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Abstract

Alzheimer's disease, the most prevalent age-related neurodegenerative disease, is characterized by a progressive deterioration in memory and cognitive function and severe neurodegeneration associated with the occurrence of two distinctive pathological hallmarks: the extracellular deposition of amyloid-β in brain parenchyma and cerebral blood vessels and the presence of intracellular neurofibrillary tangles containing hyperphosphorylated tau protein. Currently, there is still no cure or effective therapy for Alzheimer's disease.

Among the existing protective strategies, preconditioning emerges as a phenomenon that triggers brain tolerance against potential lethal insults by activating endogenous adaptive and pro-survival events. Although the exact molecular mechanisms underlying preconditioning remain unclear, the transcription factor hypoxia-inducible factor-1α and mitochondria have been proposed to be critical pieces of the preconditioning puzzle. Particularly, mitochondrial-derived reactive oxygen species and mitochondrial ATP-sensitive potassium channels were identified as specific mitochondrial mediators and targets in the preconditioning phenomenon. Within this scenario, this study was undertaken to decipher the role of mitochondrial-derived reactive oxygen species and hypoxia-inducible factor-1α signaling pathway in the protection triggered by pinacidil preconditioning, a mitochondrial ATP-sensitive potassium channel modulator, in an *in vitro* model of Alzheimer's disease induced by okadaic acid that promotes tau hyperphosphorylation by inhibiting protein phosphatases 1/2A.

Using the rat brain endothelial cell line RBE4, it was observed that non-toxic concentrations of pinacidil (≤ 10μM; 24 hours) significantly increased superoxide anion production. Confocal microscopy analysis revealed that pinacidil affects mitochondrial network by promoting a perinuclear distribution of elongated mitochondria. Pinacidil also increased the protein levels of glucose transporter-1 and vascular endothelial growth factor, two specific downstream target genes of hypoxia-inducible factor-1 α. Importantly, pinacidil preconditioning prevented the loss of cell viability and mitochondrial membrane potential, and superoxide anion overproduction promoted by okadaic acid. However, the protective effects mediated by pinacidil preconditioning were abolished by the specific mitochondrial ATP-sensitive potassium channel antagonist 5-hydroxydecanoic acid, the mitochondrial reactive oxygen species scavenger coenzyme Q_{10}, and the hypoxia-inducible factor-1 α inhibitor 2-metoxyestradiol. Finally, pinacidil preconditioning partially attenuated the effect of okadaic acid on mitochondrial shape (smaller and round mitochondria) and spatial distribution (perinulear area).

Overall, these results show that mitochondrial ATP-sensitive potassium channels activators-mediated reactive oxygen species production and induction of hypoxia-inducible factor-1α are critical mechanistic "steps" involved in the preconditioning phenomenon and suggest that mitochondrial ATP-sensitive potassium channels may represent possible therapeutic targets for Alzheimer's disease.

Keywords: Alzheimer's disease, brain endothelial cells, hypoxia-inducible factor-1α, mitochondria, mitochondrial ATP-sensitive potassium channels, okadaic acid, pinacidil, preconditioning, reactive oxygen species

Resumo

A doença de Alzheimer, a doença neurodegenerativa mais comum no idoso, é caracterizada por uma deterioração progressiva da memória e função cognitiva, e neurodegenerescência severa associada à ocorrência de duas características patológicas distintas: a deposição extracelular do peptídeo β-amiloide no parênquima cerebral e vasos sanguíneos cerebrais e acumulação intracelular de tranças neurofibrilares compostas por proteína tau hiperfosforilada. Actualmente, não existe nem cura nem tratamento eficaz para a doença de Alzheimer.

Entre as estratégias de protecção existentes, o pré-condicionamento surge como um fenómeno que confere tolerância cerebral contra possíveis insultos letais através da activação de uma resposta endógena adaptativa e de sobrevivência. Embora os mecanismos moleculares específicos associados ao pré-condicionamento permaneçam desconhecidos, o factor de transcrição induzido por hipóxia 1α e as mitocôndrias são reconhecidos como peças integrantes do puzzle do pré-condicionamento. Especificamente, as espécies reactivas de oxigénio produzidas pelas mitocôndrias bem como os canais de potássio mitocondriais sensíveis ao ATP foram identificados como intermediários e alvos mitocondriais específicos envolvidos no fenómeno do pré-condicionamento. Desta forma, este estudo foi realizado para decifrar o papel das espécies reactivas de oxigénio produzidas pela mitocôndria e da via de sinalização mediada pelo factor de transcrição induzido por hipóxia 1α na proteção desencadeada pelo pré-condicionamento com pinacidil, um modulador dos canais de potássio mitocondriais sensíveis ao ATP, num modelo *in vitro* da doença de Alzheimer induzido pelo ácido ocadaico que promove a hiperfosforilação da proteína tau, através da inibição das fosfatases 1 e 2A.

Usando as células RBE4, uma linha de células endoteliais de cérebro de rato, observou-se que concentrações sub-letais de pinacidil (≤ 10 μM, durante 24 horas) aumentaram significativamente a produção de superóxido. Recorrendo a microscopia confocal verificou-se que o pinacidil altera a rede mitocondrial nas células endoteliais, promovendo uma distribuição perinuclear de mitocôndrias alongadas. Constatou-se ainda que o pinacidil também aumenta os níveis proteicos do transportador de glucose-1 e do factor de crescimento do endotélio vascular, dois genes alvo específicos do factor de transcrição induzido por hipóxia 1α. Notavelmente, o pré-condicionamento com pinacidil impediu a perda de viabilidade celular e o potencial de membrana mitocondrial, e a produção excessiva de superóxido promovida pelo ácido ocadaico. Contudo, os efeitos protectores do pré-condicionamento com pinacidil foram inibidos na presença do antagonista específico dos canais de potássio mitocondriais sensíveis ao ATP, o ácido 5-hidroxidecanóico, do antioxidante mitocondrial coenzimaQ_{10}, e do inibidor do factor de transcrição induzido por hipóxia 1α, o 2-metoxiestradiol. Por último, o pré-condicionamento com pinacidil preveniu parcialmente o efeito do ácido ocadaico na morfologia (mitocôndrias pequenas e arredondadas) e distribuição espacial (zona perinulear) das mitocôndrias.

Em conjunto, estes resultados mostram que a produção de espécies reactivas de oxigénio mediada por activadores dos canais de potássio mitocondriais sensíveis ao ATP e a indução do factor de transcrição induzido por hipóxia 1α são eventos cruciais no fenómeno do pré-condicionamento, sugerindo que os canais de potássio mitocondriais sensíveis ao ATP podem representar possíveis alvos terapêuticos na doença de Alzheimer.

Palavras-chave: Ácido ocadaico, canais mitocondriais de potássio sensíveis a ATP, células endoteliais cerebrais, doença de Alzheimer, espécies reactivas de oxigénio, factor de transcrição induzido pela hipóxia, mitocôndria, pinacidil, pré-condicionamento

List of Abbreviations

Abbreviation	Description
2-ME2	2-methoxyestradiol
5-HD	5-hydroxydecanoate
AD	Alzheimer's disease
AIF	Apoptosis-inducing factor
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP-1	Activator protein-1
APOE4	Apolipoprotein E4
ATP	Adenosine triphosphate
Aβ	Amyloid-β peptide
AβPP	Amyloid-β-protein precursor
BBB	Blood-brain barrier
CAA	cerebral amyloid angiopathy
Ca^{2+}	Calcium ion
cAMP	cyclic adenosine monophosphate
Cdk	Cyclin-dependent kinase
CNS	Central nervous system
CoQ_{10}	Coenzyme Q_{10}
DRP 1	Dynamin-like protein 1
ERK	Extracellular regulated kinase
fAD	Familial Alzheimer's disease
FIS1	Fission 1 protein
GSK-3β	Glycogen synthase kinase-3β
GLUT	Glucose transporter
HIF	Hypoxia-inducible factor
ICV	Intracerebroventricular
Kb	kilo base pairs
Kir	Inwardly rectifying potassium channel
MAPs	microtubule-associated proteins
MAPK	Mitogen-activated protein kinase
MAPT	microtubule-associated protein tau
MEK	Mitogen-activated protein kinase kinase
Mfn 1	Mitofusin 1

Mfn 2	Mitofusin 2
MitoK_{ATP}	Mitochondrial ATP-sensitive potassium channel
ΔΨm	Mitochondrial membrane potential
MPTP	Mitochondrial permeability transition pore
mtDNA	Mitochondrial deoxyribonucleic acid
NFT	Neurofibrillary tangles
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA	N-methyl D-aspartate
NO·	Nitric Oxide
NRF-1	Nuclear respiratory factor-1
NRF-2	Nuclear respiratory factor-2
O_2·	Superoxide anion
OA	Okadaic acid
OPA 1	Optic atrophy protein 1
OXPHOS	Oxidative phosphorylation
PGC-1α	Peroxisome proliferator activator receptor gamma-coactivator 1α
PHD	Prolyl hydroxylase
PHF	Paired helical filaments
Pin	Pinacidil
PINK1	PTEN-induced putative kinase protein 1
PKB	Protein kinase B
PKC	Protein kinase C
PP	Protein phosphatase
PTEN	Phosphatase and tensin homolog
RBE	Rat brain endothelial cells
ROS	Reactive oxygen species
sAD	Sporadic Alzheimer's disease
sK_{ATP}	Surface membrane ATP-sensitive potassium channel
Ser	Serine
Sur	Sulfonylurea receptor
TCA	Tricarboxylic acid cycle
TFAM	Mitochondrial transcription factor A
Thr	Threonine
Tyr	Tyrosine
VEGF	Vascular endothelial growth factor

Chapter 1 - Introduction

1.1 Alzheimer's disease: a brief overview

First identified by Alois Alzheimer in 1906, Alzheimer's disease (AD) is the most common age-related neurodegenerative disorder that affects approximately 35 million people worldwide, being a leading cause of morbidity and mortality in elderly people (Querfurth & Laferla 2010). In Portugal, it has been estimated that approximately 90,000 individuals suffer from AD (Alzheimer Portugal 2009). Despite a massive global research effort, currently there are no cure nor effective disease-modifying drugs for AD (Tillement et al., 2011).

Clinically, AD is characterized by a progressive cognitive deterioration together with impairments in behavior, language, and visuospatial skills, culminating in the premature death of the individual (Querfurth and LaFerla, 2010). From a neuropathological point of view, this disorder is marked by the occurrence of two distinctive hallmarks: the presence of intracellular neurofibrillary tangles (NFTs) containing hyperphosphorylated tau protein and the extracellular deposition of amyloid-β (Aβ) peptide in brain parenchyma (senile plaques) and cerebral blood vessels (cerebral amyloid angiopathy, CAA) (Correia et al. 2012a). AD is also characterized by severe neuronal degeneration, which initially occurs in the entorhinal region and the temporal lobe progressing to the limbic system and, subsequently, extending to major areas of the neocortex (Braak & Braak 1995). However, cerebrovascular defects have been shown to precede neuronal changes during the course of AD pathology (Zlokovic 2011). Indeed, the anatomical footprint of cerebrovascular defects in AD is clear and manifested through a complete deterioration of the vascular architecture as a consequence of chronic cerebral hypoperfusion, neurovascular uncoupling and loss of blood–brain barrier (BBB) integrity (Storkebaum et al. 2011; Zlokovic 2011).

Even though the indistinguishable clinical symptoms, AD can be categorized into late-onset sporadic AD (sAD) and early-onset familial AD (fAD). Around 5% of the cases are familial forms of autosomal dominant (not sex-linked) inheritance, with mutations in amyloid-β protein precursor (AβPP), presenilin 1 and 2 genes, which usually have an onset before age 65 (Piaceri et al., 2012). The vast majority of cases of AD are sporadic in origin with aging, diabetes and apolipoprotein E4 (APOE4) as main risk factors (Tillement et al., 2011).

1.2 Physiological and pathological roles of Tau protein

Tau protein was one of the first microtubule-associated proteins (MAPs) to be characterized, named by Marc Kirschner when his team was searching for factors that promote the self-assembly of tubulin into microtubules (Mandelkow & Mandelkow, 2012; Morfini et al., 2009). Human tau protein is encoded by a single gene, MAPT, located on chromosome 17q21.3 (Neve et al., 1986; Andreadis, 2013) that spans ~150 kb and consists of 16 exons, 11 of which are expressed in the central nervous system (CNS) (Pittman et al., 2006). The protein is mainly localized in the axons of the CNS and the

tau transcript undergoes extensive alternative splicing that is regulated spatially and temporally and can originate 30 isoforms (Andreadis, 2005). The expression of the different tau isoforms in the brain is under developmental control, suggesting that the regulation of tau isoforms is important during brain formation (Goedert et al., 1989). This protein is constitutively expressed in several types of cells, including neurons and brain endothelial cells, playing important biological functions, such as microtubules stabilization, neurite outgrowth, membrane interactions, facilitation of enzyme anchoring, organelle trafficking and cell differentiation and polarization (Ittner 2011; Andreadis, 2009; Mandelkow & Mandelkow, 2012).

