Tunicamycin
0h
4h
12h

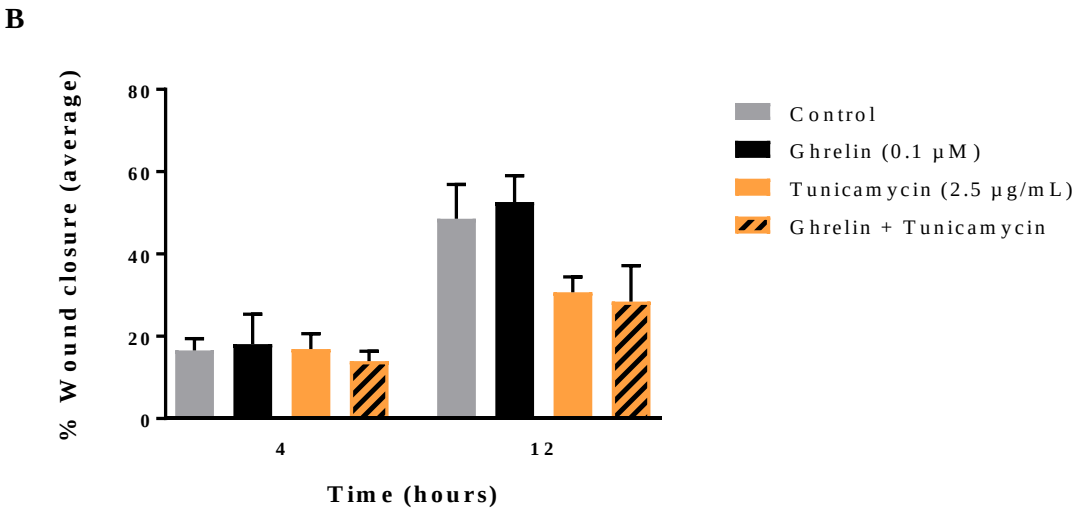


Figure 4.7 ***Effect of ghrelin on the invasion properties of cardiac ECs upon ER stress.***
Confluent monolayers of MCEC-1 cells seeded onto gelatin biofilm were scratched and the media was replaced with tested compounds-containing media (2.5 µg/mL tunicamycin in the presence or absence of 0.1 µM ghrelin). Representative phase images of pre-migration (time 0; blue line) and migration after 4 (green line) and 12 hours (yellow line) were captured by a Zeiss Axiovert microscope (100x magnification). Scale bar = 50 µm. **(B)** Depiction of the graph of three independent experiments, performed in duplicate, obtained from images scanned by Image J analysis software. Data presented as average percent closure ± SEM.

Despite a tendency to be overtly hindered by ER stressors, cell proliferation is lower in cardiac ECs, which, in turn, exhibits a less pronounced effect of GHR. We can conclude that cardiac ECs are more susceptible to ER stress rather than brain ECs, once cells fail to restore its migratory background.

We next tested the endothelial tube formation via *in vitro* angiogenesis assay, which is extensively used to evaluate the angiogenic potential of these compounds. Thus, brain and heart ECs were seeded onto a Matrigel layer and supplemented with GHR and the widely used ER inducers TG and TM, after which the formation of capillary-like network was accessed (Fig. 4.8 A, 4.9 A). The data revealed that tube formation capacity in RBE4 cells was weaker in TM-treated cells than in TG-treated ones. Moreover, GHR pre-treatment before TG or TM addition could restore the tube formation capacity of brain ECs to control levels. From a more detailed analysis, the total number of meshes, nodes and master segments length were increased in TG-treated RBE4 cells (a 0.36, 0.23 and 0.17-fold increase, respectively), when compared with control cells (Fig. 4.8 B - B''). TM-treated cells present values closer to controls (Fig. 4.8 C - C''). In conditions preceded by GHR incubation, albeit the differentiation into capillaries is occluded, the opposite seems to happen in GHR-treated ECs, which *per se* stimulates tube formation (about an increase of 0.57-fold on total number of meshes, 0.37-fold on nodes and 0.29-fold of master segments length). This outcome is sustained in TM-treated RBE4 cells.

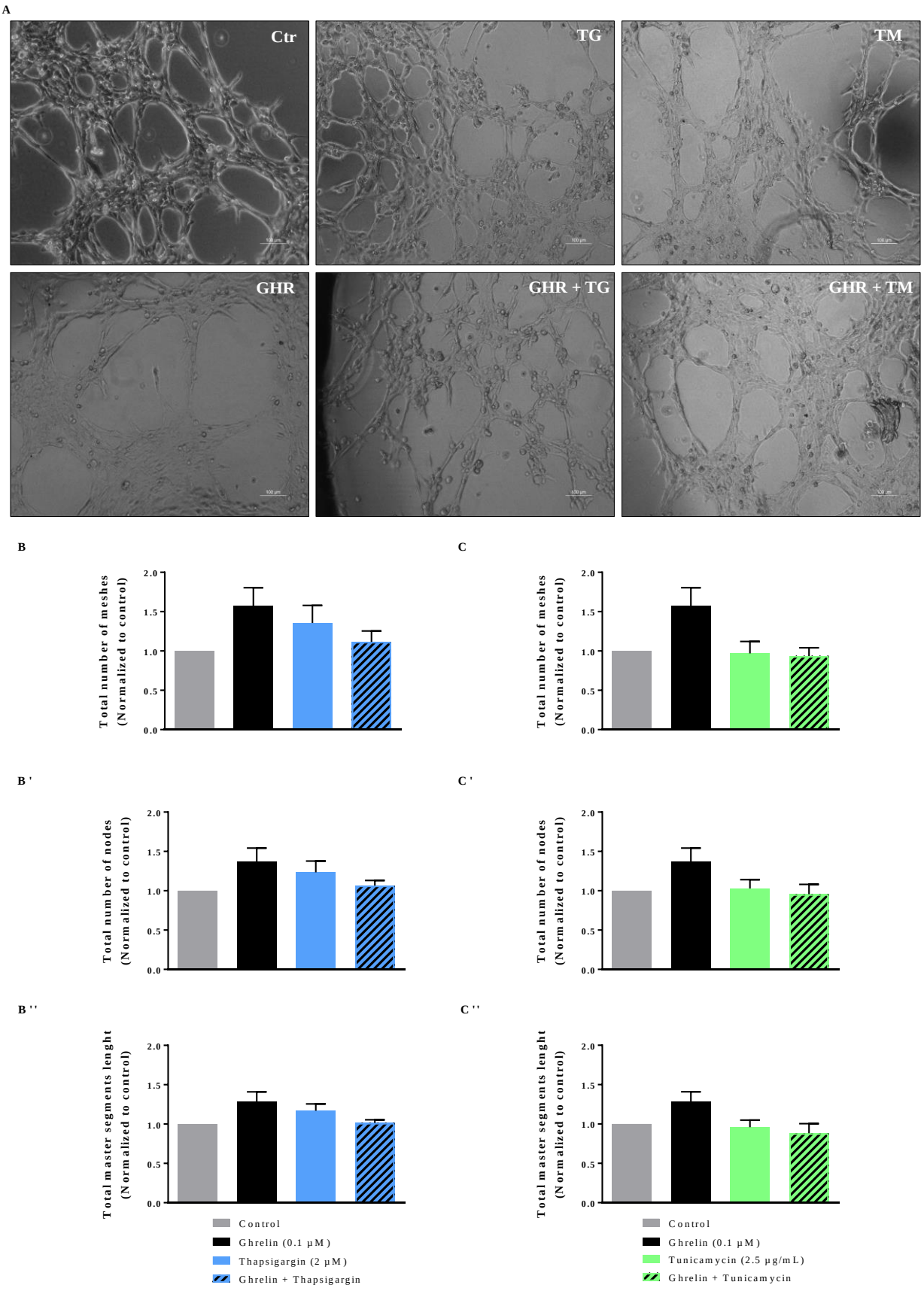


Figure 4.8 Impact of *ER stress on brain ECs differentiation into capillary-like structures in vitro.*

Capillary-like tube formation assay on Matrigel was assessed 5 hours after ECs seeding with ER stress inducers-containing medium, supplemented or not with ghrelin (0.1 µM). **(A)** Representative phase-contrast images are portrayed. All images were

taken at 100x magnification. Scale bar 100 μm. Quantitative assessment of **(B and C)** total number of meshes, **(B' and C')** nodes and **(B'' and C'')** master segments length of treatments with thapsigargin (2 μM) and tunicamycin (2.5 μg/mL), respectively, was performed using an analytical plugin for Image J software and results were normalized to the control conditions. Results represent the means ± SEM of duplicate samples from three independent experiments.

On cardiac ECs, the tested substances have a reversed profile in comparison to brain ECs. Here, capillary-like structures levels with GHR *per se* are equivalent to those from control conditions, as does for both ER stressors. Merely GHR pre-incubation suppresses differentiation, reporting to lower levels of tube formation than control MCEC-1. The total number of meshes, nodes and master segments length for TG- and TM-treated cells are illustrated in Fig. 4.9 (B - B'') and (C - C''), respectively.

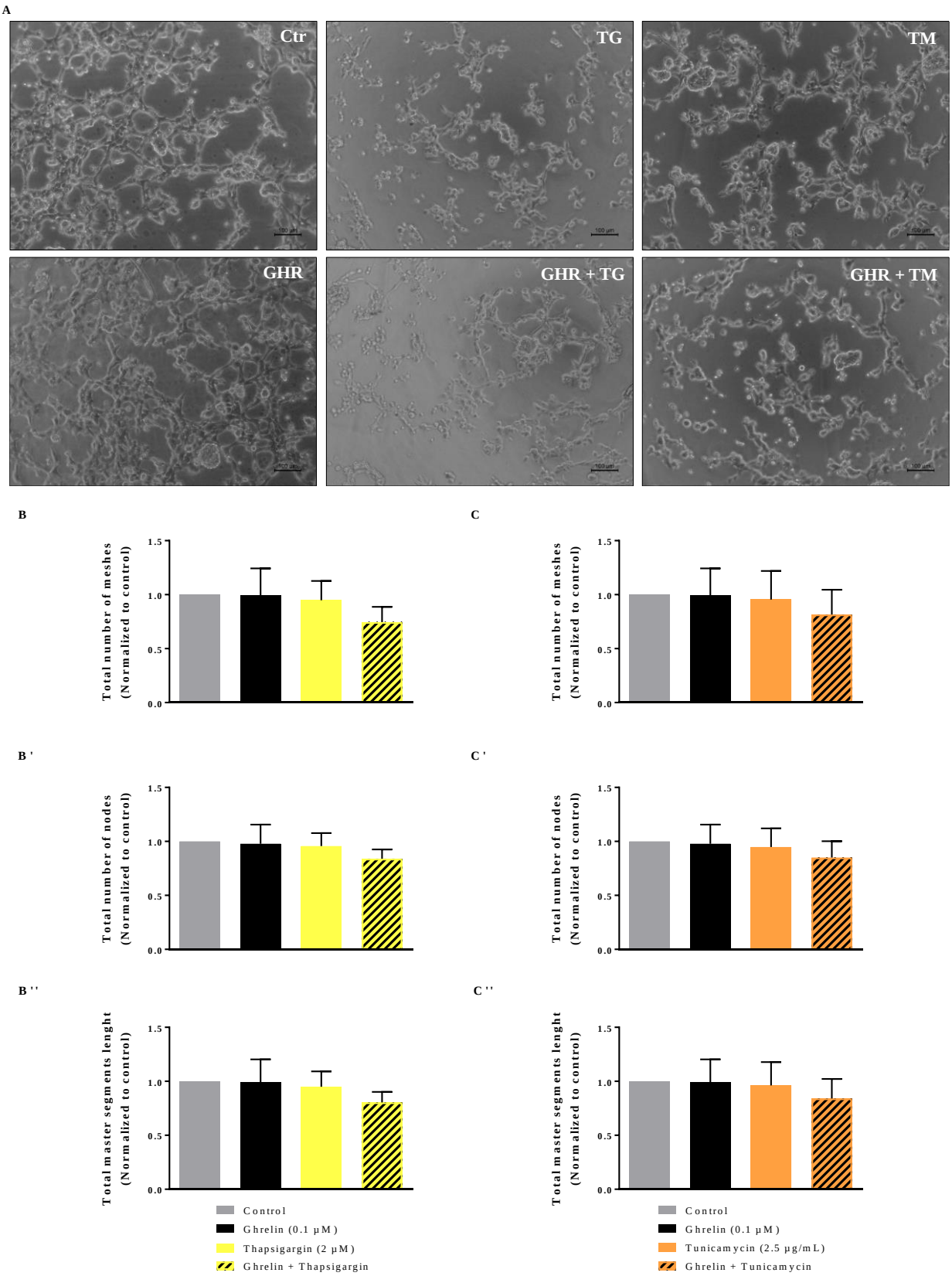


Figure 4.9 ***Role of ghrelin on cardiac ECs differentiation into angiogenic tubes under stress conditions.***

