UNIVERSIDAD POLITÉCNICA DE MADRID

ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA AGRONÓMICA, ALIMENTARIA Y DE BIOSISTEMAS

GRADO EN BIOTECNOLOGÍA

IMPACT OF β 2-ADRENERGIC RECEPTOR POLYMORPHISMS IN HEART FAILURE: Cell surface expression and internalization

TRABAJO FIN DE GRADO

Autor: **María Imbert Griñó**

Tutor: **Dra. Véronique Pons**
Dra. Eva Miedes

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María Imbert Griñó

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Tutora: Dra. Véronique Pons

CoTutora UPM: Dra. Eva Miedes

Dpto. de Biotecnología – Biología Vegetal

POLITÉCNICA

E.T.S. DE INGENIERÍA AGRONÓMICA, ALIMENTARIA Y DE BIOSISTEMAS

TITULO DEL TFG- IMPACT OF B2-ADRENERGIC RECEPTOR POLYMORPHISMS IN HEART FAILURE: Cell surface expression and internalization

Memoria presentada MARÍA IMBERT GRIÑÓ
para la obtención del título de Graduado en Biotecnología por la Universidad Politécnica de Madrid

Fdo:

VºBº Tutor UPM

Dra. Eva Miedes

Dpto. de Biotecnología - Biología Vegetal.

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ABBREVIATIONS

β2-AR: β2-adrenergic receptor

BSA: Bovin serum albumin

cAMP: Ciclic adenosine monophosphate

CMV: Cucumber Mosaic Virus

DMEM: Dulbecco Modified Eagle's Minimal

ELISA: Enzyme-Linked ImmunoSorbent Assay

Epi: Epinephrine

GPCR: G protein coupled receptor

HA: Human influenza hemagglutinin

HCl: Hydrochloric acid

HEK: Human Embryonic Kidney

HRP: Horseradish peroxydase

HF: Heart failure

I2MC : Institut de Maladies Cardiovasculaires

Iso: Isoproterenol

MCS: Multiple Cloning Site

NE: Norepinephrine

PBS: Phosphate buffered saline

PEI: PolyEthylenImine

PI3K: Phosphatidylinositol 3-kinase

PFA: Paraformaldehyde

PKA: cAMPdependent protein kinase

SEM: Standard Error of the Mean

TBM: TetraMethylBenzidine

WT: Wild Type

ABSTRACT

Heart Failure (HF) represents a major health problem throughout all over the world. One of the most cardiotoxic effects of HF is chronic catecholamine hypersecretion. In the heart, we can find β2-Adrenergic Receptors (β2-AR). β2-AR are known to be activated by catecholamines such as epinephrine and in consequence of this activation, receptors are internalized. It has been shown that certain polymorphisms of the β2-AR respond differently to ligand stimulation therefore being correlated with the severity of HF. These polymorphisms are: R16G, Q27E and T164I. We performed ELISA assays on HEK cells expressing β2-AR polymorphisms in order to determine β2-AR cell surface basal expression and internalization after epinephrine stimulation. We observed no differences with the WT on receptor cell surface expression and internalization for the β2-AR polymorphisms Q27E and T164I while polymorphism R16G showed an increased receptor cell surface expression and a decreased internalization post epinephrine stimulation.

CHAPTER 1: INTRODUCTION

Heart Failure (HF) is a condition in which the heart cannot pump enough blood to meet the body's needs. Without enough blood flow, all major body functions are disrupted. This disease has a remarkable prevalence in the world affecting approximately 1% to 2% of adult population (McMurray and Pfeffer, 2005). Particularly, in Spain, this value raises up to 5% becoming a major health problem since it represents the first reason of hospitalization of the elderly and supposes 2.5% of health care costs (Sayago-Silva *et al.*, 2013).

Heart failure can be originated by different conditions that damage heart muscle such as heart attack, coronary artery disease, hypertension, etc. Consequently, the heart is unable to pump enough blood. To counteract this situation, the organism develops a compensation phase which consists in a chronic catecholamine hypersecretion (Baker, 2014; Mahata *et al*., 2016). As a result of this, there is an increase of heart rate. In addition, during this compensation phase there is a dilatation of the left ventricle via hypertrophy of the cardiomyocytes which increases blood pressure and heart rate (tachycardia) in order to meet the body's needs. (Lymperopoulos and Koch, 2013; Bencivenga *et al.*, 2019). Most of sudden deaths occur during this first phase. The second phase is the actual heart failure and consists in a decompensation phase: the heart becomes unable to function correctly and there is a decrease of cardiac output, leading to the death of cardiomyocytes and tissue necrosis (Madamanchi, 2007).

