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Heart diseases

DIABETIC CARDIOMYOPATHY: SEARCHING FOR A THERAPEUTIC TARGET

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1. Project summary

Heart disease is the leading cause of morbidity and mortality in diabetic patients. The presence of ventricular dysfunction in a diabetic patient without underlying coronary artery disease and hypertension is now recognized as a distinct clinical entity termed diabetic cardiomyopathy (DC). The disturbances that characterize DC contribute to the high incidence of cardiac mortality in diabetic patients. Despite the added burden that diabetes poses on the heart, current therapeutic strategies do not specifically address DC. Clinical management of cardiomyopathy in affected patients is managed similarly, regardless of whether there is coexistent diabetes. Therefore, the mechanisms underlying the development of DC and the development of new drugs in order to improve disease management claim urgent investigation.

One promising therapeutic option in the treatment of DC is the use of peroxisome proliferator-activated receptor PPARβ/δ agonists. PPARβ/δ is the predominant PPAR subtype in the heart, and its activation may attenuate several of the mechanisms involved in DC development. Accordingly, the main objective of this research project consisted in studying the mechanisms by which PPARβ/δ could prevent the development of DC.

With this aim, mice were used that had suppressed PPARβ/δ expression and that had been treated with streptozotocin to induce diabetes. In addition, in vitro and in vivo studies were also performed in order to elucidate whether PPARβ/δ agonists prevent the activation of the signalling pathways involved in DC development. Finally, heart samples from patients suffering DC were also analysed to verify the potential of the findings observed in animal models.

2. Results

We first investigated whether PPARβ/δ knockout (KO) mice treated with streptozotocin (STZ) showed an accelerated DC development. With this aim, diabetes was induced by STZ administration (50 $mg \cdot kg^{-1} \cdot day^{-1}$ i.p., for 5 days) to 12-week old control (wild-type, WT) or knockout (KO) PPARβ/δ male mice. The animals were sacrificed 6 months after the diagnosis of diabetes.

STZ-induced diabetes reduced the weight of the heart, although it only reached statistical significance in PPARβ/δ KO mice. The body weight of diabetic mice, and in particular of PPARβ/δ KO diabetic mice, was also lower than that of control (WT) mice, thus accounting for the lack of changes in the HW/BW (heart weight/body weight) ratio. In contrast, the HW/TL (heart weight/tibia length) ratio decreased in diabetic mice, particularly in those with suppressed expression of PPARβ/δ (KO +STZ), which suggested some sort of atrophy in the heart. However, haematoxylin and eosin (H&E) staining analysis revealed no differences between cardiomyocyte size among groups, although there was a clear tendency to diminish in KO PPARβ/δ (both diabetic and non-diabetic) and diabetic WT mice.

Echocardiographic analysis indicated that neither diastolic nor systolic function varied among different treatment groups, although it suggested the presence of incipient dilated cardiomyopathy in diabetic WT mice and in both diabetic and non-diabetic PPARβ/δ KO mice, since LV-EDD and LV-ESD (left ventricle diameter at end diastole systole respectively) were increased. We also observed a rise in posterior wall thickness at end-diastole (PWTd) and end-systole (PWTs), and the thickening of interventricular septum at end-diastole (IVSd) and end-systole (IVSS) in PPARβ/δ KO non-diabetic mice when compared with the rest of the groups. Taken together, these data suggest that there is a thickening of the heart wall in PPARβ/δ KO mice, while the induction of diabetes with STZ would cause a thinning of the wall accompanied by expansion of the chamber especially in PPARβ/δ KO animals. On the other hand, data obtained from the area of the aortic valve (Ao VTI, aortic velocity time integral and Ao PV, aortic peak velocity) showed that PPARβ/δ KO mice developed aortic stenosis, which was much more evident in diabetic KO mice.