In vitro endothelial network formation of MCEC-1 cells seeded over Matrigel-coated wells was assessed 5 hours after incubation with two classical ER stressors into the medium, supplemented or not with ghrelin (0.1 µM). **(A)** Representative phase-contrast images of the structures are shown. All images were taken at 100x magnification. Scale bar 100 µm. Quantitative assessment of **(B and C)** total number of meshes, **(B' and C')** nodes and **(B'' and C'')** master segments length of treatments

with thapsigargin (2 µM) and tunicamycin (2.5 µg/mL), respectively, was performed using an analytical plugin for Image J software and produced values were compared against control cells. Results represent the means ± SEM of duplicate samples from four independent experiments.

Put together, all these parameters pointed that TG-induced ER stress in brain ECs promote tube formation *in vitro* and ER stress has no effect in cardiac ECs. Even though, GHR limits ER stress by decreasing the tubular network formation.

These techniques evaluate different pro- or anti-angiogenic compounds. The former results discovered that ER stress might affect both migration and angiogenesis in different manners. TG and TM are likely to decrease migration, but to improve or at least maintain angiogenesis. In respect to GHR, the outcome varies according to the cell type. Brain ECs revealed a pro-angiogenic profile of GHR, whereas GHR in cardiac ECs possesses an anti-angiogenic one. Thus, from the functional evidences, we can conclude that the angiogenic properties of GHR itself depend on the origin of the ECs (brain versus heart), and that GHR could suppress ER stress-migration/tubulation in both brain and cardiac EC cells.

4.3 Mitochondrial Ca^{2+} homeostasis and ER/mitochondria cross-talk are regulated by GHR.

Aside from protein folding, ER functions as a major intracellular Ca^{2+} store, however its dyshomeostasis can be fatal owing to the activation of cell death pathways (Pereira, 2013). Several lines of evidence demonstrate that ER stress causes a perturbation of intracellular Ca^{2+} homeostasis, especially ER- and mitochondrial-related Ca^{2+} regulation (Fonseca et al., 2013; Tadic et al., 2014; Krebs et al., 2015; Plácido et al., 2015; Xu et al., 2015; Rajakumar et al., 2016). ER stores are depleted under ER stress conditions and a rapid Ca^{2+} movement from ER to mitochondria matrix, closely connected via MAMs, overloads this organelle (Simmen et al., 2011; Kaufman and Malhotra, 2014; Volgyi et al., 2015). Our group stated that a transient accumulation of mitochondrial Ca^{2+} triggered by ER stress in brain ECs and primary cultures of cortical neurons is the cause for a later activation of the mitochondrial apoptotic pathway (Ferreiro et al., 2004, 2008a, 2008b; Plácido et al., 2015).

In order to disclose the regulation of mitochondria by GHR under stress conditions, mitochondrial Ca^{2+} content was monitored in control and ER stressed brain ECs, pre-treated with GHR. Using a specific mitochondrial Ca^{2+} fluorescent probe, Rhod-2AM, we were able to assess mitochondrial Ca^{2+} levels. After 4 hours of incubation, both classical ER stressors potentiated an increment of Ca^{2+} in mitochondrial stores, an effect that ceased after 12 hours' incubation, where these stores begin its depletion (Fig 4.10). Despite the portrayed results arise from a single experiment, if their reproducibility is confirmed, they suggest that GHR-treated brain ECs seem to minimally drop TG-induced overload (4 hours) followed by an increase in Ca^{2+} content throughout prolonged TG exposures, even though the expressed levels of mitochondrial Ca^{2+}

are lower than those from control cells (Fig. 4.10 A). Therefore, GHR might sensitize mitochondria to reestablish Ca^{2+} basal levels. Less effective results were obtained in brain ECs treated with TM, on which GHR supposedly worsened the deleterious effect of TM after 4 hours (Fig. 4.10 B).

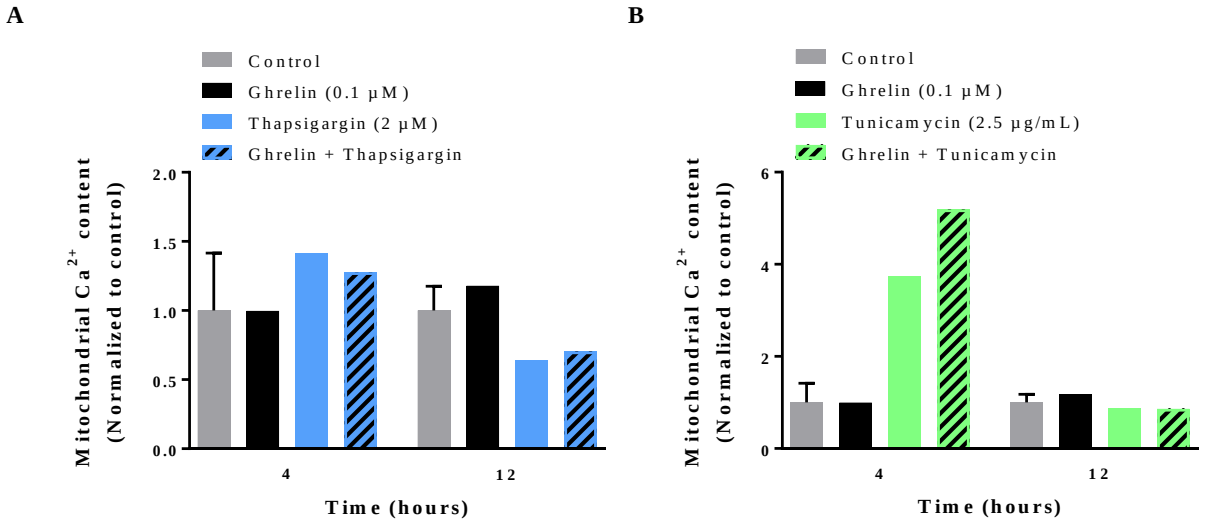


Figure 4.10 ***Effect of ghrelin on mitochondrial Ca^{2+} homeostasis in brain ECs upon ER stress.***
After treatment with widely used ER stress inducers **(A)** 2 µM thapsigargin or **(B)** 2.5 µg/mL tunicamycin for 4 or 12 hours, in the absence or presence of ghrelin, RBE4 cells lacking extracellular Ca^{2+} were loaded with Rhod-2/AM (5 µM) to measure mitochondrial calcium storage. Results are the mean of fluorescence arbitrary values corresponding to a single experiment and then, normalized to control data.

Given the deregulation of Ca^{2+} homeostasis, previous reports showed a general reduction of protein levels of Ca^{2+} regulators (Fonseca et al., 2014a, 2015) together with the upregulation of proteins in ER-mitochondria interface during situations of stress (Plácido et al., 2016). Having this in consideration, the other aspect that we examined was the role of GHR in the MAM-containing proteins Sig1R and VDAC by WB. The analysis of Sig1R revealed a significant time-dependent rise on the levels of this chaperone after TG treatment that was prevented in the presence of GHR (Fig. 4.11 A).

Concerning TM-evoked ER stress, there was a similar increment in Sig1R levels at 4 and 12 hours, which was partially reverted in the presence of GHR after 4 hours (Fig 4.11 A').

In agreement with the aforementioned results, TG fosters a progressive rise in VDAC levels, a regulator of the Ca^{2+} fluxes between ER and mitochondria in MAMs, statistically significant at 12 hours (Fig. 4.11 B). Similarly, TM upregulated VDAC in RBE4 cells, however the increase observed at 4 and 12 hours was not statistically significant (Fig. 4.11 B'). Interestingly, ECs co-treated with GHR and TM present VDAC levels similar to control values. In addition, GHR slightly decreased VDAC upregulation in TG-treated cells. Although, GHR exhibited a tendency to normalize VDAC levels in both conditions, our data was not statistically significant.

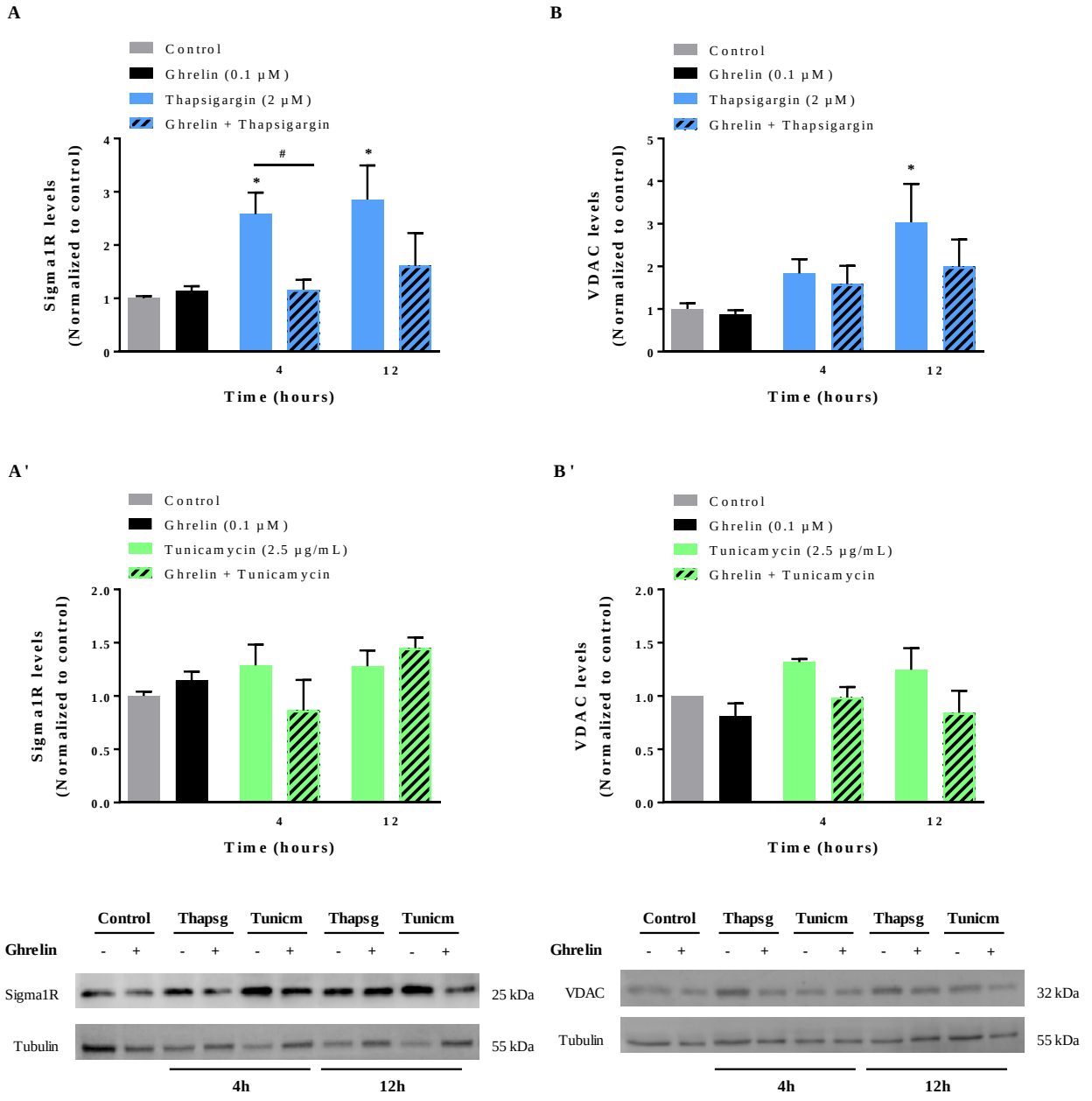


Figure 4.11 ***Role of ghrelin on the tethering between ER and mitochondria in brain ECs.***