To treat heart failure, there are several types of drugs including β-blockers, Angiotensin-Converting Enzyme inhibitors and diuretics (Miller, 2019). Among the most commonly used, β-blockers are antagonists of β-adrenergic receptors (β-AR). Although they help to reduce the complications associated such as ventricular function impairment or sudden death, β-blockers do not treat the disease itself since heart failure continues to develop (Manurung and Trisnohadi, 2007). In addition, it has been shown that 30% of the population do not respond to treatment with β-blockers (Carr *et al.*, 2016). Consequently, there is a compelling need to improve the treatments for HF and this necessarily implies a better understanding of the molecular mechanisms involved in HF.

β-blockers target β-adrenergic receptors (β-AR), which are seven transmembrane domain receptors coupled to heterotrimeric G proteins (GPCR). There are 3 types of β-AR: β1-AR, β2-AR and β3-AR (Liggett, 2000). The receptors are broadly expressed throughout the different organs. β1-AR and β2-AR achieve important functions in heart. β1-AR is responsible for chronotropism and inotropism via catecholamine activation. When ligands like adrenaline bind to β1-AR, Gs proteins promote the activation of a signaling cascade which ends up in the activation of PKA (cAMPdependent protein kinase), responsible for promoting cardiac muscle contraction (Figure 1) (Yoo *et al.*, 2009). On the other hand, β2-AR can couple to Gs as well but also to Gi protein. However, unlike the β1-AR receptor considered to be pro-apoptotic in HF, the β2-AR receptor is thought to be anti-apoptotic during HF. This mechanism could be explained, by a Gi signaling pathway that involves the activation of PI3K (Phosphatidylinositol 3-Kinase) and AKT protein leading to the transcription of cell survival genes thus conferring cardioprotection (Figure 1) (Chesley *et al.*, 2000; Berthiaume *et al.*, 2016). It is for this interesting function, that I have focused my study on the β2-AR.

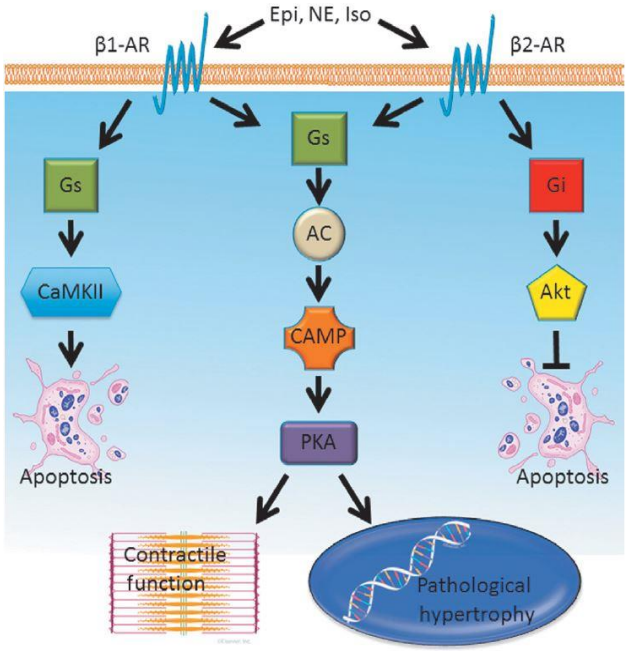

Figure 1. β1-AR and β2-AR signalling pathways during HF in cardiomyocytes after stimulating with Epi (Epinephrine), NE (Norepinephrine) or Iso (Isoproterenol) (Berthiaume *et al., 2016).* The rows express activation while the lines express inhibition.

Interestingly, β1-AR and β2-AR undergo significant modifications during HF, regarding receptor expression, location and signaling. In healthy hearts, the ratio of β1-AR/ β2-AR is 80/20, while this ratio shifts to 50/50 in HF due to a marked decrease of β1-AR expression. In addition, in normal conditions, β1-AR is distributed through the whole cell surface of the cardiomyocyte whereas β2-AR is confined into raft domains in deep transverse tubules, leading to spatially restricted cAMP (Cyclic adenosine monophosphate) response. In contrast, during HF, β2-AR is decompartmentalized causing an altered and diffuse cAMP signaling (Nikolaev *et al.*, 2010).