A morphometric study performed at the Hospital Universitario Valdecilla by researchers participating in this project also showed that diabetic PPARβ/δ KO mice displayed a more aggressive cardiac remodelling profile than diabetic WT mice, with increased wall thickening and dysregulated longitudinal systolic function, after transverse aortic constriction (TAC)-induced pressure overload (Figure 1).

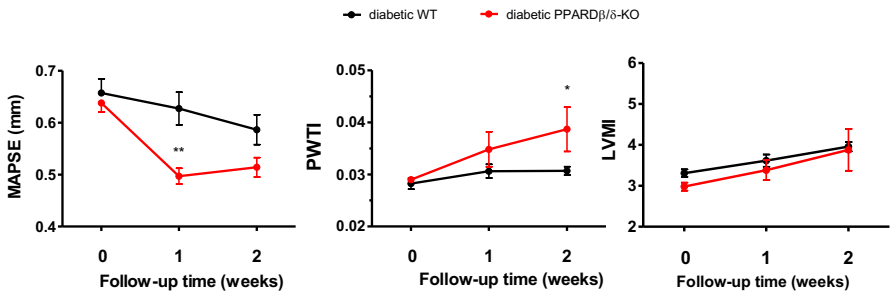

Figure 1. Echocardiographic parameters determined two weeks after performing TAC in WT and PPARβ/δ KO mice. MAPSE: mitral annular plane systolic excursion; PWTI: posterior wall thickness indexed to body mass; LVMI: left ventricular mass indexed to body mass.

*P<0.05 and **P<0.01 vs. WT STZ.

Later we investigated whether the above changes corresponded with functional cardiac structural remodelling of the heart. Masson's trichrome staining showed that diabetic WT and KO mice, as well as non-diabetic PPARβ/δ KO mice, developed cardiac fibrosis and intense fibril disorganization in perivascular and interstitial areas compared to control WT mice (Figure 2). These data also suggested that the increase in myocardial fibrosis did not account for cardiac functional changes observed in PPARβ/δ KO mice described above.

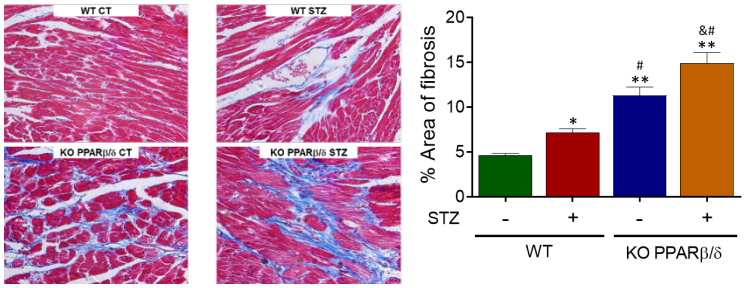

Figure 2. Masson's trichrome staining (left) and corresponding quantification of the collagen area (right) in the heart of WT and PPARβ/δ KO male mice, six months after the diagnosis of diabetes.

*P<0.05 and **P<0.01 vs. WT control; &P<0.05 vs. KO control; #P<0.05 STZ vs. WT.

To confirm the role of PPARβ/δ, we investigated whether the activation of this nuclear receptor prevented the development of DC in the same animal model of diabetes. Diabetes was induced by STZ administration to 12-week old C57BL/6 male mice. Once diabetes was diagnosed, animals were fed a diet supplemented with (+GW) or without (control) the PPARβ/δ agonist GW0742, so that each animal received an approximate daily dose of 1 $mg \cdot kg^{-1} \cdot day^{-1}$ of GW0742 for 16 weeks.

GW0742 treatment totally or partially prevented the reduction in body weight, heart weight and the HW/BW and HW/TL ratios observed in diabetic animals (STZ +GW), and in comparison with the untreated diabetic animals (STZ). Interestingly, none of these parameters was modified after treating non-diabetic mice with GW0742 (Ct +GW) compared with the control group (Ct or WT). GW0742 treatment did not cause significant changes in echocardiographic parameters in any of the groups analysed, whether diabetic or not.