Protein levels of **(A and A')** Sigma-1R and **(B and B')** VDAC were quantified by WB in cellular extracts obtained from RBE4 cells treated with **(A and B)** 2 µM thapsigargin or **(A' and B')** 2.5 µg/mL tunicamycin for 4 or 12 hours with simultaneous incubation with ghrelin (0.1 µM). Tubulin was used as loading control and to normalize the levels of proteins of interest. Results were calculated relatively to control values and represent the means ± SEM of at least three independent experiments. Statistical significance between control and treated cells was determined using the one-way ANOVA test, followed by Dunnett's post hoc test: $^{*}p < 0.05$; or between ER stress inducers and ghrelin treatment applying unpaired student's *t*-test: $^{\#}p < 0.05$.

Results demonstrated that GHR can prevent mitochondrial Ca^{2+} dyshomeostasis induced by TG, and to a lesser extent TM, perhaps by restoring the levels of Sig1R and VDAC. However, GHR was only able to significantly protect cells from TG-induced ER stress.

4.4 GHR prevents oxidative stress responses in ER stressed brain ECs.

Previous studies already proved in different models the ability of GHR to efficiently abrogate oxidative stress (Chung et al., 2007; Andrews et al., 2008; Obay et al., 2008; Martins et al., 2013; Gomes et al., 2014). At the same time, ER stress gives rise to an increment in ROS accumulation in brain ECs (Plácido et al., 2015). Here, we sought to determine the ROS production, namely hydrogen peroxide (H_2O_2), in stressed brain ECs challenged with GHR. The levels of H_2O_2 were detected by a time-dependent analysis of Resorufin Red fluorescence.

Our results support the protective role of GHR in brain ECs by decreasing the oxidizing environment implemented by TG, which stands for a time-dependent increase in ROS production (Fig. 4.12 A). In agreement with former results, TM was less efficient than TG in inducing oxidative stress. Only at 4 hours, the rise of ROS levels became statistically significant, however no effect can be seen at 12 hours. GHR prompt a minor loss of TM-induced ROS generation (Fig. 4.12 B).

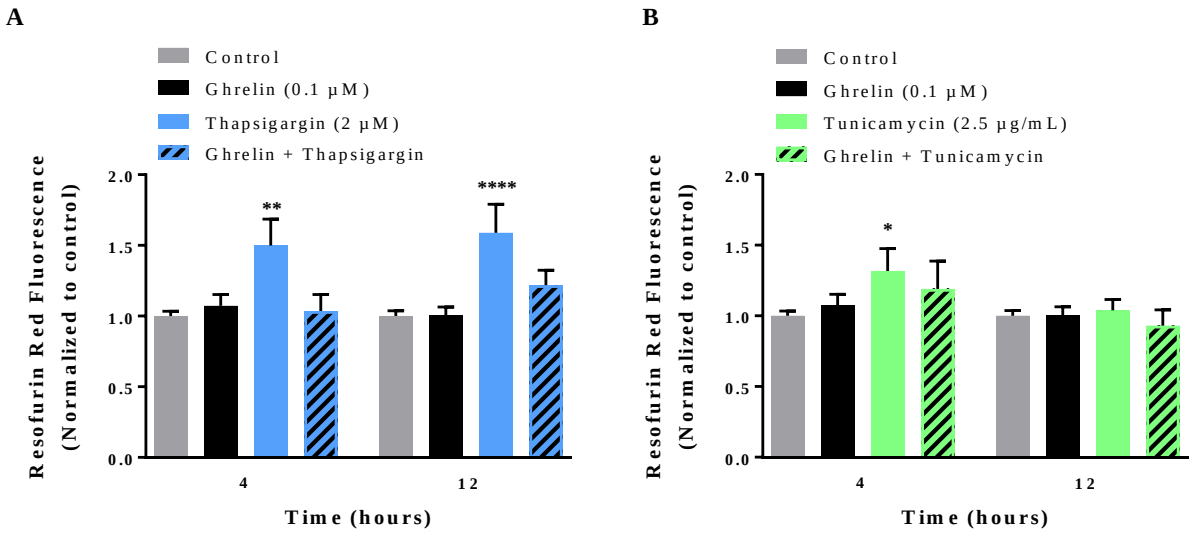


Figure 4.12 ***Effect of ghrelin on the production of ROS in brain ECs upon ER stress.***
In the absence or presence of ghrelin (0.1 µM), RBE4 cells were incubated with **(A)** 2 µM thapsigargin or **(B)** 2.5 µg/mL tunicamycin during 4 or 12 hours. Afterwards, RBE4 were loaded with Amplex Red probe and fluorescence of Resofurin Red was used to screen ROS generation – Amplex™ Red-horseradish peroxidase assay. Values from ROS accumulation are the mean ± SEM of at least three independent experiments, performed in duplicate, and then normalized to control data. *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$ significantly different from control using the one-way ANOVA test, followed by Sidak's post hoc test.

Taking into account the oxidoreductase function of ERO1α in ER, we next investigated its protein levels to infer about the redox status of RBE4 cells. Both classical ER stressors, TG and TM, time-dependently upregulate ERO1α with statistical significance, and GHR counteracts their effect, returning ERO1α levels to control levels (Fig. 4.13). As a note, GHR treatment against TM revels a significant difference in comparison to TM condition at 12 hours of exposure.

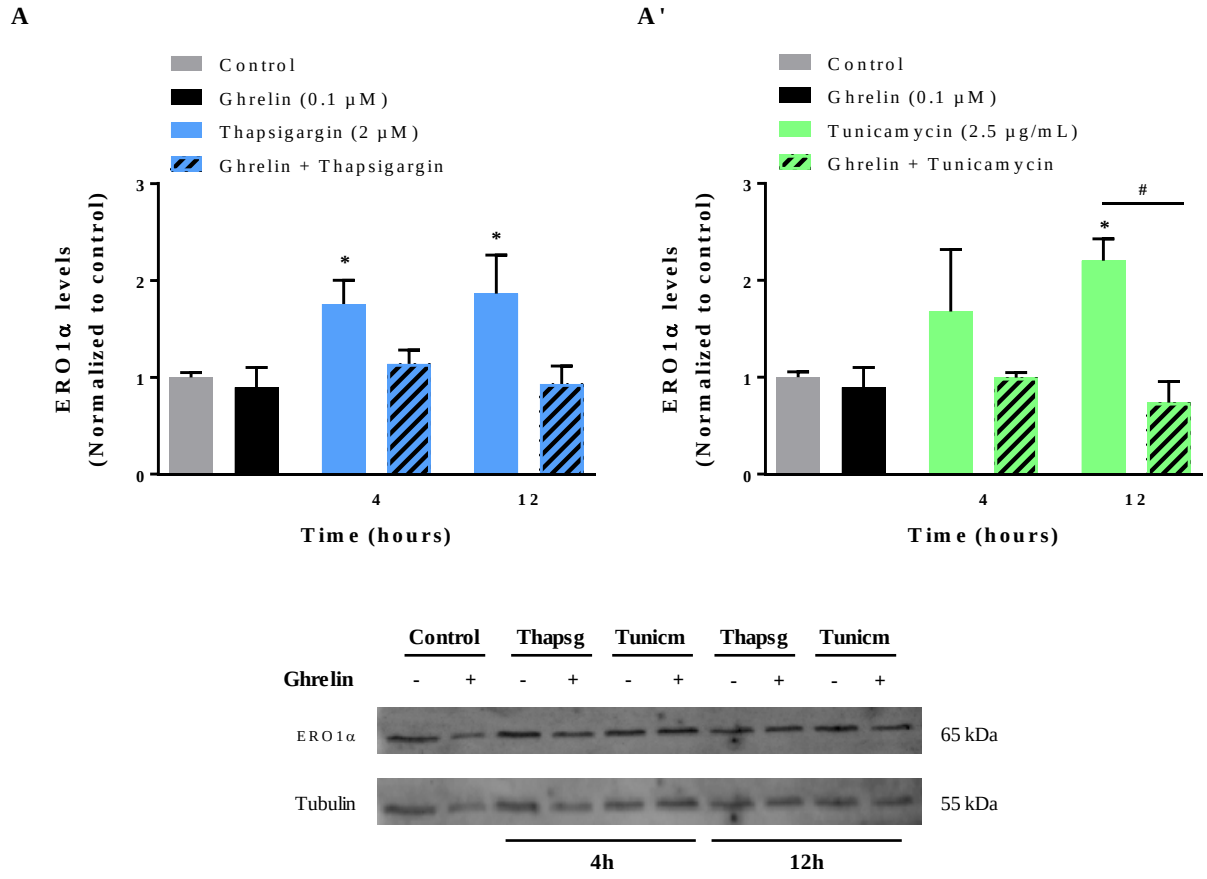


Figure 4.13 ***Effect of ghrelin on ER redox homeostasis under stress conditions.***
Lysates from brain ECs treated with **(A)** 2 µM thapsigargin or **(A')** 2.5 µg/mL tunicamycin for 4 or 12 hours, in the presence or absence of ghrelin (0.1 µM), were prepared as described in Methods. The expression of ERO1α was determined by WB analysis. Immunoreactive bands were detected after ECF reagent incubation on a Bio-Rad Versa Doc Image System. Tubulin was used as a protein loading control. The data corresponds to one representative blot of at least three independent experiments. Results are the means ± SEM of values and were normalized to control conditions. $^{*}p < 0.05$ (ANOVA with Dunnett's post hoc test) compared with control. $^{\#}p < 0.05$ significantly different from ghrelin treatment using unpaired student's *t*-test.

In conclusion, ERO1α activity leads to a hyper-oxidation of ER lumen, reflected by the great amount of ROS raised by TG. Divergent findings about TM were obtained. This ER stressor induces an early rise of ROS that was abolished 12 hours upon exposure to TM. Nevertheless, TM is able to upregulate ERO1α. For both conditions, brain ECs recovery was assured by an antioxidant action of GHR.