Tau protein has more than 45 phosphorylation sites, most of which are located in the proline-rich region (P-region; residues 172–251) and the C-terminal tail region (C-region; residues 368–441) (Hanger et al. 2009). Tau protein phosphorylation at both of these regions affects its capacity to interact with microtubules (Liu et al. 2007). As a matter of fact, early studies performed by Lindwall and Cole (1984) revealed that tau protein is more efficient at promoting microtubules assembly in a more unphosphorylated state (Lindwall & Cole 1984). In subsequent studies, tau protein was demonstrated to make up the paired-helical filaments (PHFs), which form the NFTs found in AD brains and to be abnormally phosphorylated in these structures (Wischik et al. 1988; Goedert et al. 1988; Grundke-Iqbal et al. 1986; Kosik et al. 1988). Further studies confirmed that PHF-tau phosphorylated at “pathological” sites contributes to pathological processes in AD. For instance, enhanced immunoreactivity in human AD brain tissue was observed with the phosphorylation-dependent antibodies AT8 (epitope pS199/pS202/pT205), PHF-1 (epitope pS396/pS404), and pS262 (Mondragón-Rodríguez et al. 2014; Gu et al. 2013). Furthermore, it has been proposed that under this pathological situation, tau protein is abnormally phosphorylated or dephosphorylated in specific residues, perhaps due to the activities of various protein kinases and phosphatases, including glycogen synthase kinase 3 beta (GSK-3 β), cyclin-dependent kinase-5 (Cdk-5), and protein phosphatase 1A (PP-1A) and 2A (PP-2A) (Rudrabhatla & Pant 2011). Phosphorylation of tau protein is the most well documented modification in the AD brain; however, several other biochemical modifications can occur, including oxidation, ubiquitination, nitration of tyrosine residues and acetylation of lysine residues (Morishima-Kawashima et al., 1993; Cohen et al., 2011; Reynolds et al., 2007). Furthermore, tau protein also suffers proteolytic cleavage in AD pathology, which makes this protein more prone to aggregation. Other time-dependent changes in tau protein involve deamidation, isomerization, cross-linking and non-enzymatic cleavage around aspartate residues, which is the cause of the “high molecular weight smear” that is typical in AD (Watanabe et al., 2004).

Abnormal phosphorylation of tau protein has been proposed to be the “pathological connector” underlying oxidative stress, mitochondrial dysfunction and synaptic failure in AD pathology (Mondragón-rodríguez et al. 2013). Tau protein accumulation within the cytoplasm is associated with impaired axonal transport of mitochondria from the cell body towards synapses, which leads to severe

energetic crisis and imbalance in the generation of reactive oxygen species (ROS) and nitrogen species (RNS), all together contributing to synaptic failure and neuronal loss in AD (Mondragon-Rodriguez et al., 2013).

1.2.1 Okadaic acid as an experimental tool to study Alzheimer's disease

Okadaic Acid (OA) is a novel toxin that has proven to be extremely useful for the study of gene regulation by protein phosphatases. This compound is a complex polyether derivative of a 38-carbon fatty acid which is mainly produced by marine dinoflagellates (Sugiyama et al., 2007). Importantly, OA has been found to bind and inhibit the two major intracellular phosphatases, PP-1 and PP-2A (Cohen et al., 1990; Kamat et al., 2013). Inhibition of PP-2A is more efficient than inhibition of PP-1, with 50% inhibition (IC50), occurring at 0.1–1.0 and 20–100 nM, respectively. Due to this differential effect, the consequences of treatment with low doses of OA have generally been attributed to a reduction of PP-2A activity (Kamat et al., 2014).

PP-1 and PP-2A, are present in various cell types including neurons (Kins et al. 2003; Vogelsberg-Ragaglia et al. 2001) and brain endothelial cells (Le Guelte et al. 2012; Yin et al. 2006). PP-2A is an important serine/threonine phosphatase which regulates numerous kinase cascades influencing gene expression. Among other functions, PP-2A and its substrates regulate endothelial cells function (Kása et al. 2013). Endothelial cells communicate through junctional structures such as tight junctions, adherent junctions, and gap junctions (Wallez & Huber 2008; Dejana et al. 1999) formed by transmembrane proteins (Lum and Malik 1994). Adherent junctions play a dominant role in endothelial cells barrier function, and their regulation depends on the phosphorylation state of the adherent proteins (Huber & Weis 2001). OA binding to the active site of the catalytic subunit of PP-1 and PP-2A leads to the inhibition and activation of protein kinases (Kamat et al., 2014). Although a direct effect of OA on any kinase has not been found, it may exert indirect regulatory effects on various kinases (Sassa et al., 1989). Protein kinases whose catalytic activities are regulated by reversible phosphorylation may be stimulated in the absence or reduced phosphatase activity (Haystead et a., 1989). In turn, this may lead to the accumulation of phosphorylated substrates, which themselves may be kinases and further amplify the signal (Kamat et al., 2014).

Accumulating evidence reveals that OA promotes increased BBB permeability, Aβ deposition, tau protein hyperphosphorylation, synaptic loss and consequent neuronal degeneration and cognitive deficits, all of which resembles the AD pathology (Arias et al. 1998; Lee et al. 2000; Sun et al. 2003; Kamat et al. 2012). Therefore, OA can be used as an experimental tool to study cellular and molecular mechanism of AD pathology, and PP-2A may be a target for novel therapeutics applications (Kamat et al., 2013; Kamat et al., 2014)

1.3 Mitochondria in Alzheimer's disease: not just innocent bystanders

Mitochondria orchestrate a wide range of processes, exerting a prominent role in brain physiology. Besides being major sites of ROS production, these fascinating dynamic organelles are involved in the generation of ATP through oxidative phosphorylation (OXPHOS) and regulation of intracellular calcium (Ca^{2+}) homeostasis (Correia et al., 2010). But, why brain cells critically depend on mitochondrial function? Neurons are metabolically active cells with a high energy requirement for synaptic transmission, axonal/dendritic transport, ion channels, ion pumps activity, among others, which are processes with high energy demand. However, neurons exhibit a limited glycolytic capacity, which in association with the inability of the brain to store glucose as glycogen in significant amounts and transport restrictions of the BBB, makes them extremely reliant on aerobic OXPHOS for their energetic needs (Moreira et al. 2010). Concerning brain endothelial cells, mitochondria act mainly as signaling organelles and regulate the activation of several signaling pathways by producing moderate levels of second messengers, including ROS (Zhang & Gutterman 2007). And, if mitochondria fail? What happens to brains cells? Compromised mitochondrial function promotes an excessive mitochondrial Ca^{2+} accumulation, increased generation of ROS, which disrupts the electron transport chain, resulting in decreased ATP production and cell death (Correia et al., 2010). Particularly, mitochondrial Ca^{2+} overload potentiates mitochondrial permeability transition pore (MPTP) opening and the release of pro-apoptotic factors such as apoptosis-inducing factor (AIF) and cytochrome c (Busija et al. 2009). On the other hand, high levels of ROS damage cell membranes through lipid and protein oxidation and accelerate the mutation rate of mitochondrial DNA (mtDNA). Accumulation of mtDNA mutations enhances oxidative damage, causes a mitoenergetic crisis and potentiates ROS production, in a vicious cycle (Correia et al., 2010).

Taking into account the importance of mitochondria for proper brain cells functioning, it is not surprising that mitochondrial abnormalities have been implicated in AD pathology (Correia et al., 2010). Therefore, the purpose of this sub-chapter section is to highlight the structural and functional alterations that occur in AD.

1.3.1 Mitochondrial bioenergetic deficits in Alzheimer's disease

Mitochondrial bioenergetic deficits have been documented during the early stages of AD pathology. In fact, a decline in respiratory chain complexes I, III, and IV activities has been consistently reported in postmortem AD brain tissue and in several *in vivo* and *in vitro* models of the disease (Correia et al., 2012a). Additionally, impaired activity of key tricarboxylic acid cycle (TCA) enzymes, including pyruvate dehydrogenase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase, was also found in postmortem AD brain tissue (Correia et al., 2012a).

In order to decipher the role of Aβ and tau proteins on mitochondrial respiratory machinery and energy homeostasis separately, Eckert and collaborators (2011) took advantage from the triple transgenic mouse model of AD. Notably, deregulation of complex I was found to be tau protein-dependent, while deregulation of complex IV was Aβ-dependent, at both protein and activity levels (Eckert et al., 2011). The convergent effects of Aβ and tau protein led to depolarized mitochondria in triple AD mice. Additionally, it was found that age-related oxidative stress may exaggerate the disturbances in the respiratory system and synthesis of ATP and, in turn, take part in the vicious cycle that finally leads to cell death (Eckert et al., 2011).

1.3.2 Mitochondrial ROS production in Alzheimer's disease

Mitochondrial oxidative damage has been reported as an early event in AD progression. However, the causal factors are still unclear (Reddy et al., 2009). Tau protein-induced decline in mitochondrial respiratory activity can reduce the efficiency of electron transfer, resulting not only in decreased production of ATP, but also in increased generation of ROS (Eckert et al. 2013). In addition, oxidative stress can be also achieved by a decline in the capacity of the intracellular antioxidant system (David et al., 2005). It has been reported that partial or complete deficiency in mitochondrial superoxide dismutase (SOD) promoted by Aβ results in increased mitochondrial proton leakage, inhibition of respiration, accumulation of oxidative damage, and premature induction of apoptosis (Wakayama et al., 2000). In addition, ROS can induce different types of damage including spontaneous DNA oxidation. This may produce nuclear fragmentation, chromatin condensation, DNA fragmentation, and ultimately cell death. Interestingly, increased oxidative stress *per se* also promotes tau hyperphosphorylation in a SOD-2 knockout mouse model (Melov et al. 2007).

1.3.3 Mitochondrial structure and dynamics in Alzheimer's disease

A healthy pool of mitochondria requires a balance between mitochondrial fission/fusion, biogenesis and degradation (mitophagy). Under physiological conditions, an equilibrium among these events exists. In the brain, this balance is required for the maintenance of the respiratory chain capacity and mtDNA pool, recruitment of mitochondria to critical subcellular compartments, such as synaptic terminals, and control of mitochondria quality, shape and number, among others (Swerdlow, 2004).

Mitochondrial morphology is regulated by two antagonistic processes, fission and fusion. Mitochondrial fission is regulated by dynamin-like protein 1 (DRP1) and fission protein 1 (Fis1) in the outer membrane. The core components of the mitochondrial fusion machinery are mitofusins 1 and 2 (Mfn1, Mfn2) in the outer membrane and optic atrophy protein 1 (OPA1) in the inner membrane (Correia et al., 2012a). Fusion deficient cells demonstrate uncoupled respiratory rates and reversible

interorganellar heterogeneity in membrane potential and inhibition of cell growth (Santos et al., 2010). Fission deficiency also causes a reduced rate of mitochondrial ATP synthesis due to a significant decrease in complex IV activity and an inefficient OXPHOS system. A fragmented mitochondrial network is less efficient in mitochondrial Ca^{2+} uptake and intramitochondrial Ca^{2+} diffusion, and the formation of a mitochondrial network facilitates Ca^{2+} propagation within interconnected mitochondria, suggesting that the balance of mitochondrial fission/fusion can significantly impact cellular Ca^{2+} homeostasis (Santos et al., 2010). Mitochondrial dynamics are also involved in apoptosis with mitochondrial fragmentation being an early event during this process that precedes cytochrome c release and caspases activation. Excessive mitochondrial fission is also correlated with increased ROS production (Santos et al., 2010). Abnormal mitochondrial dynamics with detrimental effects on synaptic and neuronal function have been implicated in the pathogenesis of AD (Wang et al., 2009). Ultrastructural alterations in mitochondrial morphology such as reduced size and broken internal membrane cristae were documented in AD brain tissue suggesting an excessive mitochondrial fragmentation (Baloyannis, 2006). A reduction in the expression levels of DRP1, OPA1, Mfn 1 and 2 and an increase in Fis1 levels were observed in hippocampal tissue from AD subjects (Wang et al., 2009). An abnormal interaction of hyperphosphorylated tau protein and mitochondrial fission protein DRP1 has been described suggesting a relationship with mitochondrial dynamics alteration (Manczak et al., 2011).

Mitochondria are very particular organelles because they cannot be generated *de novo*, and the biogenesis of "new" mitochondria is dependent on preexisting ones – mitochondrial biogenesis. This is a very important and regulated process, which allows the brain cells to achieve, among other factors, the required energy levels. Another important feature of mitochondria is that these organelles have proteins encoded by nuclear and mitochondrial genes (Santos et al., 2010). In this way, mitochondrial biogenesis is a highly regulated and compartmentalized process that requires a synchronized and effective crosstalk between the nucleus and mitochondria to ensure proper and coordinated expression of both genomes. Briefly, the protein nuclear respiratory factor 1 (NRF-1) and nuclear respiratory factor 2 (NRF-2) coordinate the expression of nuclear genes encoding mitochondrial proteins, and mitochondrial transcription factor A (TFAM) drives transcription and replication of mtDNA. The expression of these three proteins is regulated by the peroxisome proliferator activator receptor gamma-coactivator 1α (PGC-1α). In AD, the levels and actions of these proteins are altered. In particular, AD is associated with reduced expression of mitochondrial biogenesis-related proteins, which consequently causes a decrease of the mtDNA/nDNA ratio (Wu et al., 1999).