H&E staining showed that the PPARβ/δ agonist partially prevented the cardiomyocyte size decrease induced by STZ and, even more remarkably, Masson's trichrome staining demonstrated that GW0742 significantly prevented fibrosis in diabetic mice (STZ +GW; Figure 3).

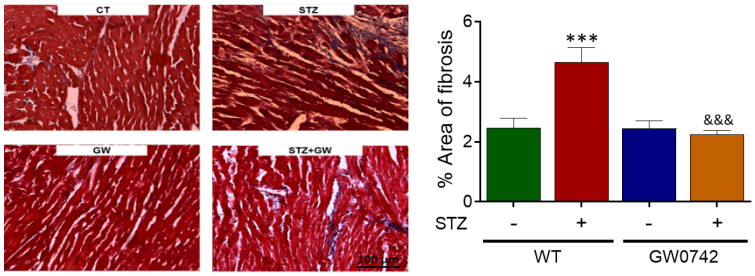

Figure 3. Masson's trichrome staining (left) and corresponding quantification of the collagen area (right) in the heart of control (Ct or WT) or diabetic (STZ) male mice treated with (+GW) or without GW0742 for 16 weeks from diagnosis of diabetes.

***P<0.001 vs. CT; &&&P<0.001 vs. STZ.

After this, the molecular mechanisms by which PPARβ/δ regulated the development of the disease were investigated. Through real-time RT-PCR analysis we examined gene expression of several molecular markers (Figure 4). STZ-induced diabetes caused an increase in the expression of inflammatory markers in the heart (MCP-1, IL-10, galectin 3/LGALS3), a fact that was further enhanced in PPARβ/δ animals (KO and KO +STZ). This inflammatory profile was not due to increased macrophage infiltration since the expression of widely recognized markers of macrophage infiltration (F4/80, CD68) was not modified. STZ-induced diabetes (STZ and STZ +KO groups), and to a lesser extent the suppression of PPARβ/δ expression in knockout mice (KO group), also induced the expression of TREM-1, a receptor involved in the amplification of the innate immune response and the recruitment of inflammatory cells after a heart attack.

We also observed an increase in the expression of the pro-apoptotic marker CHOP in STZ-induced diabetic animals, especially in those with suppressed PPARβ/δ expression (KO +STZ). In accordance with our previous histological analysis, diabetes caused an increase in the gene expression of cardiac fibrosis markers (MMP9, CTGF and collagen type I1a). This increase was clearly boosted in PPARβ/δ KO diabetic (KO +STZ) mice, even though non-diabetic PPARβ/δ KO animals also displayed an increase in cardiac fibrosis.