4.5 GHR prevents the ER stress-mediated apoptotic cell death pathway in brain ECs

So far we were able to show that, not only ER stress impairs Ca^{2+} and redox homeostasis, but also GHR is a mediator of the cellular responses to rescue ECs from these insults. Once oxidative stress is triggered along with Ca^{2+} imbalance, cell death pathways are activated, including apoptosis (Pereira, 2013). Conversely, an overwhelmed ER stress can initiate apoptotic cell death as well. In this context, GHR was appraised before as an anti-apoptotic effector in non- and neuronal cells (Baldanzi et al., 2002; Ferrini et al., 2009), in particular in neuronal cells enclosing a ER stress milieu (Chung et al., 2011).

Under the conditions studied in the present work, the protective action of GHR against ER stressors-induced apoptosis in brain ECs was evaluated by immunoblotting. Among several apoptotic mediators, caspase-12 and CHOP protein levels were measured in TG- and TM-treated RBE4 cells. As depicted in Fig. 4.14 A - A', the activation of ER stress-mediated apoptosis pathway is given by the protein levels of full-length and cleaved caspase-12. Upon TG or TM treatment, a slight increase in the ratio between the active and inactive form of this ER-resident caspase was found in RBE4 cells, which was not statistically significant. While GHR seems to not affect caspase-12 activation in TG-stressed brain ECs (Fig. 4.14 A), the increased levels of active caspase-12 upon treatment with TM for 12 hours slightly decayed in the presence of GHR (Fig. 4.14 A'), returning to control values. In addition, GHR *per se* affected caspase-12 activation.

Then, protein levels of the pro-apoptotic transcription factor CHOP were determined. CHOP contributes to ER stress-induced cell death through the rising of H_2O_2 levels (Wang et al., 1996; Marciniak et al., 2004; Oyadomari and Mori, 2004). The results obtained showed that in the presence of ER stressors, after an incubation period of 12 hours, CHOP levels climbed over the control and that GHR significantly prevented this increase by approximately 50% (Fig. 4.14 B, B'). The effect of GHR was even more evident when TM treatment occurs upon GHR pre-incubation (Fig. 4.14 B'). During 4 hours' incubation, CHOP levels were almost unchanged.

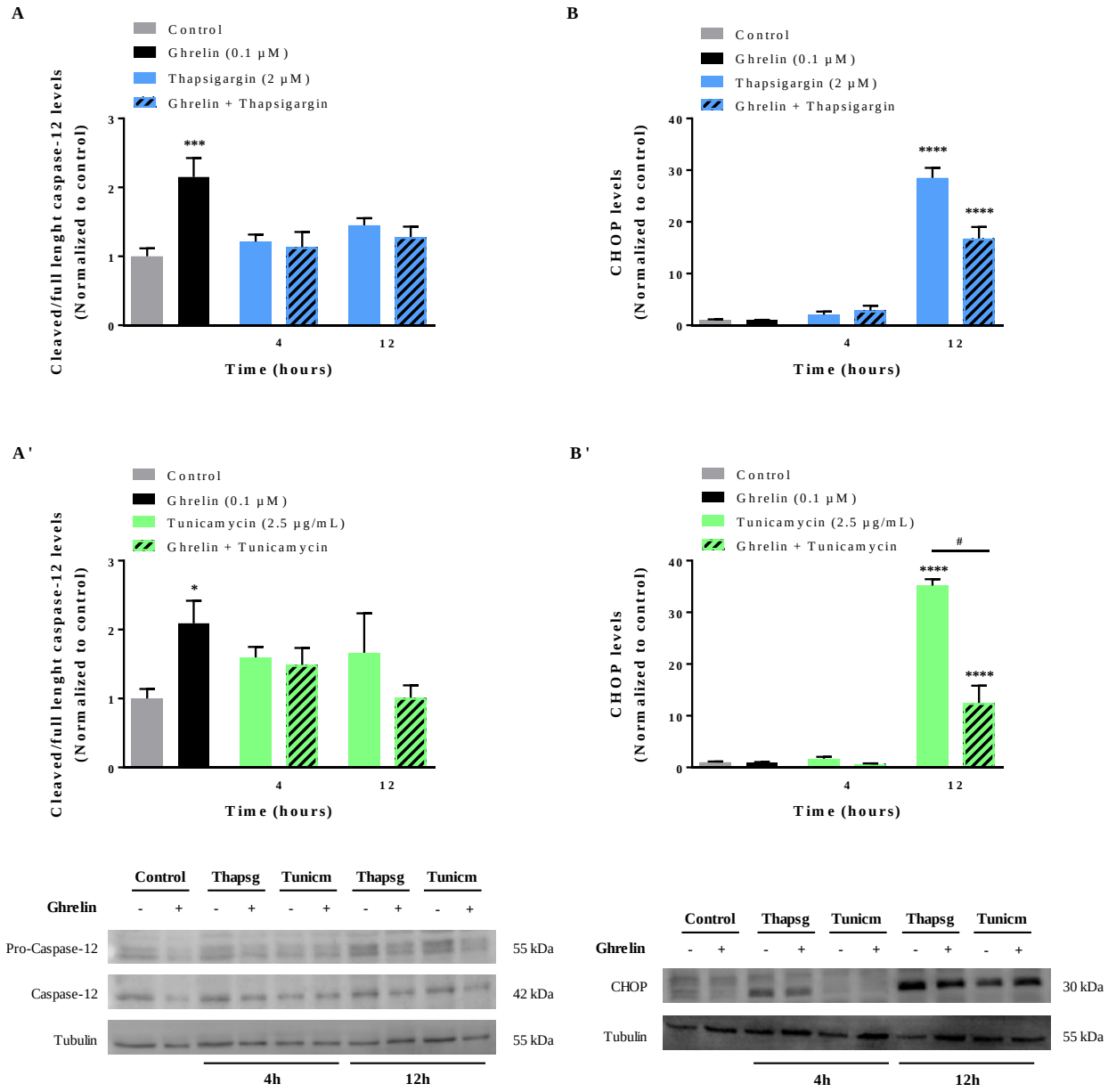


Figure 4.14 ***Role of ghrelin on ER stress-mediated apoptosis in brain ECs.***

RBE4 cells were maintained at 37°C in the presence or absence of ghrelin (0.1 µM), in parallel to the addition of ER stress inducers during 4 or 12 hours: 2 µM thapsigargin **(A and B)** or 2.5 µg/mL tunicamycin **(A' and B')**. Total cells lysates were prepared and analysed by immunoblotting using **(A and A')** anti-caspase-12 or **(B and B')** CHOP antibodies. The ratio between protein levels measured by WB analysis of cleaved and full-length caspase-12 is illustrated for both treatments. Values are the means ± SEM of at least three independent experiments, normalized to control. Tubulin was used as loading control. *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$ significantly different from control conditions using the one-way ANOVA test, followed by Dunnett's post hoc test. $^{\#}p < 0.05$ significantly different when compared to ghrelin treatment using unpaired student's *t*-test.

Overall, the results described above report the neuroprotective role that underlies GHR in the apoptotic process accomplished by the decrease of CHOP on ER stress-mediated cell death in endothelial dysfunction. In truth, GHR did not affect caspase-12 ratio levels. Thus, these evidences foreshadow the spare of RBE4 cells by GHR from apoptosis triggered by ER stress via CHOP targeting, an apoptotic preventive role that occurs independently of the caspase-12 cascade inhibition.

4.6 Modulation of UPR by GHR in brain ECs

Previous results confirmed that UPR activation was ameliorated by GHR (Chung et al., 2011; Ercan et al., 2015). UPR is indeed established by widely used ER stress inducers in brain ECs, among them, TG and TM, as described by Plácido and her co-authors (Plácido et al., 2015). In order to further investigate the direct relationship between GHR and the UPR pathway the protein levels of ER stress markers were assessed by WB, including the ER resident chaperone GRP78, eIF2α and the transcription factor XBP-1 (both spliced and unspliced forms), in TG- and TM-treated brain ECs.

For both ER stress inducers, an increased GRP78 expression occurred after 4 and 12 hours, which was partially reversed by GHR with statistical significance for 12 hours. At 4 hours' incubation, the chaperone recovers to its basal levels (Fig. 4.15 A, A'). Protein levels of phosphorylated eIF2α (peIF2α) increased significantly at 4 and 12 hours in TM- and TG-treated brain ECs, respectively, whereas in the presence of GHR, the ratio phospho-/total-eIF2α decreases during the mentioned treatments, but only prominent at 4 hours' TM exposure (Fig. 4.15 B, B'). Surprisingly peIF2α levels climbed down from basal levels after 12 hours' exposure to TM. These levels, which augmented after co-treatment with GHR, became statistically significant between each other (Fig. 4.15 B'). Finally, GHR *per se* increases peIF2α levels comparatively to that detected in control cells.