Importantly, three different human polymorphisms have been characterized for the β2-AR and it has been shown that they are directly correlated with the development and the severity of heart failure (Pereira *et al.*, 2013). These polymorphisms, which are characterized by an amino acid substitution, are: R16G, Q27E and T164I (Figure 2). R16G and Q27E polymorphisms are located in the extracellular N-amino terminus of the receptor, with a high allele frequency of 0.45 and 0.60 respectively. T164I is placed in the fourth transmembrane domain of the receptor, near the ligand interacting pocket and its allele frequency in the population is 0.04 (Pereira *et al.*, 2010)

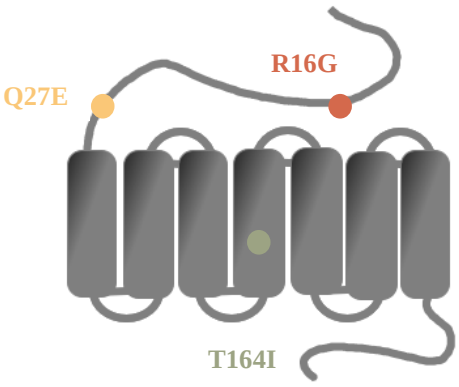

Figure 2. Human polymorphisms for β2-Adrenergic Receptor: R16G, Q27E and T164I.

Despite having been poorly characterized at the molecular level, R16G and Q27E polymorphisms have been demonstrated to affect in ligand-induced receptor downregulation, since R16G displays an enhanced downregulation while β2-AR Q27E showed an absence of downregulation (Green *et al.,* 1993; ; Kolovou *et al.,* 2018). On the other hand, T164I has been correlated with a decrease in ligand binding affinity and Gs protein coupling (Green *et al.,* 1994; Muthumala *et al.,* 2018).

Bringing the matter to the actual situation of the SARS-CoV-2 pandemic, patients with HF have shown to get a poor prognosis of COVID-19 disease. On the other hand, SARS-CoV-2 has also proved to cause heart injury in patients with no record of cardiovascular problems. SARS-CoV-2 is thought to infect host cells through ACE2. ACE2 is not only expressed in lungs but also in the heart which could be one of the reasons why COVID-19 causes heart damage in some patients. Additionally, this virus triggers an imbalanced T cell response that causes a cytokine storm and this mechanism has been suggested to cause myocardium injury (Zheng *et al.*,2020). Consequently, there is one more reason why it is urgent to find an improved treatment for HF.

In this context, my study is a small part of a project carried out by the I2MC (Institut des Maladies Cardiovasculaires/Institute of Cardiovascular Diseases) which aim is to characterize the pharmacological signature of polymorphic β2-AR receptors in order to establish their relationship with heart failure and to ultimately improve the treatment. Understanding the impact of these polymorphisms on the development and severity of HF will enable in the long term to set up new targeted therapies for patients with these polymorphisms and suffering from HF. With this objective, the Institute of Cardiovascular Diseases is studying β2-AR polymorphisms impact on:

1. Receptor location and cell surface expression
2. Membrane compartmentalization
3. Receptor activity: G protein signaling and β-arrestin recruitment

CHAPTER 2: OBJECTIVES

Thereby, the goals of my study have been to determine:

1) The impact of β2-AR polymorphism on receptor expression at the cell surface using ELISA on non-permeabilized cells.
2) The impact of β2-AR polymorphism on receptor internalization following catecholamine stimulation.

This study should allow a better characterization of the pharmacology of polymorphic receptors compared to the wild-type receptor, and it could provide a better understanding of the link between β2-AR and the development of HF.

CHAPTER 3: MATERIALS AND METHODS

1. CELL CULTURE

For this study, HEK293T/17 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM). These are human embryonic kidney cells which are easy to grow and easily transfected. This cell line is transfected with the gene for SV40 T-antigen allowing to replicate plasmids carrying the SV40 origin of replication. Cells were cultured in DMEM containing 4.5 g / L glucose (Sigma) supplemented with 10% fetal bovine serum (FBS), and 100 units / mL of penicillin and 0.1 mg of streptomycin / mL. Cells were kept in an incubator at 37 ° C in the presence of 5% CO_2 in order to maintain mediums' acid-base equilibrium. Cell maintenance is carried out twice a week in a laminar flow hood. Prior to cell plating, 24-well-plates are covered with polylysine which allows a better cell adhesion to support. Afterwards, cells are rinsed and dissociated with trypsin-EDTA. Trypsin is an endoprotease that cleaves peptide chains mainly at the carboxyl side of amino acids like lysine or arginine. EDTA sequesters metal ions such as Ca^{2+}, this is necessary for cadherin-dependent cell-cell adhesion. Cells are then diluted in DMEM to block the action of trypsin, counted and finally plated in 24-well-plates with 75,000 cells per well.