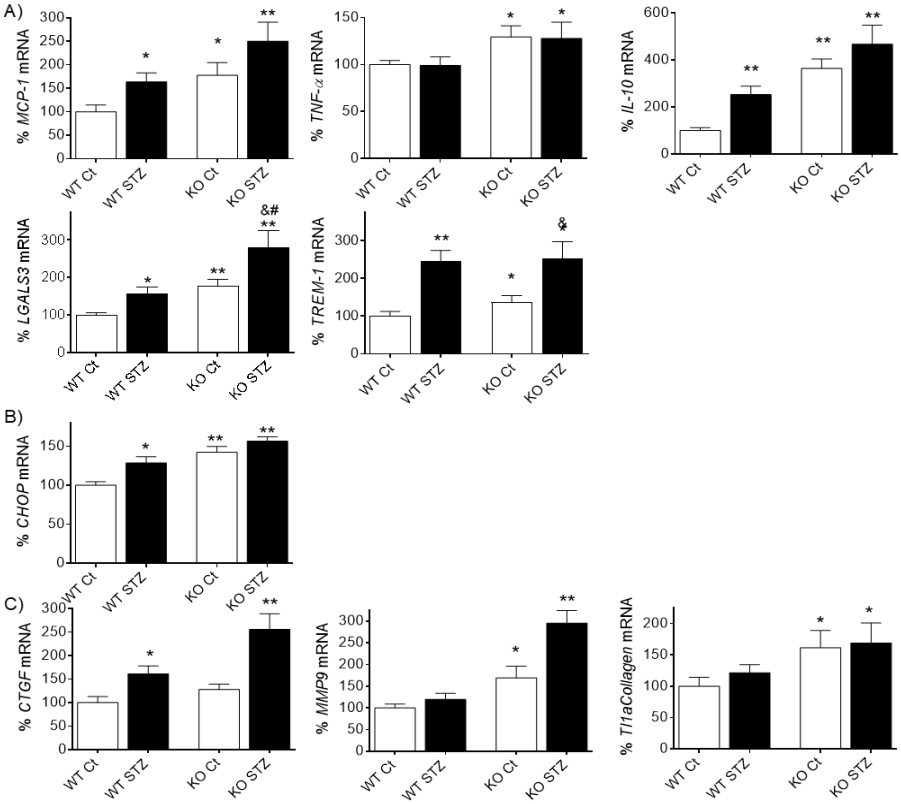

Figure 4. Gene expression analysis in heart samples obtained from WT and PPARβ/δ male mice six months after the diagnosis of diabetes. Panels show gene expression of inflammation (A), endoplasmic reticulum stress and apoptosis (B) and fibrosis (C) markers.

*P<0.05; **P<0.01 and ***P<0.001 vs. WT control; &P<0.05 vs. KO control; ###P<0.001 vs. WT +STZ.

Consistent with the above results, it was observed that the pro-inflammatory and pro-fibrotic response observed after STZ-induced diabetes was totally or partially prevented by treatment with the PPARβ/δ agonist GW0742 (Figure 5).

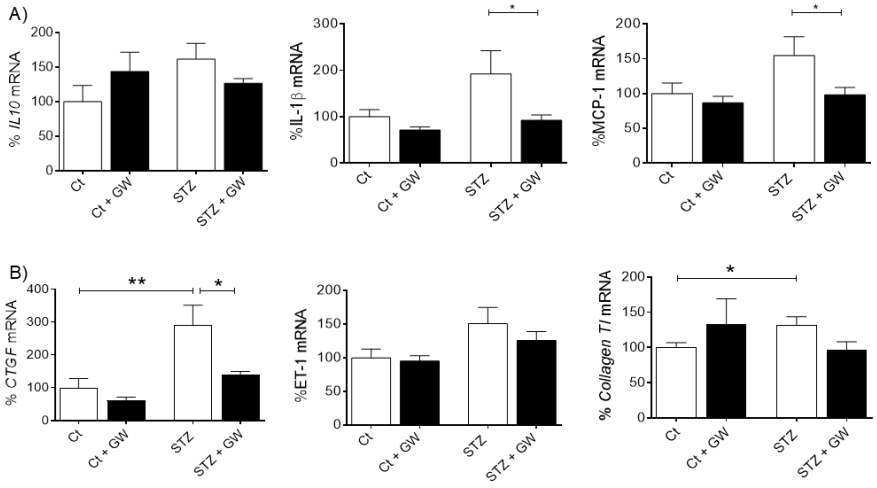

Figure 5. Gene expression analysis in heart samples obtained from control (Ct) or diabetic (STZ) male mice treated in the presence (+GW) or absence of the PPARβ/δ agonist GW0742 for 16 weeks from diagnosis of diabetes.

*P<0.05 and **P<0.01 vs. Ct.

We next investigated whether PPARβ/δ activation prevented the development of diabetic cardiomyopathy in an in vitro cell model. In order to investigate the effect of hyperglycaemia and PPARβ/δ on cardiac cells in vitro we used the mouse cardiac muscle cell line HL-1, which was exposed to low (control, 5.5 mmol/L) or high (hyperglycaemia, G30; 30 mmol/L) glucose concentrations for 72 hours. Cells were also treated in the presence or absence of GW0742 (1μmol/L) in order to confirm the results obtained in vivo. As shown in Figure 6, hyperglycaemia (G30) induced the expression of pro-inflammatory (IL-6, MCP-1), pro-fibrotic (MMP9, TGF-β, collagen type I1a) and pro-apoptotic (CHOP) markers. Hyperglycaemia also reduced the levels of IκBα, the protein responsible for the inhibition of the key pro-inflammatory transcription factor NF-κB, in these cells (Figure 7). The addition of GW0742 to the medium 24 hours before exposure to hyperglycaemia prevented most of the above-described changes.