Ultimately, the specific degradation of mitochondria occurs mainly through autophagy, a process named mitophagy. This mechanism comprises two steps: 1) dysfunctional mitochondria are engulfed by vesicles with a double membrane called autophagosomes and, 2) fusion of

autophagosomes with lysosomes allowing the degradation of the vesicles' content. Thus, mitophagy plays a crucial role in the "recycling" of these organelles. But how dysfunctional mitochondria are tagged for degradation by autophagy? The loss of mitochondrial membrane potential leads to the accumulation of phosphatase and tensin homolog–induced putative kinase 1 (PINK1) and subsequent recruitment of the E3 ubiquitin ligase Parkin to mitochondria. Parkin promotes the ubiquitination of proteins in the mitochondrial membrane, which targets the damaged mitochondria for removal by an autophagosome (Narendra et al., 2010). The elimination of dysfunctional mitochondria prevents an unmeasured increase of ROS production and induction of apoptosis. In brain cells of AD subjects there is a decrease or an impaired degradation of dysfunctional mitochondria by mitophagy, which leads to their accumulation potentiating ROS generation, energy depletion and apoptosis (Correia et al., 2012a).

1.4 Preconditioning as a potential approach to prevent Alzheimer's disease

1.4.1 What is preconditioning?

Preconditioning is a phenomenon whereby a stressful but non-lethal stimulus is able to trigger an endogenous adaptive response that allows tolerance against a subsequent potential lethal stimulus (Bhuiyan & Kim 2010). This phenomenon has been described in several organs including the brain, heart, liver, intestine, lung, skeletal muscle, kidney and bladder (Bhuiyan & Kim, 2010). It has been shown that certain stimuli such as ischemia, low doses of endotoxin, hypoxia (including brief hypoxia/ischemia episodes), opioids, acetylcholine, bradykinin, activators of protein kinase C (PKC), and pharmacological agents that activate the mitochondrial ATP-sensitive potassium channels (mitoK_{ATP}) can induce preconditioning-dependent protective responses (Lebuffe et al., 2003).

Two temporally distinct types of tolerance are afforded by sublethal pretreatments: early and delayed tolerance and the mechanisms underlying each type of tolerance may differ. Immediate preconditioning occurs within minutes after the preconditioning stimulus and involves cellular changes related to the activity or function of enzymes, secondary messengers, and ion channels. Conversely, delayed preconditioning-induced tolerance occur several hours and even days after the stimulus and involves new gene expression and *de novo* protein synthesis (Correia et al., 2010). Both types of tolerance have been found in the brain (Bhuiyan & Kim, 2010; Hanley & Daut 2005).

In general, the process of tolerance induction can be divided into the following elements: sensors of the stress signal, transducers of the stimulus, and effectors of the tolerance. First, the preconditioning stimulus must be recognized by cellular sensors, which will prepare the cells, tissues or organs for upcoming stress. Neurotransmitters such as glutamate [via N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Bhuiyan & Kim, 2010)] and acetylcholine [via mitoK_{ATP} channels or muscarinic receptors (Yao et al. 1999)],

opioid receptors (Schultz et al. 1998; Ma et al. 2005; Bhuiyan & Kim, 2010; Bell et al. 2000), ion channels and redox-sensitive enzymes (Bhuiyan & Kim, 2010) act as molecular sensors of the stressful stimuli. These sensors can activate a wide range of kinases, including Ras, Raf, mitogen-activated protein kinase (MAPK) kinase (MEK), extracellular regulated kinase (ERK), PKB, and PKC (Bhuiyan & Kim, 2010), and signaling molecules, such as nitric oxide ($NO^{\cdot}$) (Xu et al. 2004; Bhuiyan & Kim, 2010), diacylglycerol, ROS, inositol triphosphate, Ca^{2+}, and ceramide (Bhuiyan & Kim, 2010), which transduce the signal and initiate an adaptive response. Finally, effectors of the preconditioning response confer tolerance through anti-excitotoxicity, anti-apoptosis and anti-inflammation processes, protection of mitochondria and increased antioxidant mechanisms (Bhuiyan & Kim, 2010).

Transcription factors are the link between kinase cascades and gene expression. Some of the most studied transcription factors include NF-κB, cyclic adenosine monophosphate (cAMP) response element-binding protein, the activator-protein 1 (AP-1) family and hypoxia-inducible factor (HIF) (Bhuiyan & Kim, 2010; Ravati et al., 2001; Liu et al., 2005; Correia & Moreira, 2010).

Despite the molecular mechanisms underlying preconditioning remain unclear, it has been postulated that mitochondria actively participate in the preconditioning phenomenon (Correia & Moreira, 2010). Therefore the aim of this subchapter is to critically discuss the involvement of mitochondria on preconditioning-mediated brain tolerance.

1.4.2 Mitochondrial ROS and preconditioning

As abovementioned, mitochondria are one of the major sources of ROS. During normal respiration, approximately 0.4-4.0% of electron flow through the respiratory chain results in partial reduction of O_2, generating $O_2^{\bullet-}$, mainly by mitochondrial complexes I and III (Santos et al., 2010). Although exacerbated mitochondrial ROS production has been associated with mitochondrial dysfunction, and brain cells degeneration and death, it is well established that ROS at low/moderate concentrations play physiological roles (Correia et al., 2010). Notably, it has also been shown that a slight rise of mitochondrial ROS levels can trigger preconditioning-mediated brain tolerance, suggesting that mitochondria might be gateways on endogenous neuroprotection (Correia et al., 2010). For instance, preconditioning mediated by moderate ROS levels was able to protect neurons against different damaging agents and prevent against the subsequent massive oxygen radical formation. Conversely, an immediate and constant radical scavenger abolishes this ROS-induced neuronal preconditioning (Ravati et al., 2001).

1.4.3 Mitochondrial ATP-sensitive potassium (mitoK$_{ATP}$) channel and preconditioning

1.4.3.1 Mitochondrial ATP-sensitive potassium (mitoK$_{ATP}$) channel localization and structure

Two different types of ATP-sensitive potassium channels have been implicated in preconditioning: surface membrane K_{ATP} (sK$_{ATP}$) channels and mitochondrial ATP-sensitive potassium (mitoK$_{ATP}$) channels. Both types of channels are presumed to be regulated by changes in energy metabolism and have protective effects (Hanley & Daut, 2005).

The mitoK$_{ATP}$ channels are heterooctamers consisting of a 55-kDa inwardly rectifying potassium channel, mitoKIR, and a 63-kDa sulfonylurea receptor, mitoSUR, and are localized in the inner mitochondrial membrane, regulating mitochondrial function in several tissues, including the brain (Correia et al., 2010; Xie et al. 2010; Szabò et al. 2012). Within the brain, these channels exist in neurons, endothelial and glial cells, and neurons express predominantly Kir-containing channels, presumably protecting the cells during cellular stress conditions such as hypoglycemia or ischemia (Xie et al., 2010). The physiological role of mitoK$_{ATP}$ channels has been proposed to buffer potential perturbations of matrix volume and intermembrane space (O'Rourke, 2000), consequently promoting ATP synthesis and transport to fulfill cellular demands (Correia et al., 2010).

Brain mitochondria contain seven times more mitoK$_{ATP}$ channels than liver or heart mitochondria, which reflect the importance of these channels in brain functionality and integrity (Correia et al., 2010; Liu et al. 2002).

1.4.3.2 Mitochondrial ATP-sensitive potassium (mitoK$_{ATP}$) channels as both triggers and end effectors of acute and delayed neuroprotection mediated by preconditioning

Accumulating evidence suggests a key role for mitoK$_{ATP}$ channels as both triggers and end effectors of acute and delayed neuroprotection mediated by preconditioning (Correia et al., 2010). Indeed, activation of mitoK$_{ATP}$ channels with pharmacological agents (diazoxide and pinacidil) mimics the protective effects mediated by preconditioning (Liu et al. 1998; Gross & Fryer 2000). On the other hand, it has been shown that physiological or chemical preconditioning is abrogated by mitoK$_{ATP}$ channels blockers, such as glibenclamide and 5-hydroxydecanoate (5-HD) (Liu, Y., et al 1998; Fryer et al., 2000). But how mitoK$_{ATP}$ channels opening could exert brain protective effects? It has been hypothesized that mitoK$_{ATP}$ channels opening may decrease membrane potential promoting an increase in the electron transport chain rate and, consequently, increasing ATP production (O'Rourke, 2000). Additionally, the activation of mitoK$_{ATP}$ channels also attenuates mitochondrial Ca^{2+} overload

and, thus, preventes MPTP induction **(Figure 1.1)** (Correia et al., 2010; Liu, et al., 1998; Takashi et al. 1999).

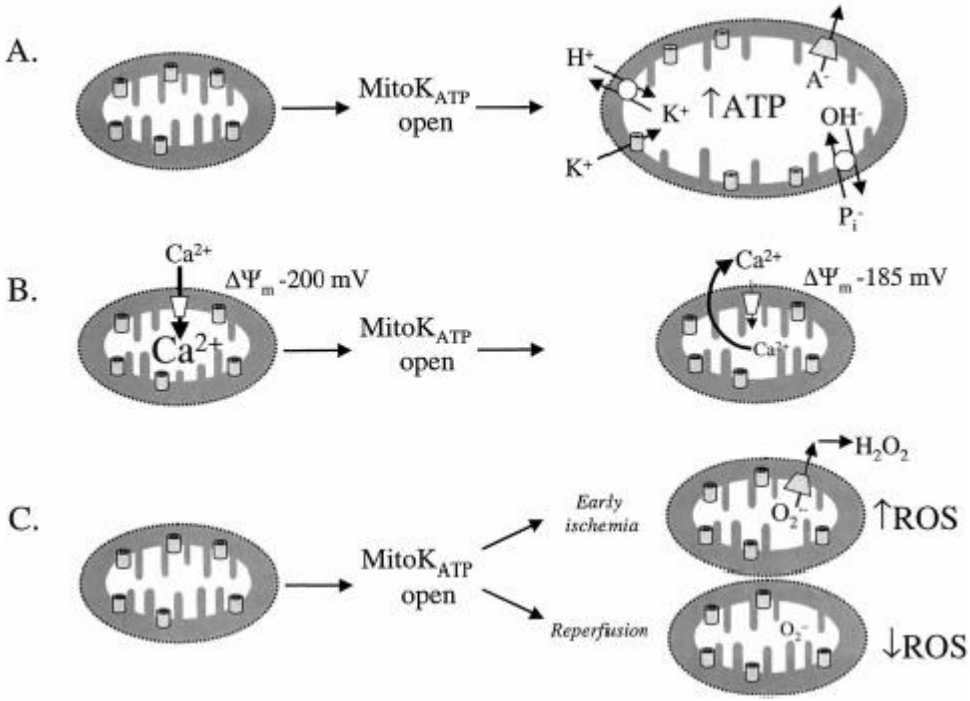


Figure 1.1 - Putative mechanisms of mitoK$_{ATP}$ channel opening-mediated protection. A, Mitochondrial volume, determined by the balance between salt influx (especially K^+ through the mitoK$_{ATP}$ channel) and efflux from the matrix, may be adjusted to optimize energy production (or perhaps minimize energy loss). B, Mitochondrial Ca^{2+} overload may be slowed by depolarization (due to mitoK$_{ATP}$ opening) of the mitochondrial membrane potential, decreasing the Ca^{2+} driving force, and Ca^{2+} release may be initiated by permeability transition pore opening. C, ROS production by the mitochondria may be enhanced by the opening of mitoK$_{ATP}$ to trigger protective pathways (adapted from O'Rourke, 2000).

1.5 Hypoxia-inducible factor-1α: the master regulator of preconditioning-mediated brain tolerance

1.5.1 Hypoxia-inducible factor composition and metabolism

A number of key molecules and signaling pathways have been proposed to participate in preconditioning. The induction of the hypoxia-mediated signaling pathway with the concomitant stabilization and transcriptional activation of the transcription factor hypoxia-inducible factor 1α (HIF-1α) has emerged as one of the major cellular pathways responsible for preconditioning-induced brain protection (Correia et al., 2010; Correia & Moreira, 2010). HIF-1α belongs to the HIF family that is involved in the regulation of cellular and molecular adaptation to hypoxia. The three isoforms (HIF-1, HIF-2, and HIF-3) are all heterodimers consisting of a constitutively expressed, stable β-subunit and an inducible α-subunit (Bergström et al., 2013). Under normoxic conditions, HIF-1α is hydroxylated by

prolyl hydroxylase enzymes (PHDs), namely PHD1, PHD2, and PHD3 and rapidly degraded by the ubiquitin-proteasome system. Briefly, prolyl hydroxylation promotes the recruitment of the tumor suppressor protein von Hippel–Lindau, which is part of the E3 ligase ubiquitination complex and primes HIF-1α for degradation in the 26S proteosome (Ogunshola & Antoniou, 2009). On the other hand, during hypoxic conditions, the enzymatic inhibition of PHDs abrogates HIF-1α proteasomal degradation and results in HIF-1α stabilization and translocation to the nucleus (Correia et al., 2010). Thereafter, HIF-1α interacts with the HIF1-β forming an heterodimeric complex, and modulates the expression of a wide range of genes involved in angiogenesis, metabolism (glycolysis), apoptosis, cell survival, cell proliferation, cell cycle control, erythropoiesis, and iron metabolism **(Figure 1.2)** (Correia et al., 2010; Correia & Moreira, 2010; Ogunshola & Antoniou, 2009; Shi, 2009).