2. CELL TRANSFECTION

Cell transfection consists in transitory expression of the DNA introduced. Cells were transfected with pcDNA 3.1 (+) (Figure 3) encoding wild type (WT) or polymorphic receptors fused to a HA tag in the extracellular region. Transfection was achieved 24 hours after plating using PolyEtylenImine (PEI), a polycationic polymer that binds to DNA's negative charges and cell surface polysaccharides also negatively charged allowing the DNA to penetrate the cell by endocytosis. The ratio used was 1:1 (µg DNA: µL PEI). An empty vector, pcDNA3.1 (+), was also transfected as negative control.

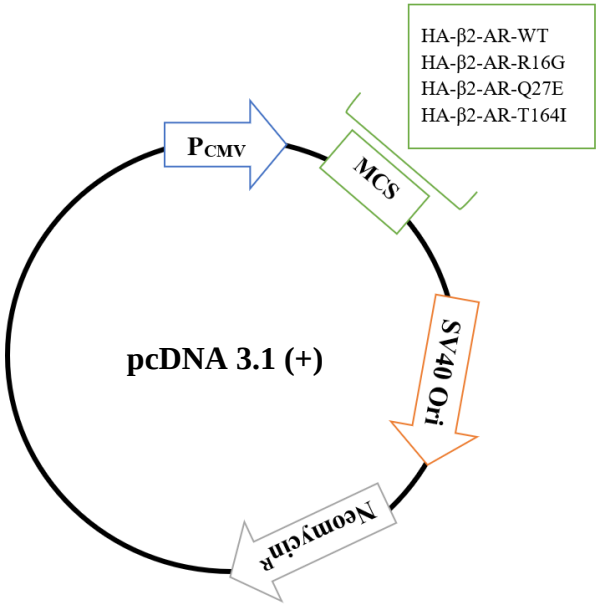

Figure 3. pcDNA 3.1 (+) plasmid map. P_{CMV} is a constitutive promotor from the Cucumber Mosaic Virus (CMV). The different HA-β2-AR are cloned inside the Multiple Cloning Site (MSC). The SV40 Ori is an origin of replication that allows the plasmid to replicate inside HEK293T cells through the T antigen. Finally, the plasmid has a Neomycin resistance gene.

3. EPINEPHRINE STIMULATION

In order to study epinephrine-induced receptor internalization, cells expressing WT or polymorphic receptors were stimulated with 10µM epinephrine during different time periods after overnight serum starvation. Cells were then rinsed with cold PBS and immediately processed for ELISA.

4. ELISA

Indirect Enzyme-linked immunosorbent assay (ELISA) was performed 36 hours after transfection to detect and quantify β2-AR at the cell surface. It is an immunoassay technique in which an immobilized antigen is detected by an antibody bound to an enzyme capable of generating a colored product quantified by spectrophotometry. The technique is carried out in non-permeabilized cells and receptors are detected thanks to an HA tag fused to their extracellular domain by a specific anti-tag antibody (Figure 4).

First, cells are fixed with paraformaldehyde (PFA) 1% for 10 minutes, then incubated with PBS-BSA 1% to saturate nonspecific binding sites thus to reduce background. Next, cells are incubated with mouse primary antibody anti-HA diluted 1/2500 in PBS-BSA 1%. Afterwards, cells are washed with PBS and incubated with secondary antibody anti-mouse IgG conjugated to HRP (Horseradish peroxydase) and diluted 1/1000 in PBS-BSA 1%. TMB (3,3′,5,5′-Tetramethylbenzidine) is HRP's substrate. When TMB is oxidized by the HRP, a soluble blue reaction product is obtained. The reaction is stopped using HCl and optic density is measured at 450 nm with a spectrophotometer.