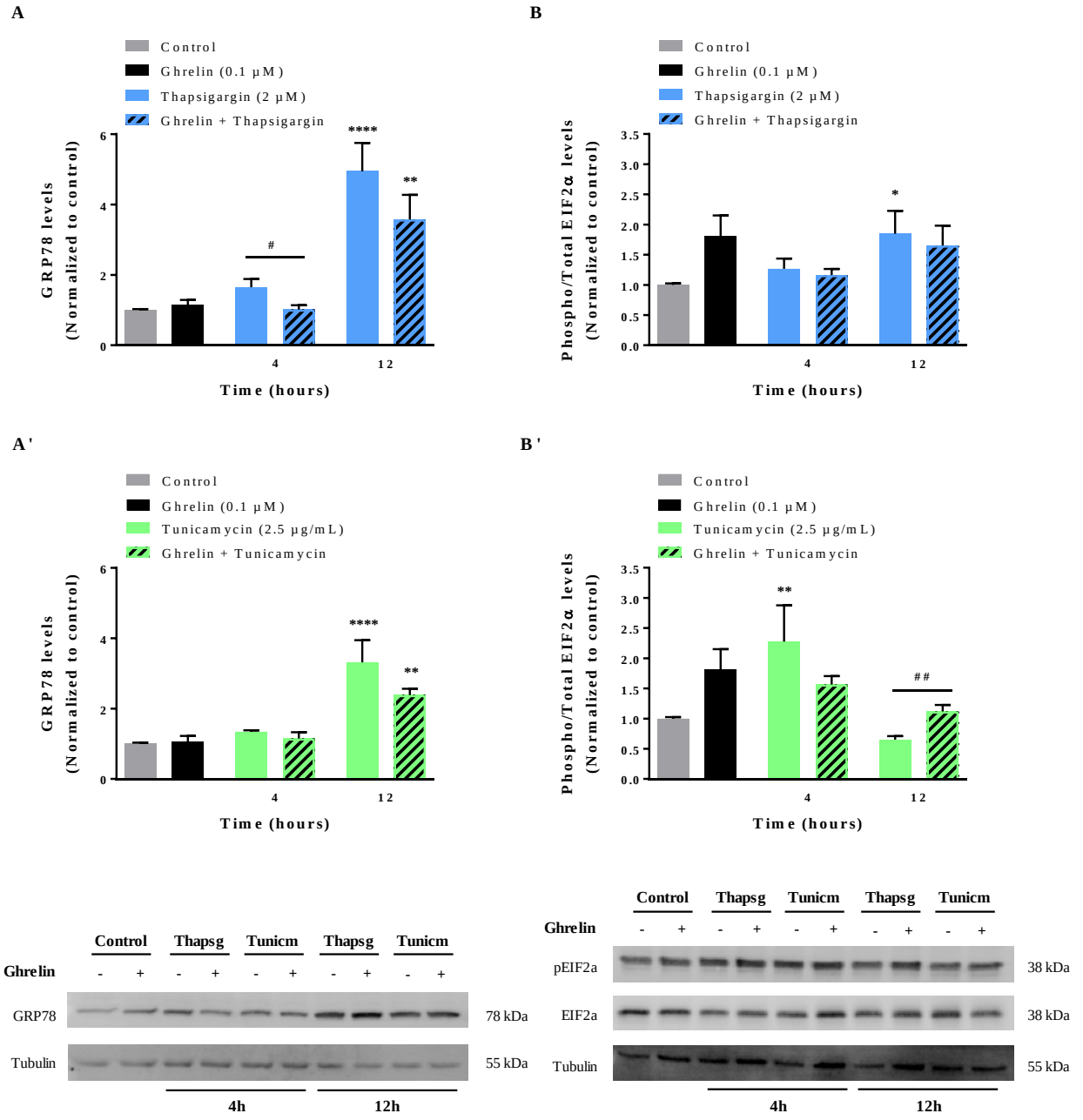


Figure 4.15 ***Effect of ghrelin on ER markers of thapsigargin- and tunicamycin-induced ER stress in brain ECs.***
ER stress was evaluated in treated and untreated RBE4 cells by Western blot. Proteins (40 µg) from total protein lysates obtained from cells incubated for 4 or 12 hours with thapsigargin/tunicamycin (2 µM, 2.5 µg/mL, respectively) alone or in combination with ghrelin (0.1 µM) were separated by SDS-PAGE and immunoblotted with antibodies against of UPR^{ER} markers, namely **(A and A')** GRP78 or **(B and B')** phospho-eIF2α (p-eIF2α^{Ser51}). The ratio between measured protein levels of phospho-eIF2α and total eIF2α was determined. An antibody specific for tubulin was applied as a protein loading control and used to normalize protein levels of ER stress markers. The results were calculated relatively to control values and represent the means ± SEM of values corresponding to at least three independent experiments and normalized to control ones. *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$ (ANOVA with Dunnett's post hoc test) compared with control. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ significantly different from ghrelin treatment using unpaired student's *t*-test.

Regarding XBP-1, uXBP-1 levels are enhanced after 4 and 12 hours' exposure to TG, and were not affected by GHR treatment (Fig. 4.16 A). For TM-mediated ER stress, it was observed a significant increment in the levels of this transcription factor after 12 hours' treatment, which returned to control levels

in the presence of GHR (Fig 4.16 A'). The spliced form of XBP-1 is formed via mRNA cleavage by the ER stress sensor IRE1α, producing an active isoform of XBP-1. Having this in consideration, sXBP-1 expression is promoted under TG and TM influence, becoming significant at 12 hours and 4 hours, respectively. Additionally, for both periods of time, when TG is subjected to GHR, sXBP-1 levels decrease (Fig. 4.16 B). In the same way, GHR displays the same effect on TM co-treatments, although more evident since control levels are replenished. Besides, after 4 hours, our data become statistically significant when compared to TM-treated brain ECs (Fig. 4.16 B').

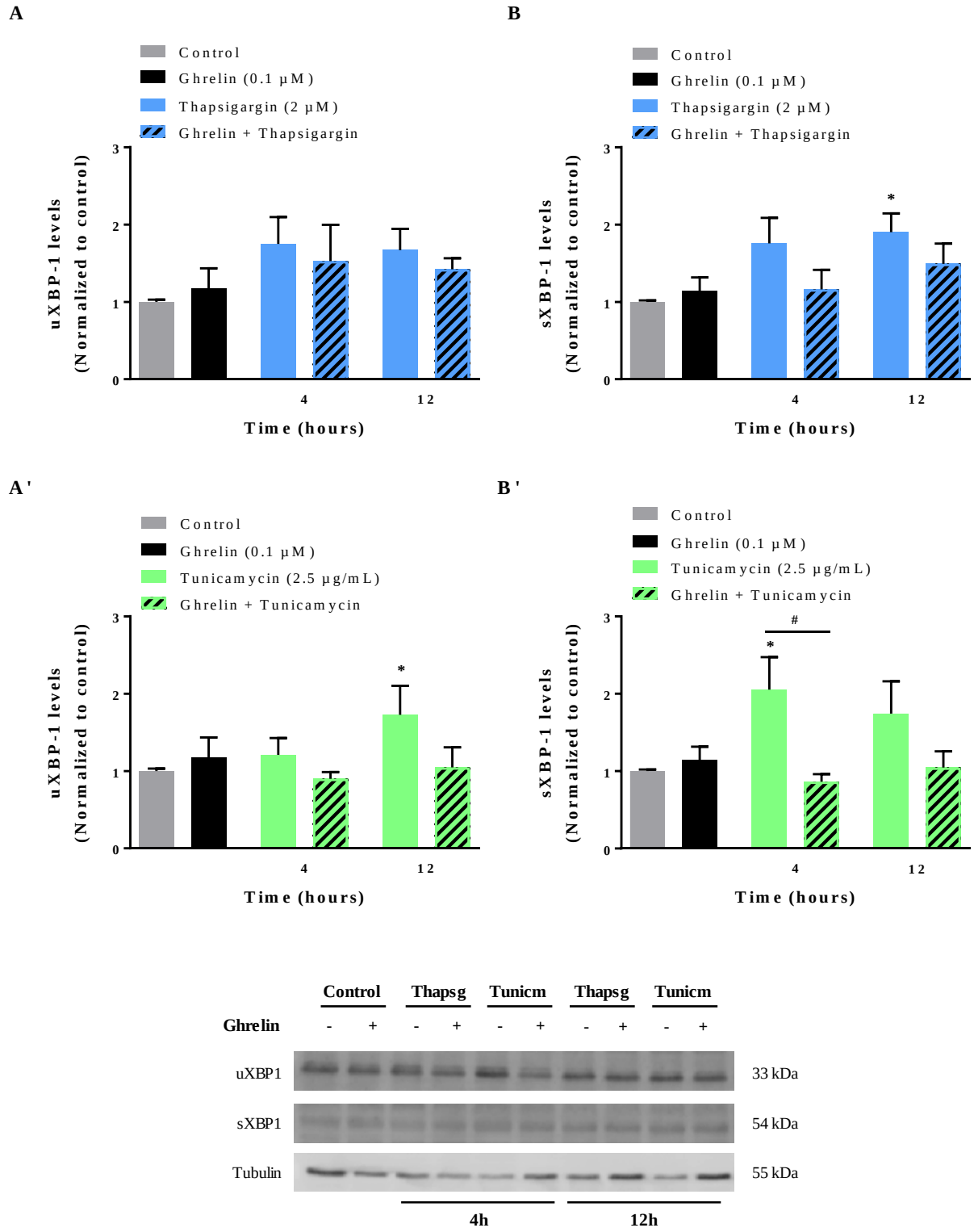


Figure 4.16 ***Effect of ghrelin on two isoforms of XBP-1 of thapsigargin- and tunicamycin-induced ER stress in brain ECs.***

Total protein lysates obtained from RBE4 cells incubated for 4 or 12 hours with thapsigargin/tunicamycin (2 µM, 2.5 µg/mL, respectively) alone or in combination with ghrelin (0.1 µM) were prepared and protein levels of the transcription factor XBP-1, a UPR markers, namely **(A and A')** unspliced or **(B and B')** spliced isoforms were analysed by Western blot. An antibody specific for tubulin was applied as a protein loading control and used to normalize protein levels of ER stress markers. The results were calculated relatively to control values and represent the means ± SEM of values corresponding to at least three independent experiments and normalized to control conditions. $*p < 0.05$ (ANOVA with Dunnett's post hoc test) compared with control. $^{\#}p < 0.05$ significantly different from ghrelin treatment using unpaired student's *t*-test.

These results demonstrate, as expected, that ER homeostasis is compromised, leading to the activation of ER stress-induced UPR in ECs exposed to toxic ER insults. Owing to its protective properties, GHR is able to partially counteract this outcome.

4.7 GHR protects brain endothelial cells from ER stress-induced macroautophagy.

The loss of proteostasis under ER stressful conditions potentiates macroautophagy as a homeostatic mechanism. Nevertheless, this pathway is compromised in brain ECs upon prolonged ER stress induction owing to the inhibition of autophagic flux (Fonseca et al., 2014b). To clarify if GHR is able to restore ECs homeostasis by modulating autophagy, the levels of the macroautophagic marker LC3II that is directly correlated with the amount of autophagosomes (Mizushima and Yoshimori, 2007), were investigated by WB in RBE4 cells treated with classical ER stress inducers, TG and TM, in the presence or in the absence of GHR. Data obtained showed that ER stress increases LC3II levels in RBE4 cells after 12 hours of incubation, which was significantly prevented by co-treatment with GHR (Fig. 4.17 A, A').

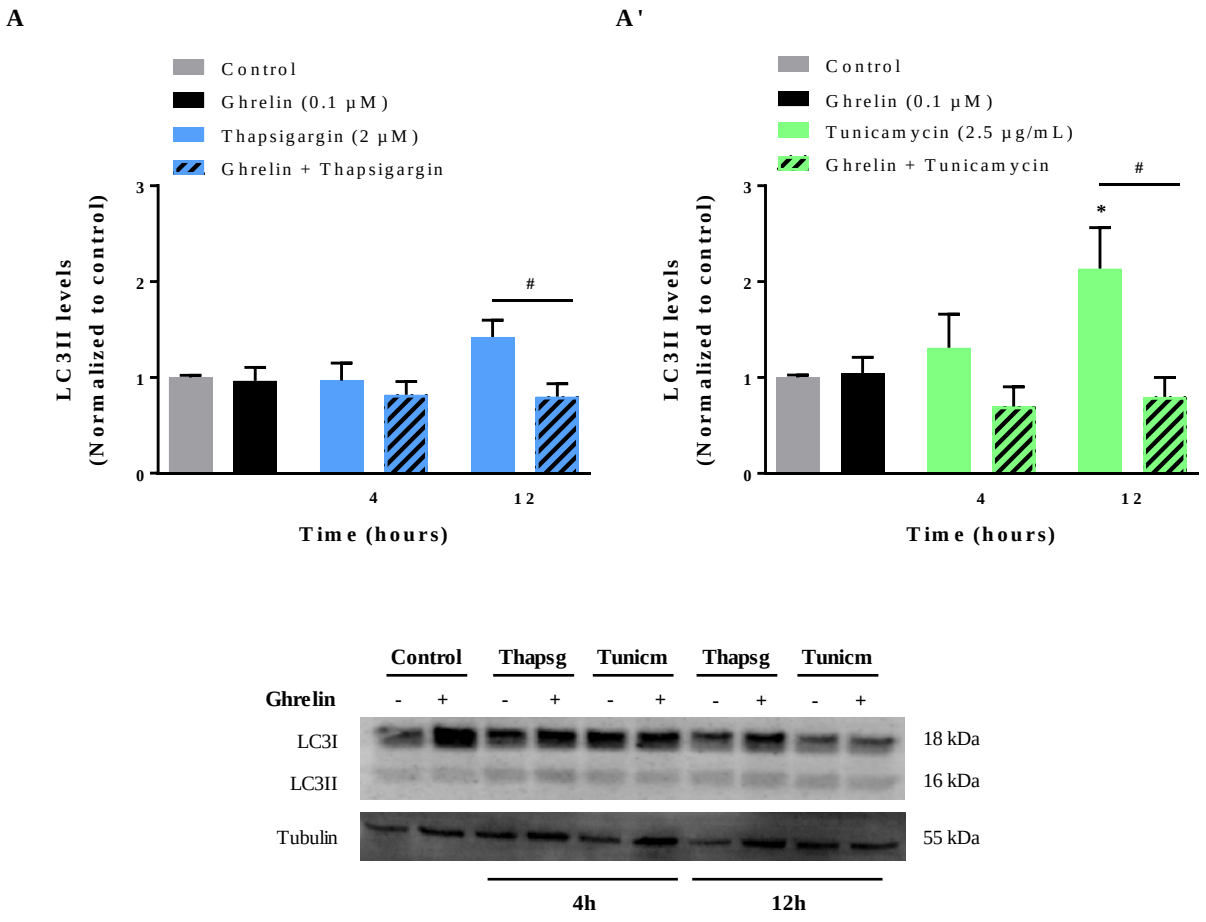


Figure 4.17 ***The protective effect of ghrelin on macroautophagy in brain ECs under prolonged ER stress conditions.*** The autophagic pathway was analysed by immunoblotting in cellular lysates from RBE4 cells treated during 4 or 12 hours with **(A)** 2 µM thapsigargin or **(A')** 2.5 µg/mL tunicamycin and co-treated with ghrelin (0.1 µM). The macroautophagy marker LC3II

levels were normalized using tubulin as a protein loading control. The final results were presented relatively to control values and correspond to the means ± SEM of at least three independent experiments. *$p < 0.05$ (ANOVA with Dunnett's post hoc test) compared with control. $^{\#}p < 0.05$ significantly different from ghrelin treatment using unpaired student's *t*-test.