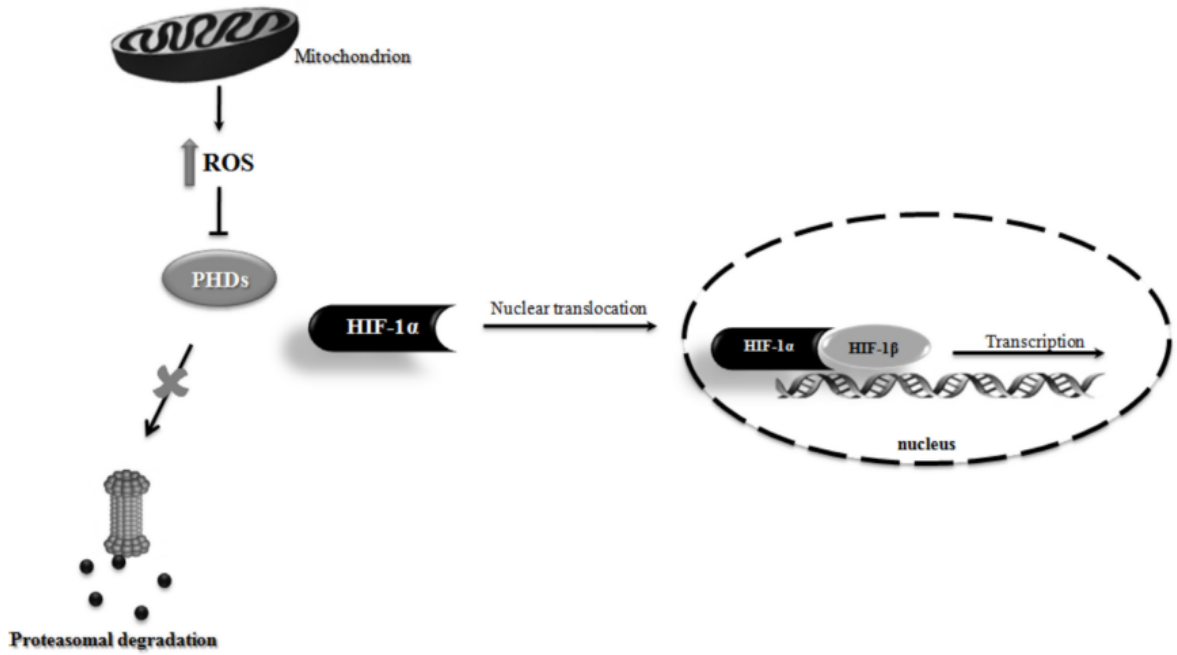


Figure 1.2 - Schematic illustration of the involvement of mitochondrial reactive oxygen species (ROS) in hypoxia-inducible factor 1α (HIF-1α) stabilization. Mitochondrial ROS production has been shown to inhibit the prolyl hydroxylase enzymes (PHDs) activity thus preventing HIF-1α proteasomal degradation. Consequently, HIF-1α is stabilized and translocated to the nucleus, where it dimerizes with HIF-1β, initiating the transcription of HIF-1 responsive genes. Adapted from Correia et al., 2010.

1.5.2 Hypoxia-inducible factor -1α and mitochondria

HIF-1α activation seems to be strictly linked to mitochondrial function. Indeed, under hypoxic conditions, mitochondria act as oxygen sensors and convey signals to HIF-1, with mitochondrial ROS being the putative signaling molecules between a cellular O_2 sensor and HIF-1. ROS generated by the Q_0 site of mitochondrial complex III have been documented to be critical in the hypoxia-mediated

survival signaling. Consistent with this, previous studies reported that mitochondrial ROS generation (that can also be due to the opening of mitoK$_{ATP}$ channels) can prevent the hydroxylation and stabilization of HIF-1α, promoting its translocation to the nucleus and dimerization with HIF-1β, initiating the transcription of target genes **(Figure 1.2)**. A crosstalk between mitochondrial ROS and HIF-1 has been proposed to underlie preconditioning-mediated protective events. Indeed, evidence from the literature demonstrates that hypoxic preconditioning-induced neuroprotection is associated with ROS production and subsequent induction of HIF-1 (Correia et al., 2010; Correia & Moreira, 2010).

1.5.3 Could Hypoxia-inducible factor -1α confer protection against Alzheimer's disease-related features?

HIF-1α levels were documented to be reduced in postmortem brain tissue from AD subjects in comparison to age-matched controls, suggesting that defective HIF-1α signaling pathways could play a role in the onset and progression of AD. Accordingly, AD subjects also exhibit reduced protein levels of brain glucose transporters GLUT1 and GLUT3, which are two common downstream targets of HIF-1α. The decrease in GLUT1 and GLUT3 levels, in turn, are negatively correlated with tau protein hyperphosphorylation and NFTs density, providing evidence for the involvement of glucose transporters in abnormal tau protein hyperphosphorylation and the formation of NFTs, these events being tightly linked to the degenerative events that occur in AD pathology (Ogunshola & Antoniou, 2009). Additionally, data derived from *in vitro* models of AD revealed that HIF-1α induction exerts protective effects whereas loss of HIF-1α increased the extent of brain cell damage (Vangeison et al., 2008). As a matter of fact, activation of HIF-1α by the overexpression of a non-degradable HIF-1α was also shown to prevent Aβ_{1-42}-induced neurotoxicity, pinpointing a neuroprotective role of HIF-1α in AD (Soucek et al. 2003). Notably, Aβ directly induces HIF-1α expression and activity in vitro (Soucek et al. 2003). Interestingly, low levels of Aβ protect neurons from a more aggressive insult via the induction of HIF-1α pathways. On the other hand, overexpression of HIF-1α has been found to be sufficient to protect neurons from Aβ neurotoxicity (Soucek et al., 2003).

Chapter 2 – Aims

The development of new and feasible disease-modifying therapies is one of the major challenges in the AD field. As outlined in the **Introduction** section, preconditioning is a powerful mechanism that affords brain protection against potential lethal insults. So far, less is known about the effectiveness of preconditioning in AD pathology.

The reprogramming of mitochondrial biology underlies the adaptive and pro-survival events triggered by the preconditioning phenomenon. In this sense, we hypothesized that a moderate rise in mitochondrial ROS induced by the opening of mitoK$_{ATP}$ channels is able to induce a pro-survival response by activating the master regulator of preconditioning, the HIF-1α. Therefore, the specific aims of this work were:

1. To evaluate the effects of preconditioning with pinacidil, a mitoK$_{ATP}$ channel modulator, *per se* on mitochondrial function and dynamics and HIF-1α signaling pathway in brain endothelial cells;
2. To explore the potential protective effects of pinacidil in an *in vitro* model of AD induced by OA;
3. To elucidate the involvement of mitochondrial ROS, mitoK$_{ATP}$ channels and HIF-1α signaling pathway on pinacidil preconditioning-mediated brain endothelial cells protection through pharmacological approaches.

Chapter 3 - Materials and Methods

3.1 Reagents

Pinacidil was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Coenzyme Q_{10} (CoQ_{10}) and 2-methoxyestradiol (2ME2) were obtained from Sigma (St. Louis, MO, USA). 5-Hydroxydecanoic acid (5-HD) was purchased from Abcam (Cambridge, UK). Okadaic acid (OA) was obtained from Calbiochem, Merck KGaA (Darmstadt, Germany). All the other chemicals were of the highest grade of purity commercially available.

3.2 Cell-line culture

The rat brain endothelial cell line (RBE4) was kindly provided by Dr. John Holy (University of Minnesota, Duluth, USA). RBE4 cells were maintained as monolayer cultures in collagen (Roche Diagnostics Mannheim, Germany)-coated T-75 flasks in a humidified atmosphere of 5% CO_2-95% air at 37°C. RBE4 cells were grown in α-MEM:Ham's F-10 nutrient mixture (1:1), supplemented with 10% FBS, 1 ng/ml bFGF and 300 μg/ml Geneticin (Roux et al., 1994). The medium was changed every 2-3 days until cells reached confluence.

3.3 Cell treatments

RBE4 cells were exposed to pinacidil (0.1-10 μM) during 24h, and then exposed to 10 nM OA for 18h. To elucidate the involvement of mitoK_{ATP} channels, mitochondrial ROS, and HIF-1α in pinacidil preconditioning-mediated protection against OA toxicity, RBE4 cells were pre-treated with 200 μM 5-HD, 10 μM CoQ_{10} and 1 μM 2-ME2, respectively, 15 min prior to the exposure to pinacidil. For all experimental procedures, controls were performed in the absence of those agents.

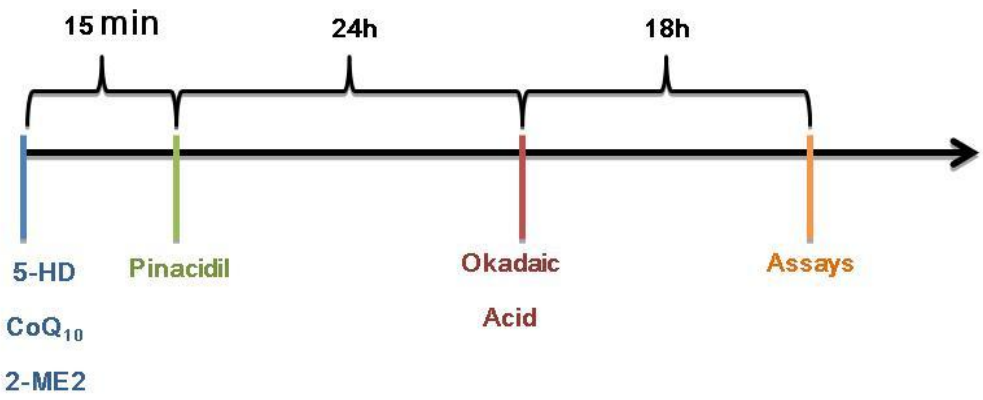


Figure 3.1 - Experimental design.

3.4 Assessment of cell viability

Cell viability was determined using the Alamar Blue assay. Briefly, RBE4 cells were seeded in 24-well plates at a density of 2×10^4 cells/well. After the incubation period, the cells' medium was aspirated and replaced by culture medium containing 10% (v/v) alamar blue. After 1-2h incubation at 37°C, supernatant was collected and the absorbance was measured at 570 nm and 600 nm using a microplate reader (SpectraMax Plus 384, Molecular Devices) (Neves et al., 2006). Cell viability (% of control) was calculated according to the formula ($A_{570}-A_{600}$) of treated cells × 100/($A_{570}-A_{600}$) of control cells.

3.5 Measurement of intracellular levels of superoxide anion

Intracellular levels of superoxide anion ($O_2^{\bullet-}$) were quantified using the probe dihydroethidium (DHE, Gibco-Invitrogen, Grand Island, NY). DHE is a cell-permeable fluorescent probe that, once internalized, is oxidized by $O_2^{\bullet-}$ to fluorescent ethidium bromide, which intercalates into DNA. RBE4 cells were seeded in 48-well plates at a density of 3×10^4 cells/well. After incubation with the experimental conditions previously outlined, cells were loaded with 10 μM DHE in sodium medium containing 135 mM NaCl, 5 mM KCl, 0.4 mM KH_2PO_4, 1 mM $MgSO_4.7H_2O$, 5.5mM glucose, 20 mM Hepes, 1.8 mM $CaCl_2$, pH 7.4, for 1h at 37 °C. Thereafter, cells were washed with phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4) and new sodium medium was added. Fluorescence was monitored for 1h, at 37 °C, using a SpectraMax GEMINI EM fluorocytometer (Molecular Devices), with excitation and emission wavelengths corresponding to 518 and 605 nm, respectively, with cutoff at 590 nm. The values were expressed as percentage of control.

3.6 Measurement of mitochondrial membrane potential

Mitochondrial membrane potential (ΔΨm) was determined by using the cationic fluorescent probe tetramethylrhodamine methyl ester ($TMRM^+$, Gibco-Invitrogen, Grand Island, NY), which accumulates predominantly in polarized mitochondria. RBE4 cells were seeded in 48-well plates at a density of 3×10^4 cells/well. After treatments, cells were washed with PBS and incubated with sodium medium containing 300 nM $TMRM^+$, for 1h at 37 °C. Basal fluorescence (540 nm excitation and 590 nm emission wavelengths, with cutoff at 590 nm) was measured using a SpectraMax GEMINI EM fluorocytometer (Molecular Devices) for 3 min, followed by the addition of carbonilcyanide p-triflouromethoxyphenylhydrazone (FCCP;1 μM) and oligomycin (2 μg/ml), which produced maximal mitochondrial depolarization. The difference between the increase of $TMRM^+$ fluorescence upon addition of FCCP plus oligomycin and basal fluorescence values was used to evaluate ΔΨm. The results were expressed as percentage of control fluorescence.

3.7 Western blot analysis

For whole cell extracts, RBE4 cells were plated in petri dishes (10 cm) at a density of 6×10^5 cells/dish. After washing, cells were scraped and resuspended in ice-cold lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin) supplemented with 0.1 M phenylmethanesulfonylfluoride (PMSF), 0.2 M dithiothreitol (DTT) and protease and phosphatases inhibitors cocktails (Roche Applied Science). The cellular extracts were frozen and defrozen three times to favor cells disruption, centrifuged at 13600 rpm (5417R, Eppendorf) for 10 min, at 4 °C, and the resulting supernatant collected. Protein content was determined by the bicinchoninic (BCA) protein assay using the BCA kit (Pierce Thermo Fisher Scientific, Rockford, IL), and the samples were denaturated with six times concentrated denaturating buffer at 100 °C, for 5 min.