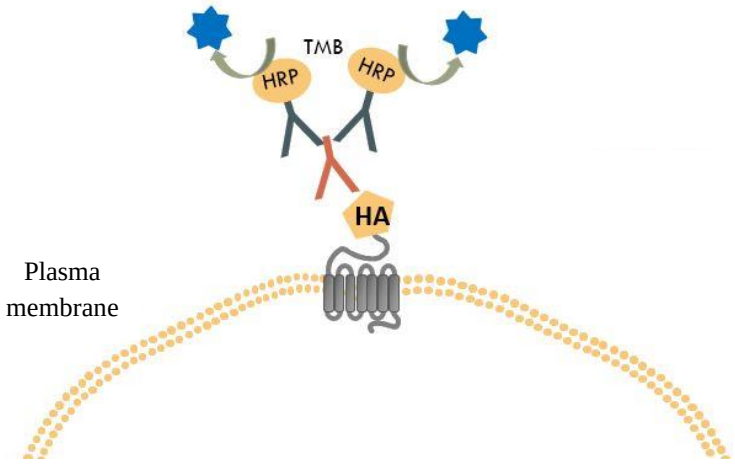

Figure 4. Enzyme Linked Immunosorbent Assay. Indirect ELISA assay was carried out on non-premetallized HEK 293T cells in order to quantify cell surface β2-AR expression. By using a primary and secondary antibody coupled to the HRP, a coloured product directly proportional to receptor level in cell surface was generated.

5. STATISTICAL ANALYSIS

The statistical analysis here refers to the comparison of the densities of β2-AR on the cell surface of each of the polymorphism with the density of the WT receptors. To carry out this scientific experiment, the ELISA experiment was repeated 6 times (n = 6). We use mean values +/- SEM (Standard Error of the Mean). We therefore chose to carry out a One-Way ANOVA test with the PRISM software to determine significant differences in mean density of receptors on the cell surface of the wild receptor and polymorphic receptors.

CHAPTER 4: RESULTS

RECEPTOR CELL SURFACE EXPRESSION

To investigate the impact of β2-AR polymorphisms on receptor pharmacology, we first studied the impact of β2-AR polymorphisms on receptor cell surface expression to determine the possible effects of the polymorphisms on receptor stability or cell surface trafficking.

Thus, we carried out an ELISA assay on non-permeabilized HEK293T/17 cells expressing WT or polymorphic receptors fused to an HA tag in the extracellular domain to accurately quantify receptor density at the plasma membrane.

We observed that the detection of WT receptor at the cell surface increased when transfecting cells with increasing amounts of β2-AR-encoding vector, until reaching saturation plateau. This plateau corresponds to receptor maximal expression at the cell surface. Interestingly, we obtained a similar plateau for Q27E and T164I polymorphisms, thereby indicating that these two polymorphisms do not impact on receptor's receptor maximal expression at the plasma membrane (Figure 5). Conversely, when increasing β2-AR R16G quantities were transfected, we observed that the polymorphism R16G caused a significant increase of receptor maximal expression in at the cell surface (OD_{450nm}=0,49) (Figure 5).