Nevertheless, our study lacks descriptive information about macroautophagy. An experimental approach to determine the autophagic flux might be fundamental to conclude accurately about GHR action over this disposable system during ER stress. Since LC3 expression levels can vary markedly between different cell types and in response to different stresses, in concordance with the highly dynamic nature of macroautophagy, it is not sufficient to monitor static levels of autophagy. Fitting techniques to measure autophagic flux are: the LC3II turnover using WB analysis in the presence and absence of lysosomal degradation inhibitors, such as pepstatin; reporter systems using tandem fusion of LC3 to the fluorescent proteins, long-lived protein degradation assay or LC3B subcellular localization by immunocytochemistry (Barth et al., 2010; Zhang et al., 2013c; Fonseca et al., 2014b).

Severe ER stress yields a great production of LC3II marker, eventually an excessive degree of macroautophagy that, at this point, redirects ECs to an irreversible state of apoptosis. To achieve a (re)homeostasis status, GHR secure cytoprotection, lessening LC3II levels, and thus macroautophagy as well, that might be harmful and deathly.

4.8 Contribution of GHR on RBE4's exosome profile under ER stressful conditions.

Exosomes are known key players in intercellular communication. In order to better understand its involvement in protein homeostasis, we took advantage of the cultured medium of brain ECs exposed to ER stress conditions to isolate released exosomes, which were characterized through size and concentration analysis. Regarding exosomes' size, it was not affected by TG or TM treatment (Fig. 4.18 A, B). Interestingly, the concentration of released exosomes is affected by ER stress induction but it changes according to the type and duration of the treatment. In general, GHR *per se* keeps a steady concentration of released exosomes. As depicted in Fig. 4.18 A', TG decreases the concentration of exosomes in the extracellular medium, which is more pronounced at 12 hours of exposure and seems to be reverted in cells co-incubated with GHR. 4 hours upon TM-evoked ER stress, exosomes concentration reaches its maximum, which is not affected by GHR, and at 12 hours, the amount of released exosomes drops and is moderately reverted by GHR (Fig. 4.18 B').

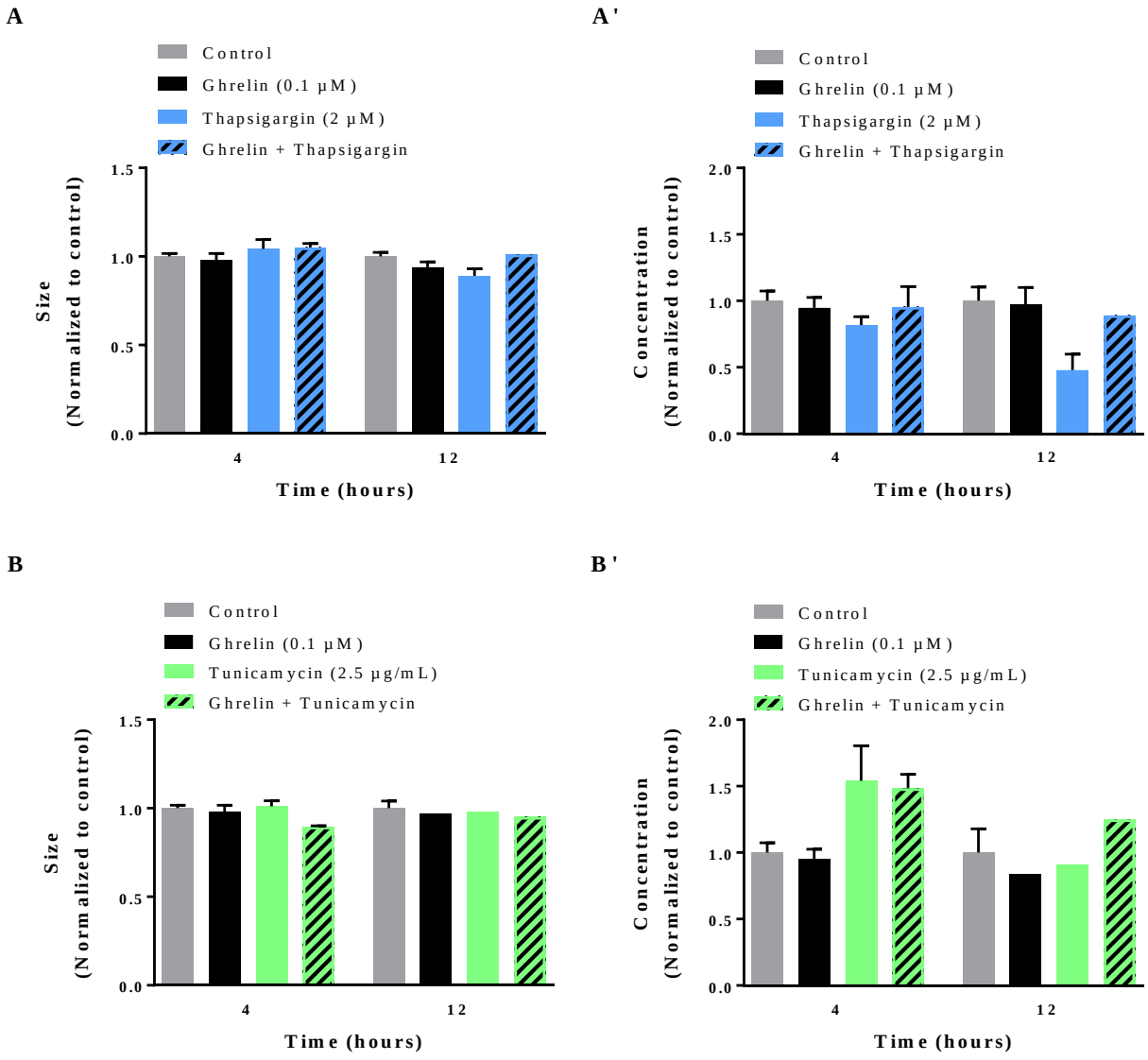


Figure 4.18. ***Characterization of exosomes isolated from control and treated brain ECs.***
Exosomes isolated from controls and RBE4 cells exposed to ER stress were ressuspended and analysed by Nanosight tracking analysis (NTA): treatments consisted in incubations with **(A and A')** 2 µM thapsigargin or **(B and B')** 2.5 µg/mL tunicamycin for 4 or 12 hours, in the presence or absence of ghrelin (0.1 µM). Representative graph of **(A and B')** size distribution and **(A' and B')** exosomal concentration are illustrated as measured by NTA. Results were calculated relatively to control values and represent the means ± SEM of one to three independent experiments.

To sum up, GHR might orchestrate exosomes release as a compensatory mechanism due to the impairment of protein control mechanisms arising from a prolonged ER stress.

CHAPTER 5 – Discussion

Loss of balance between apoptosis and survival pathways in endothelial cells (ECs) results in cellular dysfunction, which favors initiation and progression of different vascular tissues abnormalities. Amongst the causative factors, ER and oxidative stress appear to be major players in endothelial dysfunction (Pober et al., 2009; Witte and Horke, 2011; Placido et al., 2015; Cimellaro et al., 2016). Moreover, ER stress itself comprises ROS generation, being related to several cardiac and brain disorders (Glembotski, 2007; Bhandary et al., 2012). Regarding these cellular noxious effects, GHR arises as a promising rescue strategy since it was demonstrated to improve vascular endothelial function due to ER stress attenuation (Chung et al., 2011; Yang et al., 2012; Virdis and Taddei, 2016).

On this study, to explore the effect of GHR against ER stress-induced toxicity in brain and cardiac ECs, two cell lines were exposed to classic ER stress inducers, namely TG, an agent that blocks the SERCA/Ca^{2+} ATPase (Lytton et al., 1991), and TM, an inhibitor of N-linked glycosylation (Bassik and Kampmann, 2011), in the presence or absence of GHR. Herein, we pretend to evaluate different lines of evidence regarding the protective role of GHR against loss of cell survival or death, ROS accumulation and mitochondrial and Ca^{2+} deregulation. Additionally, the GHR's modulation of homeostatic systems as UPR and macroautophagy, functional endothelial parameters and intercellular communication mediated by exosomes were also investigated.

With respect to cardiac ECs (MCEC-1 cells), our results show that there was a gradual cytotoxic effect induced pharmacologically with ER stressors, emphasizing the lethal effect of chronic ER stress, which was slightly protected by GHR, shown by a slight increase in cell viability. In accordance, MCEC-1 cells migratory phenotype diminished after prolonged ER stress, however GHR did not reveal any influence on cell proliferation. Here lies a contradiction: MCEC-1 cells were more sensitized by ER stress, but that was not translated in diminished cell viability. Despite presenting a minor cardioprotective effect on cell viability upon chronic ER stress, GHR stimulation does not induce recovery from scratch, as seen by the wound healing assay. Furthermore, upon GHR treatment, the differentiation into capillary-like tubes was negatively affected under ER stressful conditions.

Albeit a similar time-dependent cytotoxicity was observed in brain ECs (RBE4 cells), as demonstrated by a significant impairment of cell viability after prolonged ER stressors' exposure, GHR cannot protect cells from such stress. Additionally, GHR did not revert the 50% slower migration rate observed in these cells after prolonged ER stress. However, tube formation was compromised during ER stress and was restored by GHR to control levels. We can conclude that cardiac and brain ECs are differently regulated either by ER stress or GHR, resulting in different angiogenic properties of GHR itself according to ECs origin, and that GHR could regulate ER stress-migration/tubulation in both brain and cardiac EC cells. Brain ECs revealed a pro-angiogenic profile of GHR (surprisingly, GHR itself promotes ECs differentiation), whereas GHR in cardiac ECs possesses an anti-angiogenic effect. Given this, the apparent paradox between cell survival, ER stress and GHR might be explained by the adaptive angiogenic functional and morphological roles according to the cellular typology. More insights about cell behavior in these conditions must be undertaken, especially in cardiac ECs. In fact, evidences pinpointing ER stress and GHR

implications on migration and capillary-like tube formation of ECs are controversial. To date, GHR markedly antagonized tube formation of rat brain ECs and HUVECs, besides its inhibitory action over angiogenic molecules (Baiguera et al., 2004; Conconi et al., 2004), while other authors stated its involvement on vascularization, including on cardiac ECs (Li et al., 2007; Ahluwalia et al., 2009; Wang et al., 2012a, 2012b). Conversely, GHR was reported to promote angiogenesis and to control ECs migration by inhibition of angiotensin-II, becoming an indirect angiostatic molecule (Rossi et al., 2007; Wang et al., 2011, 2012b; Chen et al., 2013; Katare et al., 2016). ER stress seems to modulate angiogenesis/migration according to the surrounding environment, whether it is a physiological or a pathological context. Usually, EC migration and angiogenesis is prompted under physiological conditions. The reversed effect happens ensuing a pathological mechanism. Moreover, the magnitude of ER stress might induce different outcomes as well (Kimura et al., 2001; Pereira et al., 2010; Nakamura et al., 2013; Ying et al., 2016).