Equivalent amounts of protein were resolved by electrophoresis in 8-12% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF, Millipore, Billerica, MA, USA) membranes. Non-specific binding was blocked by incubation with blocking buffer [5% bovine serum albumin (BSA) in Tris-buffered saline (TBS)] for 1h at room temperature, with gentle agitation. The blots were subsequently incubated overnight at 4 °C with gentle agitation with the specific primary antibodies. Blots were washed three times (5 min), with TBS containing 0.05% Tween (TBS-T) and then were incubated with the secondary antibodies for 2h at room temperature with gentle agitation. After three washes with TBS-T (5 min), specific bands of immunoreactive proteins were visualized after membrane incubation with enhanced chemifluorescence reagent (ECF) for 5 min in a VersaDoc Imaging System (Bio-Rad), and the density of protein bands was calculated using the Quantity One Program (Bio-Rad). The antibodies used in this study are listed in Table 1.

Table 1. List of primary and secondary antibodies used in Western blot analysis and Immunocytochemistry.

Antibody	kDa	Dilution	Host specie	Catalog number	Company
Actin	42	1:5000 (WB)	mouse	A5441	Sigma
p^{Ser9}-GSK-3β	46	1:1000 (WB)	rabbit	9336	Cell Signaling
p^{Tyr216}-GSK-3β	46	1:500 (WB)	rabbit	sc-135653	Santa Cruz Biotechnology
GSK-3β total	46	1:500 (WB)	mouse	sc-81462	Santa Cruz Biotechnology
GLUT1	46	1:1000 (WB)	rabbit	CBL242	Millipore
HIF-1α	120	1:500 (WB)	mouse	Ab1	Abcam
p^{Ser396}-Tau	46-80	1:750 (WB)	rabbit	sc-101815	Santa Cruz Biotechnology
p^{Thr181}-Tau	46-68	1:250 (WB)	rabbit	sc-101816	Santa Cruz

					Biotechnology
TOM20	20	1:200 (ICC)	rabbit	sc-11415	Santa Cruz Biotechnology
VEGF	34-50	1:200 (WB)	rabbit	#PC315	Calbiochem
Anti-mouse IgG alkaline phosphatase conjugate	-	1:20000 (WB)	goat	NIF1316	Amersham Pharmacia Biotech
Anti-rabbit IgG alkaline phosphatase conjugate	-	1:20000 (WB)	goat	NIF1317	Amersham Pharmacia Biotech
Alexa Fluor 488 anti-rabbit IgG conjugate		1:200 (ICC)	goat	A-11005	Molecular Probes

WB - Western Blot

ICC - Immunocytochemistry

3.8 Immunocytochemistry

RBE4 cells were grown on collagen-coated coverslips at a density of 2.5×10^4 cells/well. After a washing step, cells were fixed with 4% paraformaldehyde for 30 min at room temperature. Then, cells were permeabilized for 2 min at room temperature with 0.2% Triton-X100 in PBS and blocked for 30 min in PBS containing 3% BSA. Cells were incubated for 1h with primary antibodies prepared in PBS containing 3% of BSA and then washed with PBS and incubated with secondary antibodies conjugated with Alexa Fluor for 1h at room temperature. Then, cells were washed with PBS and incubated with Hoechst (1 µg/ml) for 3 min. Finally, the cells were washed twice with PBS and treated with Dako Cytomation Fluorescent mounting solution on a microscope slide. Images were acquired on a Zeiss LSM510META confocal microscope (63× 1.4NA plan-apochromat oil immersion lens) by using Zeiss LSM510 v3.2software. The antibodies used in this study are listed in **Table 1**.

3.9 Statistical analysis

Results are presented as mean ± SEM of the indicated number of experiments. Statistical significance was determined using the one-way ANOVA test for multiple comparisons, followed by the posthoc Tukey-Kramer test with the program prism 5 (GraphPad Software, San Diego, CA).

Chapter 4 - Results

4.1 Pinacidil stimulates superoxide anion generation in brain endothelial cells without affecting cell viability and mitochondrial membrane potential

In order to stimulate mitochondrial ROS production, RBE4 cells were exposed to pinacidil (0.1 – 10 μM) for 24h. As shown in **Figure 4.1**, pinacidil promoted a concentration-dependent increase in $O_2^{\bullet-}$ levels that was statistically different only at 10 μM. Notably, the pre-treatment of RBE4 cells with the specific inhibitor of mitoK_{ATP} channels 5-HD or with the mitochondrial ROS scavenger CoQ_{10} abrogated this effect on $O_2^{\bullet-}$ levels, which suggests that mitoK_{ATP} channels opening is required for the generation of mitochondrial $O_2^{\bullet-}$ mediated by pinacidil **(Fig. 4.1)**. All the concentrations of pinacidil tested (0.1-10 μM) did not significantly affect either cell viability **(Fig. 4.2)** or ΔΨm **(Fig. 4.3)**.

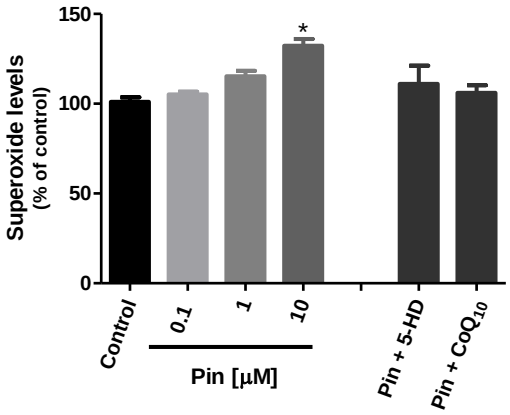


Figure 4.1. Effect of pinacidil on intracellular superoxide anion ($O_2^{\bullet-}$) levels in brain endothelial cells. RBE4 cells were incubated with increasing concentrations of pinacidil (0.1–10 μM) for 24h at 37°C. In some experiments cells were pretreated with 5-HD (200 μM) or CoQ_{10} (10 μM) 15 min before pinacidil (10 μM) exposure. $O_2^{\bullet-}$ production was measured using the DHE probe, as described in Materials and Methods section. Data are expressed as mean ± SEM of five to six independent experiments, performed in triplicate. * $p<0.05$ as compared with control cells.

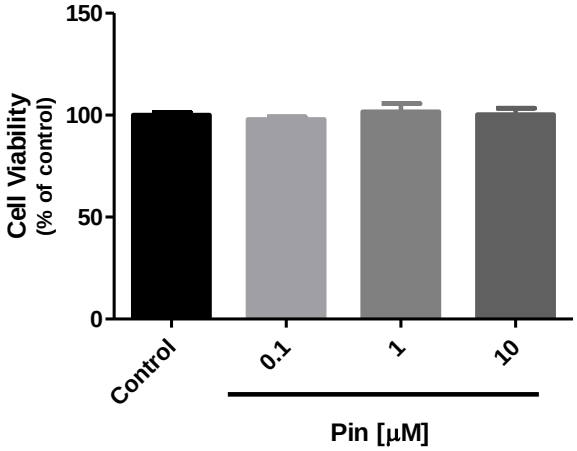


Figure 4.2. Effect of pinacidil on brain endothelial cells' viability. RBE4 cells were incubated with increasing concentrations of pinacidil (0.1–10 μM) for 24h at 37°C. Cell viability was determined by following the changes in cell reduction capacity by the Alamar Blue assay as described in Material and Methods section. Data are expressed as mean ± SEM of five to six independent experiments, performed in triplicate.

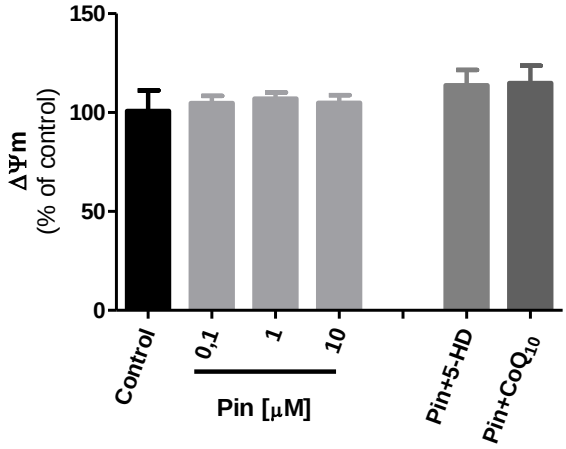
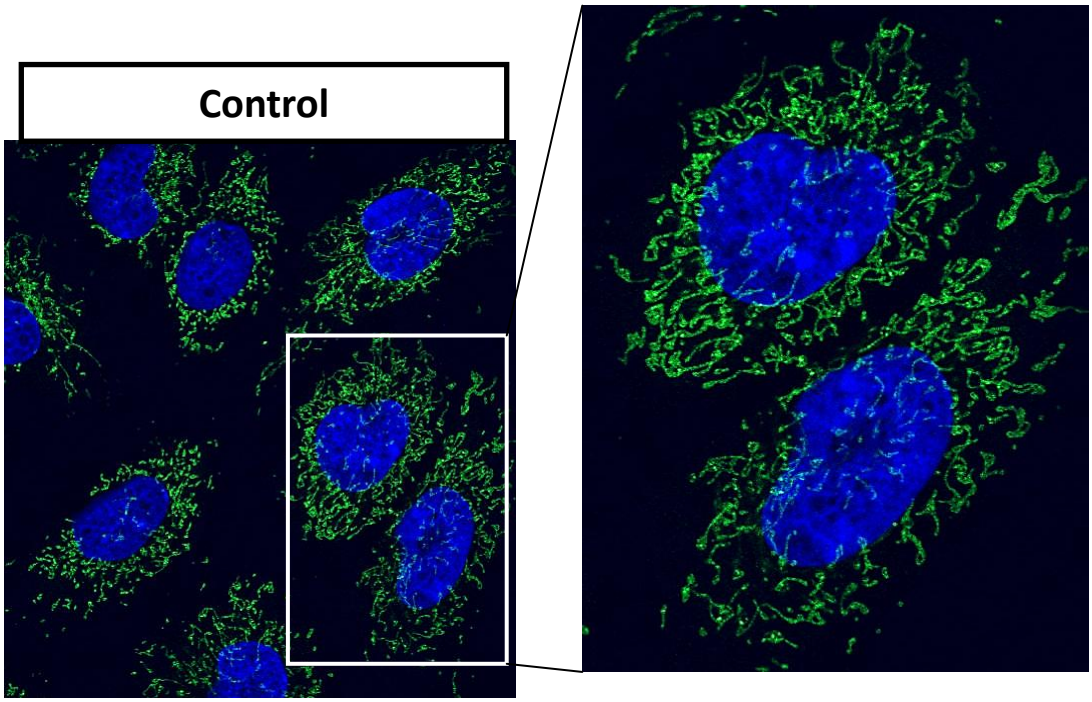


Figure 4.3. Effect of pinacidil on mitochondrial membrane potential (ΔΨm) in brain endothelial cells. RBE4 cells were incubated with increasing concentrations of pinacidil (0.1–10 μM) for 24h at 37 °C. In some experiments cells were pretreated with 5-HD (200 μM) or CoQ_{10} (10 μM) 15 min before pinacidil (10 μM) exposure. ΔΨm was expressed as the percentage of control cells. Data are expressed as mean ± SEM of five to six independent experiments, performed in triplicate.

4.2 Pinacidil induces spatial and structural mitochondrial network reorganization in brain endothelial cells

To further explore the effects of pinacidil on mitochondria, confocal microscopy was used to evaluate mitochondrial distribution and morphology. As shown in **Figure 4.4**, non-treated RBE4 cells contained mostly tubular mitochondria, distributed evenly throughout the whole cell. However, RBE4 cells treated with pinacidil (10 μM) presented mitochondria with an elongated morphology distributed mostly in the perinuclear region **(Fig. 4.4)**.



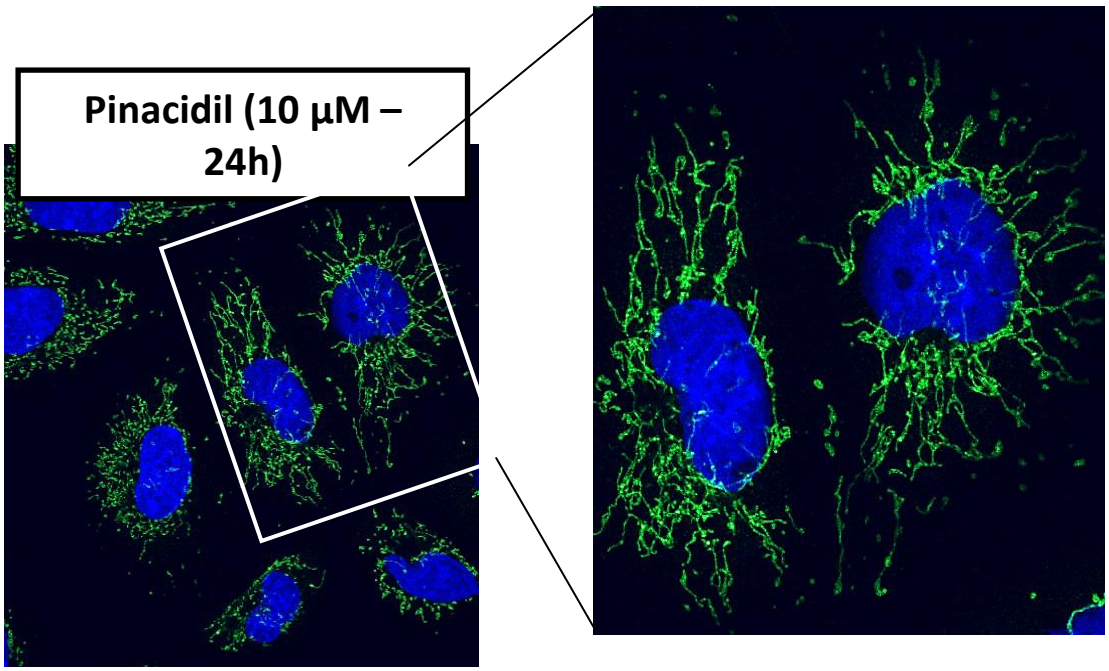


Figure 4.4. Effect of pinacidil on spatial and structural mitochondrial network reorganization in brain endothelial cells. RBE4 cells were treated with pinacidil (10 µM) for 24h at 37 ºC, stained for TOM20 (green) and visualized by confocal microscopy. Images are representative of two independent experiments.