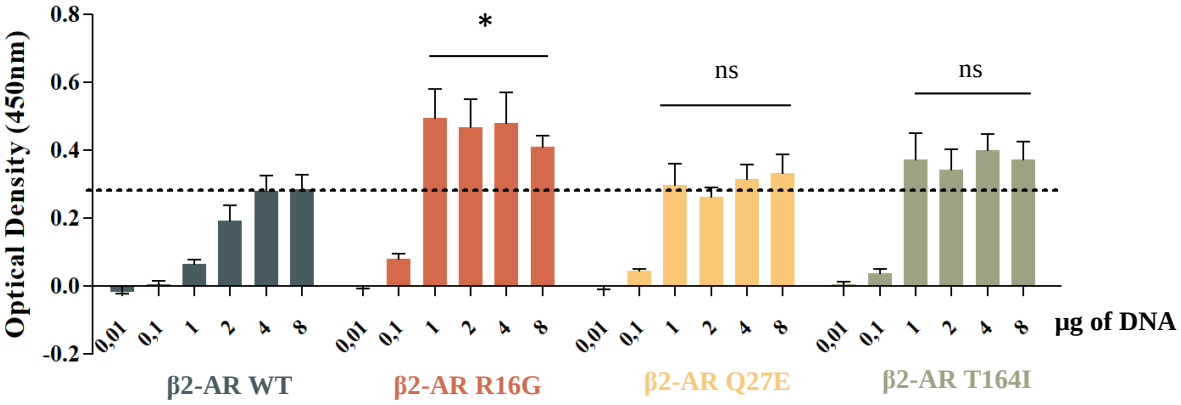

Figure 5. Cell surface expression of WT and polymorphic receptors. HEK293T/17 cells were transfected with increasing HA-β2-AR-encoding vectors and cell surface receptor density was measured by ELISA in non-permeabilized cells. Results are is expressed as optical density value (OD_{450nm}) and represent the mean ± SEM of 6 independent experiments. Significance was assessed using a One Way ANOVA test comparing each polymorphism (R16G, Q27E and T164I) with the wild-type receptor (WT). * = $p < 0.05$. ns = non-significant.

These results showed that R16G polymorphism significantly increases cell surface expression of β2-AR receptor. We then hypothesized that R16G polymorphism might alter receptor internalization.

RECEPTOR INTERNALIZATION

For this reason, we secondly studied the impact of β2-AR polymorphisms on ligand-induced receptor internalization (Figure 6). Again, we carried out an ELISA assay on non-permeabilized HEK293T/17 cells after stimulation with 10 µM of epinephrine during different time periods. We observed that after epinephrine stimulation, the WT β2-AR undergoes internalization as cell surface receptor level decreases approximately 80% throughout time. We remarked that β2-AR Q27E and β2-AR T164 also experienced a comparable diminution of cell surface receptor density, whereas R16G seemed to display a decreased ligand-induced internalization (Figure 6).

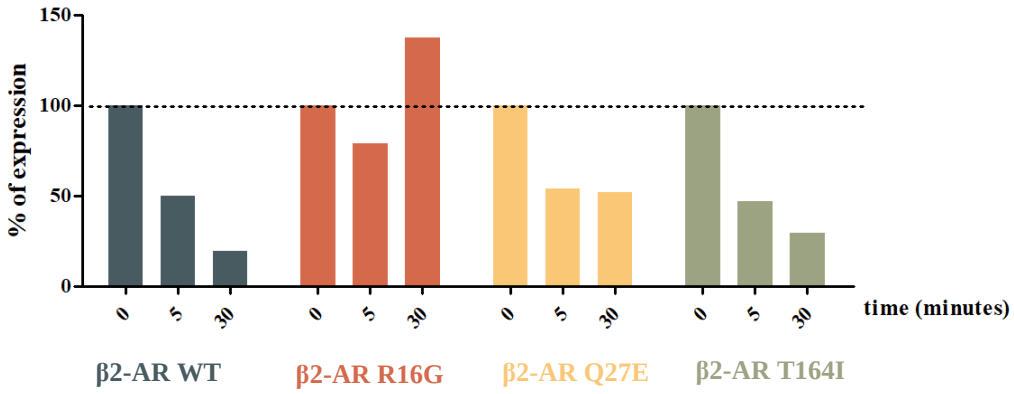

Figure 6. Cell surface expression of WT and polymorphic receptors after epinephrine stimulation. HEK293T/17 cells were transfected with HA-β2-AR-encoding vectors and cell surface receptor density was measured by ELISA in non-permeabilized cells after stimulation or not with epinephrine 10 µM during the indicated time periods. Results are expressed as optical density values (OD_{450nm}) and represent 1 independent experiment. n=1.

However, these are preliminary data and more independent experiments need to be done.

Altogether, my results demonstrated that the R16G polymorphism altered β2-AR cell surface expression as well as epinephrine-induced internalization.

CHAPTER 5: DISCUSSION AND CONCLUSION

The goal of this study was to determine the impact of three polymorphisms of β2-AR in cell surface receptor expression and receptor internalization to better decipher the pharmacological signature of these polymorphic receptors and understand the correlation of these polymorphisms with heart failure.

Firstly, our results show that only R16G polymorphism causes a significant increase of basal cell surface expression in comparison with the WT receptor, whereas the other two polymorphisms show similar expression levels to the WT.

Cell surface receptor expression is the consequence of a balance between the traffic of the receptor to the plasma membrane from the endoplasmic reticulum and its internalization (Moore *et al.*, 2007). Since β2-AR is well known to exhibit a constitutive activity (Denis *et al.*, 2012), we can hypothesize that WT as well as Q27E and T164I polymorphisms undergo constitutive internalization and recycling to the cell surface while R16G receptor fails to be internalized, leading to an increased expression at the cell surface at steady state.