From now on, all experimental consideration referred to events underlying brains ECs.

First, ER stress-triggered apoptosis was evaluated. For that, the levels of the ER stress-dependent apoptotic mediators' caspase-12 and CHOP were measured. From the results, it is evident the protective role of GHR against chronic ER stress-mediated apoptosis, mostly when GHR efficiently reduced CHOP levels in this stressed context. Interestingly, cell death could not be brought about by caspase-12 dependent cascade, due to ineffective action of GHR during ER stress on caspase-12 levels. This differential regulation of apoptotic mediators might enlighten the reason why a fraction of GHR-treated RBE4 cells still display apoptosis features. Perhaps caspase-12 conveys a more preponderant apoptotic signal during ER stress in brain ECs, i.e. neither overexpression nor downregulation of caspase-12 affected susceptibility of the cells to ER stress-induced cell death. So GHR might regulate apoptotic protease activating factor-1 (Apaf-1) levels instead of caspase-12 (Shiraishi et al., 2006), and likely protect RBE4 cells from a pathway independent from caspase-12 inhibition. However, this hypothesis raises some questions, due to the widely described protective role of GHR against caspase-12 in acute ER stress (Sun et al., 2008; Yang et al., 2012; Zhang et al., 2013a). As previously said, ER stress duration and the cellular context are key factors to have in consideration. Nevertheless, CHOP-derived apoptosis during extensive ER stress is reversed significantly by GHR, as reported by other authors (Chung et al., 2011; Ercan et al., 2015).

In addition to balance the regulation of pro- and anti-apoptotic mediators, prolonged ER stress-induced CHOP-mediated cell death occurs through increased protein synthesis and oxidative stress induction that are necessary signals for cell death (Han et al., 2013). The induction of CHOP is also associated to the activity of the oxidoreductase ERO1α (Marciniak et al., 2004). Therefore, CHOP can lead to ERO1α-mediated oxidative stress, yielding H_2O_2 generation (Han et al., 2013). In RBE4 cells largely exposed to ER stressors, the increase in CHOP levels was followed by a significant augmentation of ROS levels, in this case H_2O_2. Moreover, ERO1α appears to be upregulated as does ROS, suggesting that CHOP is involved in oxidative stress prevalence in brain ECs under stress conditions. GHR reverted both ERO1α upregulation and subsequent ROS production, although only ERO1α levels were statistically significant, being an indicative claim for GHR protective role against oxidative stress-mediated apoptosis response in ECs from

brain. A plausible underlying mechanism for GHR protective effect involves the transcription of UCPs by ROS, mainly UCP-2, to further buffer its own levels (Andrews et al., 2008; Liu et al., 2009; Toda and Diano, 2014). Other putative defensive function of GHR might be related to the upregulation of Bcl-2 (anti-apoptotic mediator), possibly by the decreased CHOP levels, but also as consequence of a diminishing phosphorylation of NF-κB transcription factor (Zhang et al., 2011). Moreover, GHR might antagonize ER stress and H_2O_2-mediated apoptosis by protecting the mitochondria. Moreover, last year, GHR was pointed out to ameliorate endothelial dysfunction through abrogation of H_2O_2-induced NOX activity (Virdis et al., 2015). So, GHR antioxidant effect on NOX subsequently restored NO bioavailability. Therefore, it is possible that these mechanisms are operating under the tested conditions herein, and UCP-2, Bcl-2 upregulation in partnership with fewer activity of NOX and NF-κB may be involved in protection afforded by GHR against prolonged ER stress-induced toxicity.

Another focus of this work was the ER-to-mitochondria communication and Ca^{2+} trafficking between these two organelles since the stabilization of mitochondrial function is crucial in ER stress-challenged RBE4 cells. Despite no evidences about GHR modulation of ER Ca^{2+} store under ER stress are here depicted, mitochondrial Ca^{2+} regulation by GHR under ER stressed environment was accessed. The obtained date, however, stands for a single experiment. If the tendency continues, our main conclusions are: Ca^{2+} fluxes into mitochondrial are transiently decreased spanning the progressive severity of ER stress, and GHR is able to slightly revert Ca^{2+} content established by ER stress in mitochondria, in treated RBE4 cells. Furthermore, Ca^{2+} released from ER can be rapidly accumulated in the mitochondria via MAM. It is believed that ER-mitochondria interaction is modulated by MAMs-localized proteins, such as Sig1R and VDAC (Hayashi et al., 2009). Next, we uncovered their roles on the tethering between Ca^{2+} flux from ER to mitochondria. Both molecules are upregulated in a time-dependent manner upon ER stress, which is decreased or even normalized to control levels by GHR. Previous studies from our group in RBE4 cells treated with TG supported the gradual decrease in mitochondrial Ca^{2+} content, because of the primarily overload of mitochondria matrix with Ca^{2+} due to its depletion from ER (Plácido et al., 2015). Additionally, Sig1R chaperone establishes a link with GRP78, causing its inactivation (Nguyen et al., 2015). This interaction is abrogated when ER Ca^{2+} is released, activating and promoting an interaction with a new partner, IP_3R leading to its stabilization. At the same time, Ca^{2+} flows into the mitochondria until a certain level (Su et al., 2010). When mitochondria start to deplete its content of Ca^{2+}, VDAC interacts with IP_3R, triggering an apoptotic signal. In accordance, VDAC is also a member of the mitochondrial permeability transition pore, involved in apoptosis, and caused by ROS generation (De Stefani et al., 2012). Once again, GHR anti-apoptotic capacity prevails over ER stress stimulus, by decreasing the levels of Sig1R and VDAC, probably localized on MAMs, and by that preventing VDAC to induce apoptosis and connect to IP_3R, at the same time that Sig1R reattaches to GRP78. This mechanism seems to overcome the Ca^{2+} dysregulation in mitochondria, inhibiting an overload to its matrix space. Besides, recent evidences suggest that Sig1R modulates ER stress by induction of cell survival signaling via ERK1/2 and Bcl-2 (Ha et al., 2014) and also support the involvement of GHR-mediated protection against mitochondrial dysfunction/apoptosis by

inhibiting caspase-3 (Martins et al., 2013; Yu et al., 2016), which could corroborate the protective effect of GHR. Despite all these conclusions, these data needs further clarification, at least the performance of additional sets of experiments.

During ER stress, deregulated amounts of Ca^{2+} inside cells can lead to activation of various Ca^{2+}-regulated pathways, including autophagy. Next, we sought to determine the effect of GHR on macroautophagy in brain ECs subjected to ER stress. Our results show that severe ER stress yields a great production of LC3II marker, whether this expression is lessened by GHR as a cytoprotective mechanism. Indeed, ER stress triggers macroautophagy, at first as a homeostatic mechanism to recover from stress, however, a durable stimulus switches the homeostatic to a noxious system that conducts to cell death (Katsiougiannis et al., 2015). When GHR plays its role against chronic ER stress as a ECs warden, it blocks excessive macroautophagy by downregulation of LC3II (Nikoletopoulou et al., 2013). Since ER stress can also be a consequence of extracellular Ca^{2+} influx, therefore, it is likely that ER Ca^{2+} is the main regulator of autophagy mediated by the UPR as an adaptive response to secure cell survival. In fact, the modulation of UPR branches modulates autophagy (Ogata et al., 2006; Katsiougiannis et al., 2015; Rashid et al., 2015). As a consequence, ER Ca^{2+} perturbation function as a modulator itself. Additionally, IP_3R modulation by ER Ca^{2+} can induce autophagy, thus MAMs possibly can be also involved in autophagy (Decuypere et al., 2011a).

Other aspect about intracellular Ca^{2+} mobilization is the activation of CAMKK, which in turn triggers autophagy via AMPK-dependent mTOR inhibition (Rashid et al., 2015). This pathway highlights and supports the protective role of GHR by blocking macroautophagy. AMPK has a dual role in autophagy. It functions as a switch between the final objective of triggering this protein quality control machinery, i.e. upregulation of AMPK leads to macroautophagy (Yuan et al., 2014; Mao et al., 2015; Yu et al., 2015), whereas its inhibition causes its abrogation (Wang et al., 2014). The last sentence clearly sustains the role of GHR in chronic ER stress-derived macroautophagy, because CAMKK as well as the AMPK/mTOR pathway are downstream effectors of GHS-R1α signal transduction (Zhang et al., 2013b). So GHR can be a regulator of macroautophagy by balancing its fate towards a cytotoxic or cytoprotective role, corresponding to activation or inhibition of GHR-induced AMPK. Other mechanisms could involve macroautophagic modulation by GHR through activation of the p38-MAPK pathway, inhibition of ROS generation that acts as a trigger of macroautophagy (Wang et al., 2014), and inhibition of NF-κB translocation into the nucleus (Mao et al., 2015).

If we extend this way of reasoning, some authors proposed AMPK cascade as an effective protector versus ER stress and apoptosis through downregulation of GRP78, an ER chaperone and precursor of the UPR response, CHOP and caspase-12, in cells treated with GHR (Zhang et al., 2009, 2013a). We then evaluated the levels of ER stress markers in order to conclude about GHR protection against ER stress-induced UPR on brain ECs. GRP78 levels increased in a time-dependent manner, as did peIF2α, both significantly, upon ER stress induction in brain ECs. Both markers were modulated by GHR to basal levels. Regarding XBP-1, the active form was upregulated by ER stress, and GHR counteracted this increase. Short

exposures to ER stressors increased the uXBP-1 levels but only at prolonged exposure it become significant. GHR had no effect after 4 hours' treatment, but decreased the level of this XBP-1 isoform during prolonged ER stress in RBE4 cells. This may suggest that GHR sustains UPR response via IRE1α during mild ER stress, which correlates to lower levels of CHOP and consequently of ERO1α. On the literature, this was supported by the view that suppression of CHOP activation via inhibition of the eIF2α/ATF4 pathway, which prevents Foxo1 activation and nuclear translocation, can mechanistically explain GHR-mediated protection during ER stress responses. Besides, PI_3K/Akt-mediated inactivation of GSK-3β, BAD and Foxo1 may be associated with the anti-apoptotic effect of GHR, as well (Chung et al., 2011), that may also involve MAPK, as previously mentioned, PKC and PKA (Chung et al., 2007, 2008).