4.3 Pinacidil increases the protein levels of GLUT-1 and VEGF

The stabilization and activation of HIF-1α by mitochondrial ROS is a key pro-survival event involved in the preconditioning phenomenon. Therefore, the next step of this study was to investigate whether pinacidil affects the protein levels of GLUT-1 and VEGF, two specific downstream targets of HIF-1α. Western blot analysis revealed that the exposure of RBE4 cells to 10 µM pinacidil induced a significant increase GLUT-1 **(Fig. 4.5A)** and VEGF **(Fig. 4.5B)** protein levels when compared with control cells. Furthermore, the effect of pinacidil on GLUT-1 **(Fig. 4.5A)** and VEGF **(Fig. 4.5B)** protein levels was abrogated by the specific inhibitor of mitoK$_{ATP}$ channels 5-HD, the mitochondrial ROS scavenger CoQ$_{10}$, and the HIF-1α inhibitor 2-ME2, implying mitoK$_{ATP}$ channels opening and mitochondrial-derived ROS in the regulation of HIF-1α signaling pathway by pinacidil.

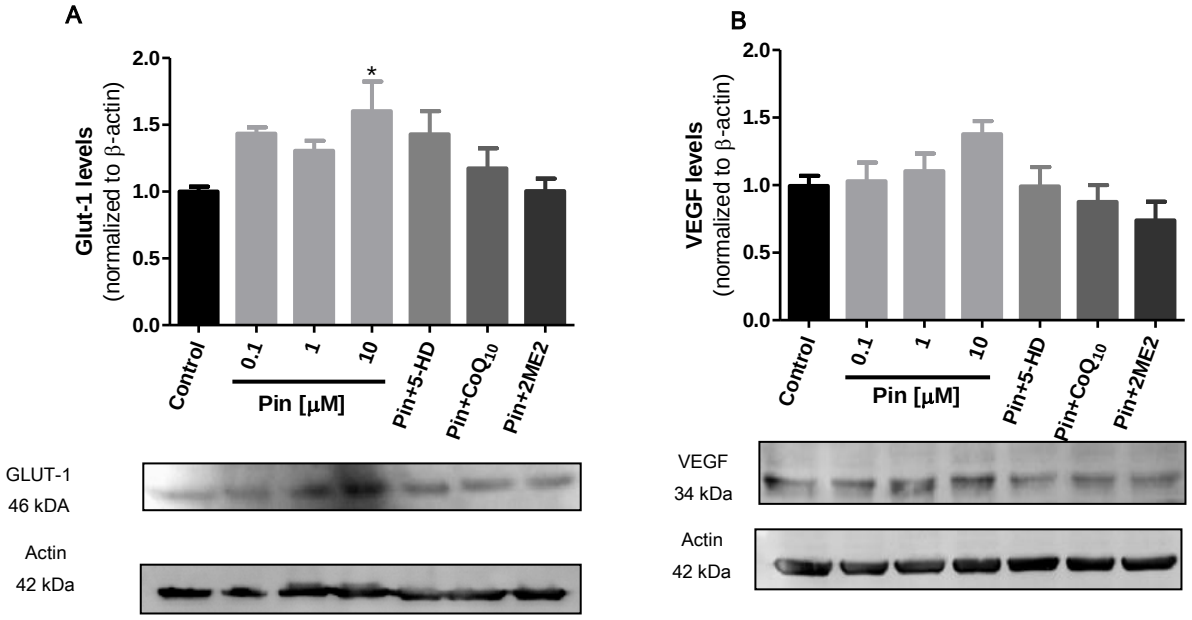


Figure 4.5. Effect of pinacidil on GLUT-1 (A) and VEGF (B) protein levels in brain endothelial cells. RBE4 cells were treated with increasing concentrations of pinacidil (0.1–10 μM) for 24h at 37 °C. In some experiments cells were pre-treated with 5-HD (200 μM), CoQ_{10} (10 μM) or 2-ME2 (1 μM) for 15 min before pinacidil (10 μM) exposure. Protein levels were determined by Western blot as described in Materials and Methods section. Data are expressed as mean ± SEM of five to six independent experiments. * $p<0.05$ as compared with control cells.

4.4 Okadaic acid induces brain endothelial cells loss and tau phosphorylation

To evaluate the potential protective effect of pinacidil preconditioning, OA was used as an experimental tool to mimic AD pathology *in vitro* (Kamat et al., 2014). In order to validate our model, the first approach of this set of experiments was to evaluate the levels of phosphorylated tau protein at ser396 and thr181 residues in brain endothelial cells at different time points. OA promoted a significant increase in the levels of phosphorylated tau protein at ser396 residue at 6h and 18h **(Fig. 4.6A)**, while a modest, but not statistically significant, increase in the levels of phosphorylated tau protein at thr181 residue was observed at 18h **(Fig. 4.6B)**. Importantly, OA induced a significant decrease in cell viability **(Fig. 4.7)** and ΔΨm **(Fig. 4.8)** at 18h, reinforcing the idea that increased tau phosphorylation has an important role in the deleterious events associated to this *in vitro* model of AD.

To better understand the molecular mechanisms underlying OA-triggered tau protein phosphorylation, the levels of the inactive (p^{ser9}-GSK-3β) **(Fig. 4.9A)** and active (p^{tyr216}-GSK-3β) **(Fig. 4.9B)** forms of glycogen synthase kinase 3β (GSK-3β) were also evaluated. As shown in **Figure 4.9A**, an increase in protein levels of p^{ser9}-GSK-3β levels promoted by OA was observed at 18h, whereas a decrease in p^{tyr216}-GSK-3β protein levels was noticed at 1h **(Fig. 4.9B)**.

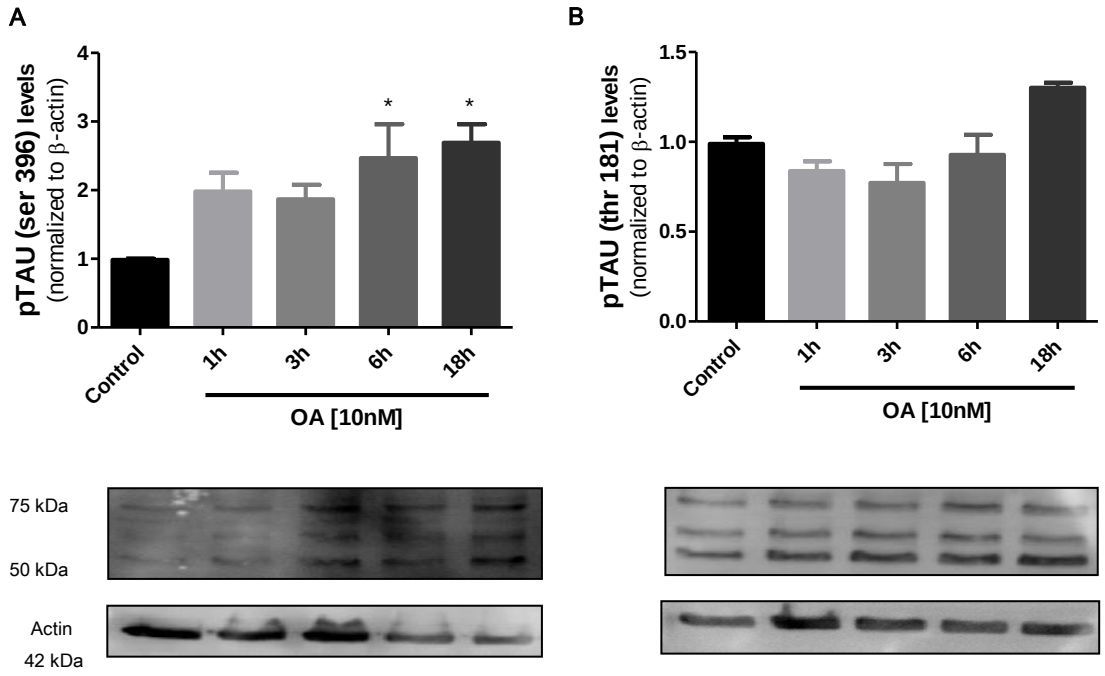


Figure 4.6. Effect of OA on tau protein phosphorylation levels on brain endothelial cells. RBE4 cells were treated with OA (10nM) for 1, 3, 6 and 18h at 37 °C. Protein levels were determined by Western blot as described in Material and Methods section. Data are expressed as mean ± SEM of five to six independent experiments. * $p<0.05$ as compared with control cells.

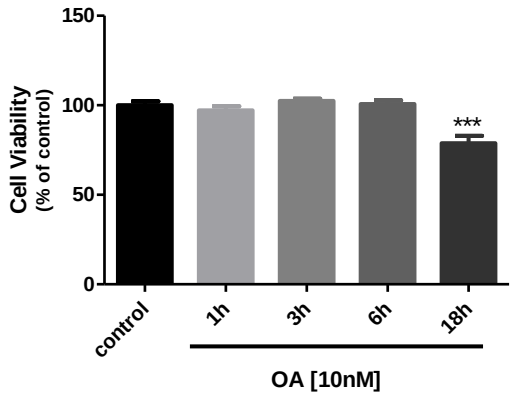


Figure 4.7. Effect of OA on brain endothelial cells' viability. RBE4 cells were incubated with 10 nM OA for 1, 3, 6 and 18h at 37 °C. Cell viability was determined by following the changes in cell reduction capacity by the Alamar Blue assay as described in Material and Methods section. Data are expressed as mean ± SEM of five to six independent experiments, performed in triplicate. *** $p<0.001$ as compared with control cells.

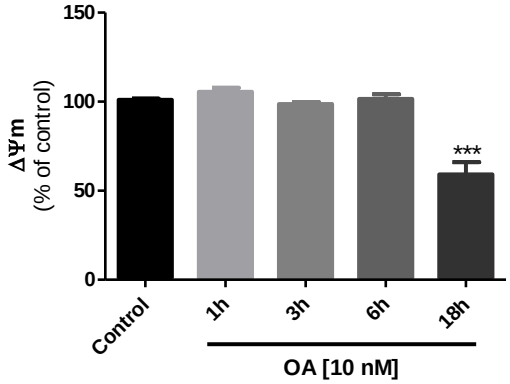


Figure 4.8. Effect of OA on mitochondrial membrane potential (ΔΨm) in brain endothelial cells. RBE4 cells were incubated with 10nM OA for 1, 3, 6 and 18h at 37ºC. ΔΨm was expressed as the percentage of control cells. Data are expressed as mean ± SEM of five to six independent experiments, performed in triplicate. *** $p<0.001$ as compared with control cells.

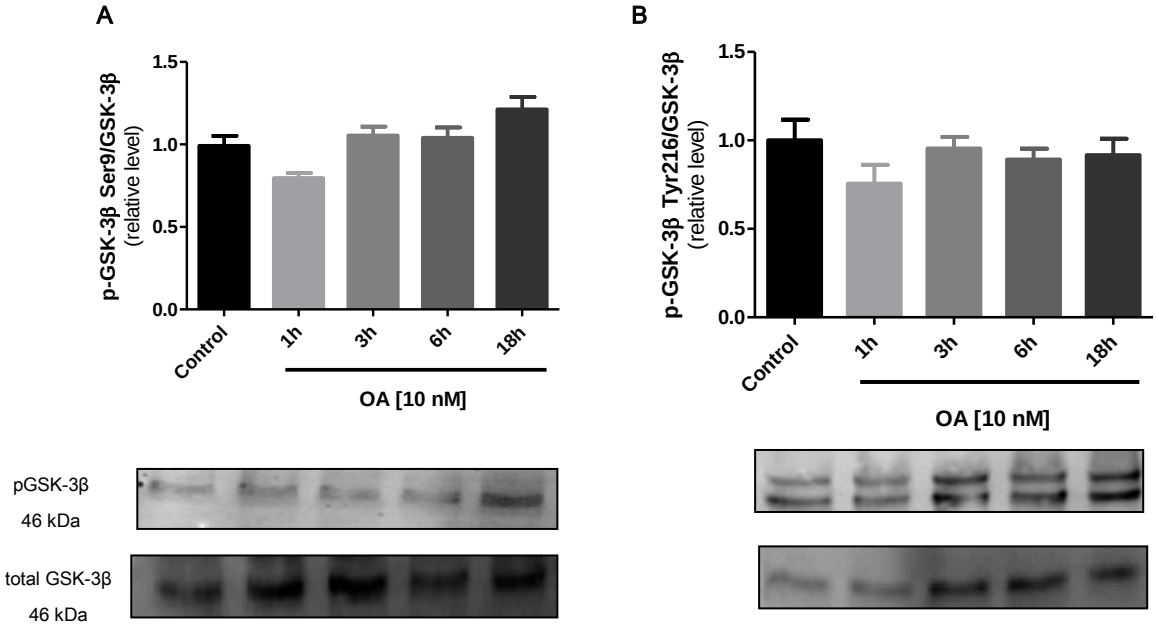


Figure 4.9. Effect of OA on GSK-3β phosphorylation levels on brain endothelial cells. RBE4 cells were treated with OA (10nM) for 1, 3, 6 and 18h at 37 °C. Protein levels were determined by Western blot as described in Material and Methods section. Data are expressed as mean ± SEM of two to four independent experiments

4.5 Pinacidil avoids brain endothelial cell viability loss promoted by okadaic acid

As shown in **Figure 4.10**, pinacidil prevented the loss of brain endothelial cell viability induced by OA. Conversely, the pre-treatment with 5-HD, CoQ_{10} and 2-ME2 suppressed the protective effects of pinacidil against OA-induced cell viability loss **(Fig. 4.10)**.