For this reason, we secondly studied–polymorphism impact on ligand-induced receptor internalization. After stimulating with epinephrine during different time periods, we observed that epinephrine triggers internalization of the WT receptor as well as Q27E and T164I receptors. This internalization is classically associated with a signalling arrest. Contrarily, β2-AR R16G seems to experience a decrease of epinephrine-induced internalization. In this context, our results suggest that R16G polymorphism might increase cell surface residence time, thereby causing a sustained signalling that could be detrimental in heart failure which is characterized by catecholamine hypersecretion (Baker, 2014). Indeed, an increased cell surface density of β2-AR and agonist hypersecretion might be responsible for sustained and abnormal signalling that could cause a direct impact on cardiac output.

In the literature, R16G polymorphism showed to enhance receptor downregulation after isoproterenol stimulation while Q27E polymorphism has been proved to be resistant to downregulation (Green *et al.,* 1994). In this context, it would be interesting to also investigate ligand-induced internalization of β2-AR polymorphisms with norepinephrine and isoproterenol as it is now well established that these three agonists are biased agonists and do not trigger similar responses (Onfroy, 2017).

Moreover, similarly, it would be of interest to study polymorphisms impact on β-blocker-induced receptor internalization, and in particular on carvedilol-induced β2-AR internalization. Indeed, among all the β-blockers, carvedilol exhibits interesting features in the treatment of HF since it improves left ventricular ejection fraction and reduces mortality in heart failure patients (Ruffolo and Feuerstein, 1997). Interestingly, carvedilol behaves as a β2-AR biased agonist by promoting anti-apoptotic pathway through β arrestin 2 recruitment. (Carr *et al.,* 2016; Dwivedi *et al.,* 2018; Bencivenga *et al.,* 2019). In addition, carvedilol but not propranolol promotes β2-AR internalization in HEK293 cells, suggesting that down-regulating β2-AR with carvedilol may be beneficial in heart failure patients. Furthermore, carvedilol was also shown to trigger persistent downregulation of β2-AR through receptor internalization and degradation via ubiquitination (Han *et al.,* 2012). Taking this into account, treating patients carrying the R16G polymorphism with carvedilol might be beneficial since carvedilol would trigger receptor internalization and degradation terminating the deleterious effects of the increased cell surface residence time and abnormal signalling. Moreover, carvedilol could act as a biased ligand recruiting β arrestin 2 and activating an antiapoptotic pathway. Carvedilol would then considerably improve the treatment for R16G patients by blocking cardiotoxic effects caused by persistent Gs protein signalling and by enhancing cardioprotective effects which include receptor internalization and degradation and activation of antiapoptotic signalling. Nevertheless, these hypotheses need to be investigated and validated. Furthermore, Carvedilol stimulates very weakly the activation of β arrestin 2 dependent pathways, therefore carvedilol could act as a prototype for the design of new and more efficient drugs (Wisler *et al.,* 2007).

Finally, the ultimate outlook would be to be able to perform personalized medicine depending on the patient's polymorphism. Ideally, elucidating the impact of the polymorphisms on the molecular mechanisms of β2-AR would allow designing new drugs specialized for each polymorphism. Then, by genotyping by sequencing patients suffering HF a personalized an efficient therapy could be set up. Personalized therapy is expanding during the last years and it is already being performed for the treatment of diseases such as cancer where chemotherapy is based on the patients genotype (for example in the treatment with irinotecan, it has been shown that in 15% of patients there is a mutation that causes severe side effects like neutropenia being this drug unsuited for patients with this genotype (Zhang *et al.*, 2017)). In addition, numerous pharmacogenetic studies are also being developed for several cardiovascular conditions like hypertension where it has been shown that polymorphisms exert a major influence on drug therapeutic response.

CONCLUSION

This study investigated the impact of β2-AR polymorphisms on basal cell surface expression and epinephrine-induced internalization. In the future, a better characterization of the pharmacological signature of β2-AR would allow determining the correlation of the β2-AR polymorphisms with heart failure in order to perform personalized medicine depending on the patient's genotype.

The main conclusions that can be outlined from this research are:

1) R16G polymorphism significantly increases cell surface expression of β2-AR receptor.
2) β2-AR R16G polymorphism experiences a reduced epinephrine-induced internalization.

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