XBP-1 has also been correlated to endothelial function, due its pro-survival and pro-angiogenic features. Moreover, UPR was found to induce the expression of VEGF, which is regulated by XBP-1 (Duan et al., 2016). This possibly means that inactive and active XBP-1 isoform ratio is unchanged by GHR in order to develop its function as a pro-angiogenic molecule. These evidences also support the differentiation into capillary-like tube in the angiogenesis assay in brain ECs, since GHR itself is a pro-angiogenic agent. Here, Akt is the signaling pathway that prevails, reinforcing the VEGF and Bcl-2 activation (Katare et al., 2016). AMPK also appears to modulate angiogenic function when prompted by GHR, increasing also VEGF and activating eNOS (Wang et al., 2015). This might explain the replenishment of NO bioavailability brought about by GHR antioxidant effect against NOX. Besides, GHR induces cell migration through GHS-R, CaMKII, AMPK, and NF-κB signaling pathways (Chen et al., 2011), corroborating our previous results.

Finally, in brain ECs, the concentration of released exosomes was affected upon ER stress induction but it changed according to the type and duration of the treatment. In prolonged ER stress, GHR-treated RBE4 cells effectively released higher amounts of exosomes, when compared to brain ECs only stimulated by ER stressors. It is interesting that the cargo type may change upon stimuli, and thus, endothelial exosomes might transfer stress factors to the other cells in the vicinity (András and Toborek; de Jong et al., 2012) or function as a disposable system to lessen the degree of the stress, such as oxidative stress reported in a brain endothelial dysfunction model (Kalani et al., 2014). Another study showed that human ECs undergoing apoptosis and autophagy release exosomes enriched with autophagosomes and mitochondria (Pallet et al., 2013). Altogether, our results suggest that GHR could keep proteostasis in a population of brain ECs by releasing more exosomes after prolonged stressors stimuli as a pathway to relief RBE4 cells from macroautophagy, apoptosis and/or oxidative stress. From formerly presented data, low levels of LC3II could result from the release of exosome-containing autophagosomes by GHR, instead of decreasing LC3II expression. Further experiments must be done in order to characterize the content of brain ECs-derived exosomes in these conditions, and according to that, infer about the mechanism of action through which GHR protects RBE4 cells. In conclusion, GHR might orchestrate exosomes release to alleviate dying autophagic ECs from ER stress.

Taken together, it can be concluded that GHR exerts a partial protective effect in brain ECs against ER stress-induced toxicity. Specifically, GHR can prevent chronic ER stress by a two-front mechanism. First,

we might assume crosstalk between PI_3K/Akt and AMPK signaling pathways, then we might speculate a putative enrollment of exosome, released by stimulation of GHR. Therefore, GHR could act (or not) through a synergetic mechanism between exosomes and PI_3K/Akt-AMPK cascades. However, further research should be done to better understand the molecular mechanisms underlying such role.

CHAPTER 6 – Conclusions

Vascular abnormalities associated with several human pathologies have been implicated in endothelial dysfunction that in turn may result from ER stress. Prompting as a cardio and neuroprotective hormone, GHR might reverse ER stress and antagonize early endothelial dysfunction. In order to elucidate the mechanisms behind the beneficial effect of GHR on endothelial cells (ECs), we investigated the GHR-mediated modulation of impaired protein quality control mechanisms, Ca^{2+} and redox dyshomeostasis in brain ECs, in parallel to morphological and functional derangement in both cardiac and brain ECs, as a result of ER stress resultant from exposure to TM and TG.

The major findings of this work are:

- cardiac and brain ECs are differently regulated by ER stress and GHR – **MCEC-1 cells** are more sensitized by ER stress in migration assay than in cell viability, but when these ECs are extensively stressed, GHR does not reestablish MCEC-1 cells' migratory rate, neither their capillary-like tube formation (provokes its undifferentiation); **RBE4 cells'** are not protected by GHR from chronic ER stress regarding cell survival and migration, but GHR prevents a compromised tube formation. Summing up, GHR roles depends on the type of ECs, as a result cardiac ECs promotes GHR anti-angiogenic properties in opposition to the pro-angiogenic excelled in brain ECs.
- Prolonged ER stress-induced apoptosis cell death is abrogated by GHR in RBE4 cells, through a mechanism independent of caspase-12 inhibition, but that requires CHOP blockage and Apaf-1 regulation.
- GHR reverts oxidative stress-mediated ER stress in RBE4 cells, possibly by protecting the mitochondria via inhibition of CHOP-mediated apoptosis, thus downregulating ERO1α and NOX activities, ROS levels and NF-κB phosphorylation and upregulating of Bcl-2; UCP-2 may function as a ROS scavenger.
- In ER stressed RBE4 cells, GHR tries to reestablish basal mitochondrial Ca^{2+} levels, supposedly by downregulation of MAMs-containing proteins, and by that preventing VDAC to induce apoptosis and connect to IP_3R (an indicator of the mitochondrial permeability transition pore formation), at the same time that Sig1R reattaches to GRP78 and induces signals from cell survival pathways (ERK1/2 and Bcl-2) by contrast of cell death ones (caspase-3).
- Throughout ER stress in RBE4 cells, macroautophagy turns out to be cytoprotective at first, but in excess promotes cytotoxicity. Under these conditions, GHR seems to block macroautophagy, as showed by low levels of LC3II. Both extracellular and ER Ca^{2+} modulates macroautophagy, as a consequence Ca^{2+}-dependent proteins from the cytosol or MAMs. CAMKK is a possible target, that once activated, triggers autophagy via AMPK-dependent mTOR inhibition. UPR might also function as regulator.
- GHR diminishes UPR response via eIF2α during ER stress, which correlates to lower levels of CHOP leading to PI_3K/Akt-mediated inactivation of Foxo1, associated with the GHR's anti-apoptotic effect. GRP78 is also downregulated. Under these circumstances, inactive and active XBP-1 isoform ratio is unchanged by GHR in order to develop a pro-angiogenic function, evident

on capillary-like tubes above mentioned in RBE4 cells. Probably, GHR-mediated Akt and AMPK might function together, favoring ECs' VEGF production.

- AMPK triggered by GHR could provoke cell migration in RBE4 cells during ER stress.
- Under prolonged ER stress, GHR promotes exosomes release from brain ECs to prevent apoptosis, macroautophagy and/or oxidative stress. Possibly, these exosomes contain autophagosomes, which explains their upregulation.

To sum up, GHR protective role against endothelial dysfunction could be characterized by a crosstalk between PI_3K/Akt and AMPK cascades, and/or the elimination of harmful mediators of ER stress by exosomes, strongly supported by evidences regarding exosomes release and prevention of oxidative stress, macroautophagy and apoptosis (Fig. 6.1).

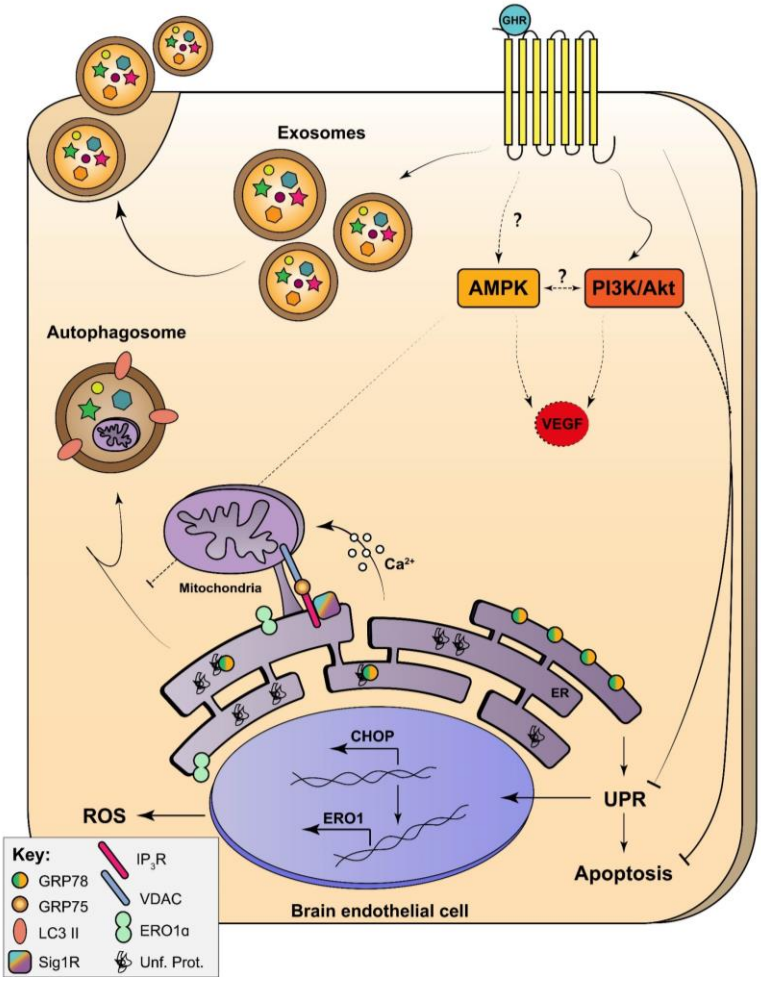


Figure 6.1 ***Hypothetical mechanism through which ghrelin (GHR) partially ameliorates chronic endoplasmic reticulum (ER) stress-induced endothelial dysfunction.***

On brain ECs, the GHR's protective features are described to result from the downregulation of mediators of the ER stress-induced unfolded protein response (UPR) pathway, possibly via activation of AMPK and/or PI3K/Akt cascades. Subsequently, CHOP-mediated apoptosis is declined, thereby influencing the expression of ERO1α and the production of ROS. Other mechanisms, namely macroautophagy and ER-to-mitochondria crosstalk (both MAM components and Ca^{2+} transport), are modulated by GHR in order to relief ER from the burden of misfolded proteins. Moreover, exosomes release from ECs can be prompted by GHR, ensuing the reestablishment of the proteostasis by cleansing cells from harmful mediators. Given the pro-angiogenic properties exacerbated by GHR, AMPK and PI3K signaling could induce vascular endothelial growth factor (VEGF) secretion. **Ca^{2+}**, calcium ions; **CHOP,** CAAT/enhancer binding protein homologous protein; **ERO1,** ER oxidoreductin 1; **GRP75/78,** Glucose-related protein; **LC3II,** microtubule-associated protein light chain 3II; **ROS,** Reactive Oxygen Species; **Sig1R,** Sigma 1 Receptor; **Unf. Prot.,** Unfolded proteins; **VDAC,** Voltage-dependent anion channel.

Further investigation regarding the mechanism of GHR action, namely unraveling of AMPK signaling and ER stress connection, might provide valuable information for downstream molecular mechanisms, in addition to PI_3K/Akt cascade involvement. Particularly, our next approaches will aim the GHR effects on ER Ca^{2+} trafficking, UPS (a promising suggestion, owing to earlier reports that showed UPS potentiation by GHR in neuronal cells (Cecarini et al., 2016)) and inflammation upon ER stress. Under these conditions, it is also crucial to evaluate and characterize endothelial exosomes, either its content and its membrane. Besides, it will be interesting to test GHR in models that resembles physiologically best ER stress instead of pharmacological induction, that could lead to some toxicity. Furthermore, it is necessary to explore the other possible implications of signaling pathways that are activated downstream of GHR receptor in brain ECs. We are also interested in exploring all the tasks that we could not perform in cardiac ECs.

Nevertheless, a complement of morphological and functional data here collected, in particular the examination of molecular mechanisms in cardiac ECs concerning ER stress must be developed in order to understand the already attributed cardioprotective role of GHR.

In conclusion, these findings are relevant because GHR can function as a protective agent and may have therapeutic potential for the treatment of neurodegenerative and cardiac diseases where ER stress plays a major role.

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