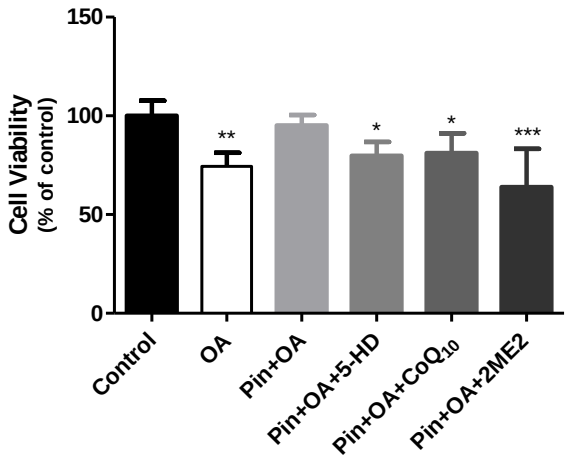


Figure 4.10. Effect of pinacidil and OA on brain endothelial cells' viability. RBE4 cells were preconditioned with pinacidil (10 μM) for 24h and then exposed to OA (10 nM) for 18h at 37º C. In some experiments cells were pre-treated with 200 μM 5-HD, 10 μM CoQ_{10}, and 1 μM 2-ME2 for 15 min before pinacidil exposure. Cell viability was determined by following changes in cell reduction capacity by the Alamar Blue assay as described in Material and Methods section. Data are expressed as mean ± SEM of five to six independent experiments, performed in triplicate. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ as compared with control cells.

4.6 Pinacidil prevents okadaic acid-induced ROS overproduction and mitochondrial depolarization

To gain further insights on the potential protective effects of pinacidil preconditioning in this in *vitro model* of AD, $O_2^{\bullet-}$ levels and mitochondrial membrane potential were evaluated by fluorometry. OA induced a significant increase in $O_2^{\bullet-}$ levels **(Fig. 4.11)** and a drastic reduction in $\Delta\Psi m$ **(Fig. 4.12)**, these effects being prevented by pinacidil **(Figs. 4.11 and 4.12)**. On the other hand, the protective effects of pinacidil were suppressed by the pre-treatment with 5-HD, CoQ_{10} and 2-ME2 **(Figs. 4.11 and 4.12)**, fostering the idea that mitoK_{ATP} channel opening, mitochondrial-derived ROS and HIF-1α activation are required for the effectiveness of pinacidil preconditioning to protect against OA.

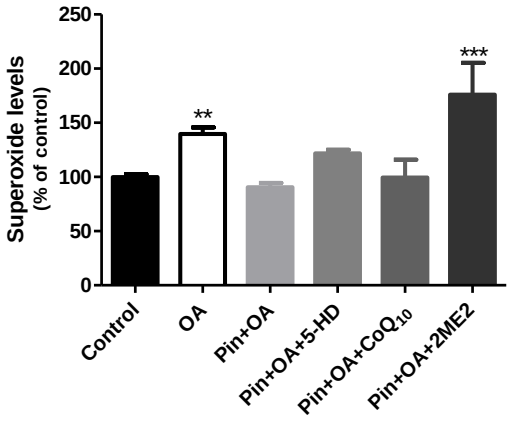


Figure 4.11. Effect of pinacidil and OA on intracellular superoxide anion ($O_2^{\bullet-}$) levels in brain endothelial cells. RBE4 cells were preconditioned with pinacidil (10 μM) for 24h and then exposed to okadaic acid (10 nM) for 18h at 37ºC. In some experiments cells were pre-treated with 200 μM 5-HD, 10 μM CoQ_{10}, and 1 μM 2-ME2 for 15 min before pinacidil preconditioning. ROS production was measured using the DHE probe, as described in Materials and Methods section. Data are expressed as mean ± S EM of five to six independent experiments, performed in triplicate. ** $p<0.01$; *** $p<0.001$ as compared with control cells.

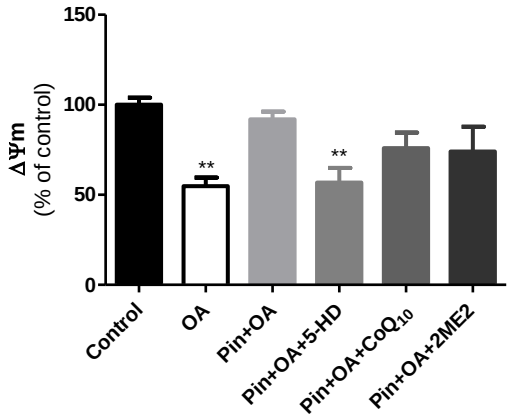


Figure 4.12. Effects of pinacidil and OA on mitochondrial membrane potential (ΔΨm) in brain endothelial cells. RBE4 cells were preconditioned with pinacidil (10 μM) for 24h and then exposed to OA (10 nM) for 18h at 37ºC. In some experiments cells were pre-treated with 200 μM 5-HD, 10 μM CoQ_{10}, and 1 μM 2-ME2 for 15 min before pinacidil preconditioning. ΔΨm was expressed as the percentage of control cells. Data are expressed as mean ± SEM of five to six independent experiments, performed in triplicate. ** $p<0.01$ as compared with control cells.

4.7 Pinacidil partially attenuates mitochondrial network disarrangement promoted by okadaic acid

As shown in **Figure 4.13**, OA affected spatial and structural mitochondrial network, being observed the distribution of smaller and rounder mitochondria in the perinuclear region when compared with control cells. Once again, pinacidil was able to partially prevent the effects of OA on mitochondrial shape and distribution **(Fig. 4.13)**.

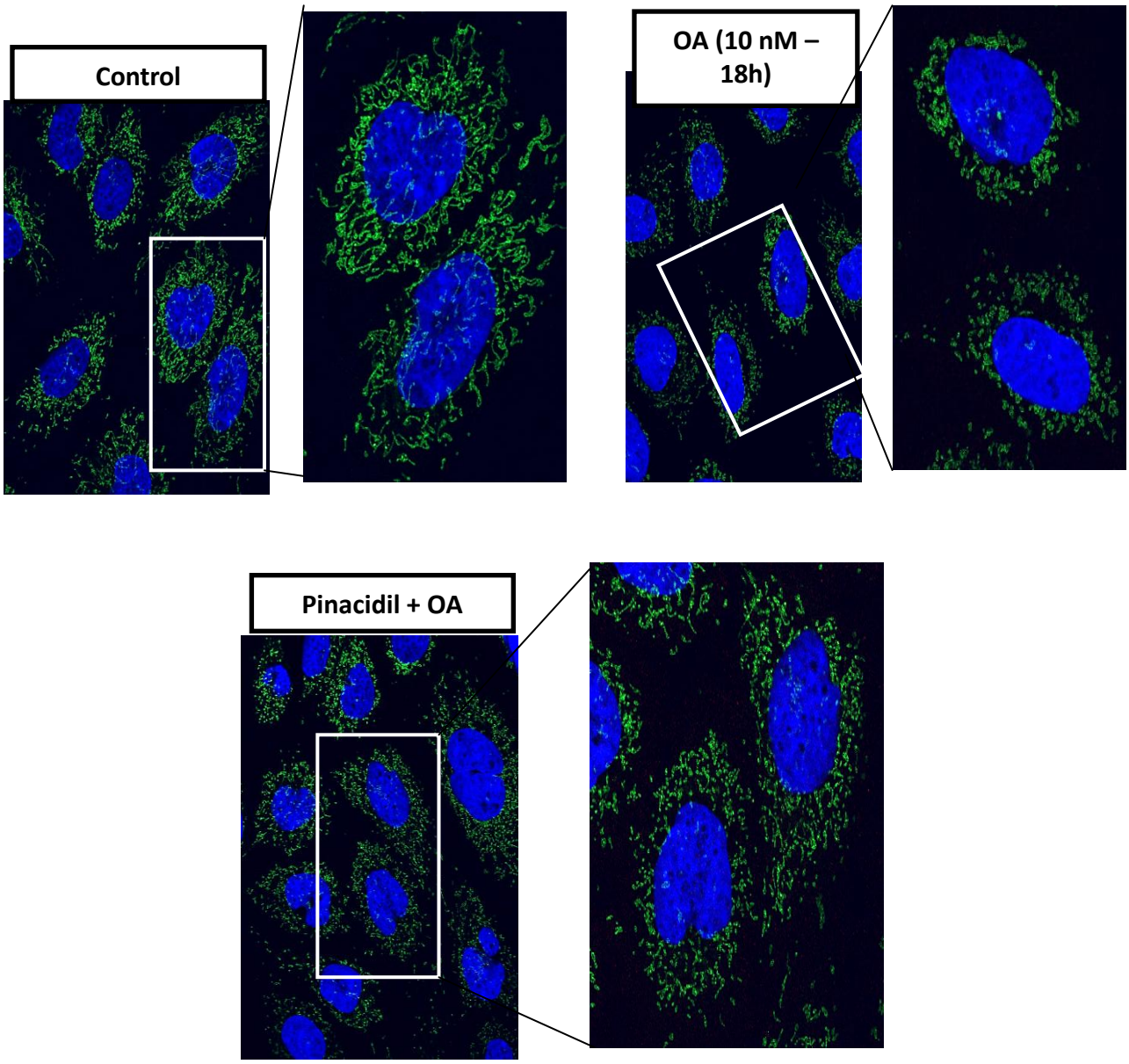


Figure 4.13. Effect of pinacidil and OA on spatial and structural mitochondrial network reorganization in brain endothelial cells. RBE4 cells were preconditioned with pinacidil (10 μM) for 24h and then exposed to OA (10 nM) for 18h, stained for TOM20 (green) and visualized by confocal microscopy. Images are representative of two independent experiments.

Chapter 5 - Discussion

The present study shows for the first time that preconditioning of brain endothelial cells with pinacidil exerts protective effects in an *in vitro* model of AD induced by OA. Importantly, the opening of mitoK$_{ATP}$ channels, the generation of mitochondrial-derived ROS and the concomitant induction of HIF-1α signaling pathways are critical mechanistic events underlying pinacidil preconditioning-triggered brain endothelial protection.

Neuronal and endothelial dysfunction occurs before the first symptoms of AD pathology, playing an important role in the degenerative events and consequent cognitive deterioration that characterize this devastating disorder (Kalaria, 1999; Zlokovic, 2011; Sagare et al., 2012). Abnormal phosphorylation of tau protein has been proposed to be a driving force behind neurodegeneration in AD; however, the pathological role of tau protein in endothelial dysfunction remains enigmatic. Tau protein phosphorylation state appears to be in part determined by the coordinated actions of two key enzymes: GSK-3β and PP-2A (Iqbal et al., 2009 ; Planel et al. 2001; Zhou et al. 2009). Of note, PP-2A accounts for more than 70% of tau phosphatases activity in the human brain (Liu et al. 2005). As shown in **Figure 4.6A**, the exposure of brain endothelial cells to OA promoted a significant increase in the levels of phosphorylated tau protein at residue ser396 in a time-dependent manner. Consistently, previous *in vitro* studies revealed that the pharmacological inhibition of PP-2A activity by OA resulted (directly or indirectly) in tau protein hyperphosphorylation in neurons at the same residues observed in the AD brain (Iqbal et al., 2009; Martin et al. 2009). A strong connection between PP-2A and GSK-3β pathways have been reported (Lin et al. 2007; Liu et al. 2008; Meske et al. 2008). GSK-3β is a proline-directed serine/threonine kinase constitutively active and highly expressed in the CNS (Woodgett 1990; Zhao et al. 2012). This protein is involved in various cellular processes including tau protein phosphorylation (Anderton et al., 2001; Brion 2001). GSK-3β phosphorylation at residue ser9 results in its inhibition, whereas phosphorylation at residue tyr216 activates GSK-3β (Abdillahi et al. 2012; Zhao et al. 2012; Martin et al., 2011). Our results demonstrated that OA induces a decrease in the levels of active GSK-3β (p^{tyr216}-GSK-3β) **(Fig. 4.9B)** and an increase in the levels of inactive GSK-3β (p^{ser9}-GSK-3β) **(Fig. 4.9A)**. Despite the supposed importance of GSK-3β action in the AD brain, the level of enzyme activity, and the role played by GSK-3β in the initial pathogenesis of early-stage AD patients remains controversial because some authors have found increased levels of GSK-3β phosphorylated at ser9 in postmortem samples (Ferrer et al. 2002; Swatton et al. 2004). This form of GSK-3β, known to be inactive (Abdillahi et al. 2012; Zhao et al. 2012; Martin et al., 2011), does not support the notion that GSK-3β is involved in molecular changes in the AD brain, including hyperphosphorylation of many tau epitopes followed by aggregation of tau into NFTs (Lim et al. 2010). It was previously reported that GSK-3β phosphorylated at ser9 was increased in an OA model relevant to AD brain (Yoon et al. 2005). This increase in phosphorylated GSK-3β is similar to findings in AD brain. Thus, it is necessary to investigate whether drugs targeting GSK-3β are appropriate under the rather paradoxical circumstances in which levels of the inactive form of GSK-3β and phosphorylation of GSK-

3β substrates are simultaneously increased (Lim et al. 2010). Furthermore, our results demonstrated that increased tau protein phosphorylation induced by OA is followed by the loss of cell viability **(Fig. 4.7)** and mitochondrial abnormalities, including loss of ΔΨm **(Fig. 4.8)**. Similarly, *in vitro* findings showed that OA evokes tau protein phosphorylation to initiate neuronal loss, which is accompanied by reduced ΔΨm and increased mitochondrial swelling (Yoon et al. 2006). Mitochondrial dysfunction also emerged as a key factor underlying OA-induced memory impairment and apoptotic cell death in the rat brain (Kamat et al., 2011). Increased Ca^{2+} and ROS as well as decreased ΔΨm, mitochondrial respiratory activity and ATP were reported in mitochondrial preparations from rats submitted to an intracerebroventricular (icv) administration of OA (Kamat et al., 2011).

But the foremost question of this thesis is: can pinacidil preconditioning counteract the deleterious effects of OA on brain endothelial cells? As abovementioned, preconditioning is a phenomenon whereby a sub-lethal condition protects against a subsequent potential lethal situation, in part by reprogramming mitochondrial biology and inducing HIF-1α. As hypothesized, our data demonstrated that pinacidil preconditioning protects brain endothelial cells against OA by preventing loss of cell viability **(Fig. 10)** and ΔΨm **(Fig. 12)**, and $O_2^{\bullet-}$ overproduction **(Fig. 11)**. Previous studies showed that the mitoK_{ATP} channels modulator diazoxide ameliorates Aβ and tau pathologies and improves memory in the triple transgenic mouse model of AD (Liu et al., 2010). Protective effects of diazoxide against Aβ-induced cytotoxicity were also documented in endothelial cells (Chi et al. 2000). Ma and Chen (2004) reported that diazoxide counteracts the effects of $Aβ_{1-42}$, protecting neurons against the increased intracellular ROS levels induced by this amyloidogenic peptide (Ma & Chen 2004). Together with previous findings, our results foster the therapeutic potential of drugs that activate mitoK_{ATP} channels, including pinacidil, in the treatment of AD pathology.

Since the functional state of mitochondria is accompanied by alterations in mitochondrial dynamics, the effects of OA and pinacidil preconditioning on mitochondrial network dynamics in brain endothelial cells was investigated. OA exposure affects the mitochondrial network in brain endothelial cells promoting a perinuclear distribution of smaller and rounder mitochondria, when compared with control cells **(Fig. 4.13)**. Mechanistically, increased phosphorylation of DRP1 was shown to underlie mitochondrial fission in OA-treated neurons (Cho et al. 2012). It was already evidenced that decomposition of mitochondrial filaments/networks to numerous single roundish mitochondria takes place under some physiological conditions (e.g. collapsed ΔΨm) or cellular pathologies (Skulachev 2001). Gilkerson and collaborators (2000) reported that the decomposition of mitochondrial reticulum to numerous single mitochondria is due to depletion of mitochondrial DNA (Gilkerson et al. 2000). Sometimes, breakdown of the mitochondrial reticulum is followed by a movement of the small mitochondria from the cell periphery to the nucleus(Collier et al. 1993; De Vos et al. 2000), as observed in brain endothelial cells treated with OA **(Fig. 4.13)**. Importantly, pinacidil preconditioning partially prevents the effects of OA on mitochondrial shape and distribution **(Fig. 4.13)**.

But, what are the adaptive and pro-survival events underlying pinacidil preconditioning? By using pharmacological approaches, this work demonstrates that mitoK$_{ATP}$ channels opening, mitochondrial-derived ROS and HIF-1α are required for pinacidil-mediated brain endothelial protection against OA. ROS signaling is a two-edged sword. While an exacerbated production of mitochondrial ROS induces mitochondrial dysfunction potentiating cell degeneration and death, moderate ROS levels appear to have protective actions, being involved in the activation of pro-survival adaptive signaling pathways (Ravagnan et al., 2002; Correia et al., 2010; 2012b). In this sense, mitochondria have been suggested to act as signaling organelles during preconditioning, with mitochondrial-derived ROS representing key messengers in this phenomenon (Correia et al., 2010; 2012b). Of note, pinacidil induced a concentration-dependent increase in mitochondrial $O_2^{\bullet-}$ levels **(Fig. 4.1)**, without affecting cell viability **(Fig. 4.2)** and ΔΨm **(Fig. 4.3)**. Similarly, a previous study demonstrated that pinacidil is able to induce a sub-lethal generation of ROS (Krenz et al., 2002). Data derived from our laboratory demonstrated that preconditioning with the mitochondrial modulator cyanide protects brain endothelial and neuronal cells against diabetes-mediated deleterious effects, this phenomenon being reliant on moderate mitochondrial ROS generation, since the protective effects of cyanide were abolished in mitochondria DNA depleted (ρ0) cells and in the presence of the antioxidant N-acetyl-L-cysteine (NAC) (Correia et al., 2012b). Additionally, mitoK$_{ATP}$ channels have also been implicated in the preconditioning phenomenon (Busija et al., 2008; Hanley & Daut, 2005). It was demonstrated that antioxidants and mitoK$_{ATP}$ channel blockers abolish preconditioning-induced protection (Vanden Hoek et al., 1998; Oldenburg et al., 2002), showing the pivotal necessity for mitoK$_{ATP}$ channels activation and ROS production in this protective phenomenon. Consistently, our results demonstrates that the use of the specific mitoK$_{ATP}$ channels antagonist 5-HD and the mitochondrial antioxidant CoQ$_{10}$ abrogated the protective effects of pinacidil preconditioning in preventing loss of cell viability **(Fig. 4.10)** and ΔΨm **(Fig. 4.12)**, and $O_2^{\bullet-}$ overproduction **(Fig. 4.11)** promoted by OA.

Although the specific mechanism of action of pinacidil has not been completely elucidated yet, it has been proposed that the protective events mediated by mitoK$_{ATP}$ channels openers include alterations in mitochondrial morphology resulting in increased fatty acid oxidation, mitochondrial respiration and ATP production (Halestrap, 1989) and changes in ROS levels - sublethal ROS production caused by an increase in the rate of OXPHOS activity (O'Rourke, 2000). Brain endothelial cells treated with pinacidil present mitochondria with an elongated morphology mainly distributed in the perinuclear region **(Fig. 4.4)**. But, what is the functional consequence of these alterations in mitochondrial network induced by pinacidil? Mitochondrial plasticity allows mitochondria to quickly react to cellular signals (e.g. ROS), which regulate their cellular position, interconnectivity and function (Lackner, 2014). These conserved activities are coordinately regulated and fully integrated with cellular physiology to respond to the rapidly changing needs of the cell (Lackner, 2014; Chan, 2012). Mitochondria frequently form tubular structures or networks which are crucial for the inheritance of

mitochondrial DNA, enhancement of ΔΨm and an increase in the rate of oxidative phosphorylation (Skulachev et al., 2001; Hoppins, 2014). Furthermore, due to the versatility of mitochondrial functions, it is critically important for cells to monitor the functional state of mitochondria and adjust nuclear gene expression accordingly to achieve functional homeostasis of mitochondria (Zhang et al., 2013). This is achieved via the coordination of mitochondria-to-nucleus signaling pathways, known as the retrograde response (Liu & Butow, 2006; Jazwinski, 2013). The retrograde response adapts cells to changes in the functional state of mitochondria, such as respiratory defects, by mediating an assortment of cellular processes that include metabolic reconfiguration, nutrient sensing, aging and stress response pathways (Biswas et al., 2005; Jazwinski & Kriete, 2012). HIF-1α is one of the most important genes involved in these pathways (Correia et al., 2010; Correia et al., 2012), being responsible for the regulation of several genes involved in cell survival, angiogenesis, and glucose transport, among others (Date et al., 2005; Melstrom et al., 2011; Wang et al., 2014). Mitochondrial ROS have been implicated as signaling molecules involved in preconditioning and its generation was shown to induce the stabilization of HIF-1α protein (Comito et al., 2011; Sena & Chandel, 2012). This notion is corroborated by studies demonstrating that antioxidants prevent HIF-1α protein stabilization (Klimova & Chandel, 2008). Our results revealed that pinacidil increases the protein levels of two specific downstream targets of HIF-1α, the GLUT-1 and VEGF **(Fig. 4.5)**. GLUT-1 is highly expressed in brain endothelial cells and is responsible for transporting glucose from blood into the extracellular space of the brain (Schubert, 2005; Qutub & Hunt, 2005), whereas VEGF is a hypoxia-inducible secreted cytokine that interacts with receptor tyrosine kinases on endothelial cells (Del Bo et al., 2009; Ferrara et al., 2003). Recent evidence indicates that VEGF is involved in neuronal survival, neuroprotection, regeneration, cell growth and differentiation (Del Bo et al. 2009). In this study, it was found that the inhibitor of HIF-1α, the 2-ME2, abrogates the protective effects of pinacidil preconditioning against the deleterious effects of OA **(Figs. 4.10-4.12)**. Accordingly, previous studies show that HIF-1α inhibition abolishes the protective events of preconditioning (Correia et al., 2012; Belaidi et al., 2008; Eckle et al., 2008)

Overall, our results demonstrate that pinacidil preconditioning protects brain endothelial cells against the deleterious effects of OA. Mechanistically, the protective effects of pinacidil seem to be reliant on mitoK_{ATP} channels activators-mediated ROS production and induction of HIF-1α. However, further studies, both *in vitro* and *in vivo*, are required to understand the specific adaptive mechanism triggered by pinacidil preconditioning and the involvement of mitochondria in this phenomenon. However, our results support the ideia that mitoK_{ATP} channels can be considered therapeutic targets in AD.

Chapter 6 - Conclusions

Past disease-modifying approaches focused on reducing Aβ levels in the AD brain have failed repeatedly in clinical trials, claiming the urgent need to uncover new therapeutic targets in this disease. The work presented in this thesis provides evidence that mitoK$_{ATP}$ channels are potential therapeutic targets to counteract AD pathology. Notably, this work reveals that preconditioning with the mitoK$_{ATP}$ channels modulator pinacidil is able to protect brain endothelial cells against the deleterious effects of OA, this phenomenon being reliant on mitoK$_{ATP}$ channels opening, moderate mitochondrial ROS generation and induction of HIF-1α signaling pathways.

So far less is known about the pathophysiological role of hyperphosphorylated tau protein in brain endothelial cells in AD. However, it is well known that the integrity of endothelial is highly regulated by components of the cytoskeleton, namely microtubules that interact directly with the tau protein. So, alterations in tau phosphorylation levels will impact endothelial cells integrity and function. To extend our knowledge and to evaluate the potential protective effects of pinacidil preconditioning, OA was used as an experimental tool to mimic AD *in vitro*. Importantly, the exposure of brain endothelial cells to OA increased the levels of phosphorylated tau protein in a time-dependent fashion. Importantly, this event was followed by alterations in mitochondrial physiology and dynamics including mitochondrial depolarization, ROS overproduction and distribution of fragmented mitochondria in the perinuclear region.

By targeting mitoK$_{ATP}$ channels, pinacidil preconditioning was shown to protect brain endothelial cells against the loss of cell viability and ΔΨm, exacerbated ROS generation and altered mitochondrial shape and spatial distribution induced by OA. However, it is important to emphasize that mitoK$_{ATP}$ channels opening, moderate ROS generation and induction of HIF-1α during the initial phase of preconditioning are critical mechanistic events to confer brain endothelial cells resistance against OA, since the protective effects of pinacidil were abrogated by the pre-treatment with the specific mitoK$_{ATP}$ channel antagonist 5-HD, the mitochondrial ROS scavenger CoQ$_{10}$, and the HIF-1α inhibitor 2-ME2.

Although the present findings strongly suggest that mitoK$_{ATP}$ channels are potential therapeutic targets in AD, further studies are required to better understand the adaptive and pro-survival mechanisms underlying pinacidil preconditioning. Particularly, we are interested in exploring the role of mitochondrial quality control events, including mitochondrial biogenesis, fusion and fission and autophagy in the preconditioning phenomenon mediated by mitoK$_{ATP}$ channels modulators. Moreover, *in vivo* studies performed in animals models of sporadic and familiar forms of AD will reinforce the potential of mitoK$_{ATP}$ channels as therapeutic targets to “fight” this devastating neurodegenerative disease.

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