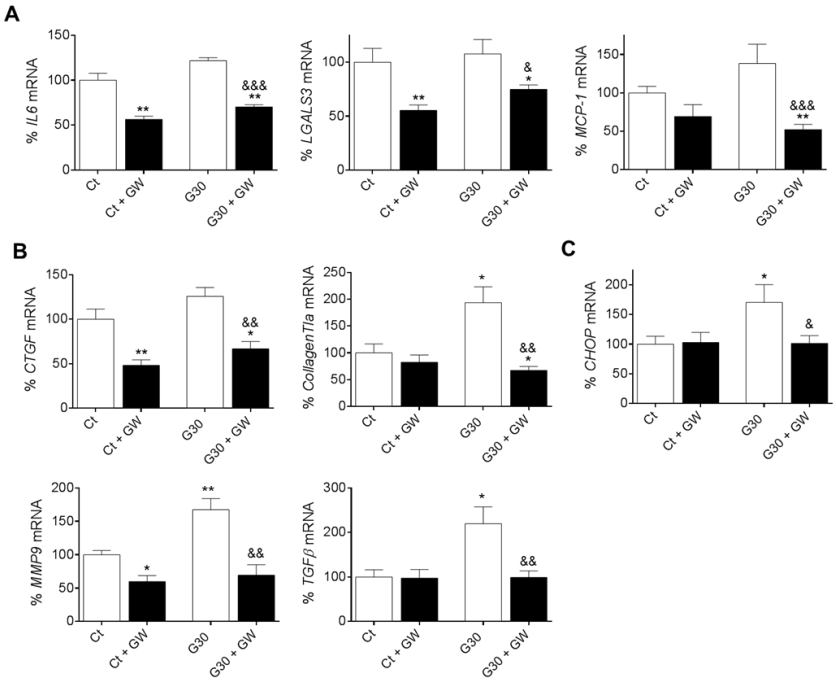

Figure 6. Gene expression analysis in HL-1 cell samples treated low (5.5 mmol/L) or high (hyperglycaemia, G30; 30 mmol/L) glucose concentrations in the presence or absence of GW0742 (GW).

**P*<0.05 and ***P*<0.01 vs. Ct; &*P*<0.05, &&*P*<0.01 and &&&*P*<0.001 vs. G30.

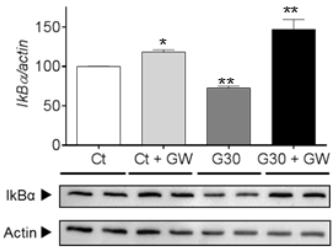

Figure 7. Western-blot analysis of IκBα levels in samples obtained from HL-1 cells treated with low (5.5 mmol/L) or high (hyperglycaemia, G30; 30 mmol/L) glucose concentrations in the presence or absence of GW0742 (GW).

**P*<0.05 and ***P*<0.01 vs. Ct.

To confirm these in vitro results, we repeated the experiments in induced pluripotent stem cells (iPSC)-derived cardiomyocytes, which confirmed that GW0742 addition to the culture media 24 hours before high glucose exposure prevented the increase in some inflammatory markers.

Studies at the Hospital Universitario Valdecilla were carried out with left ventricle biopsies obtained from diabetic (n = 23) and non-diabetic (n = 48) patients with aortic stenosis, obtained prior to transplantation and after performing the corresponding

echocardiographic analysis. There were no significant differences in PPARβ/δ gene expression between diabetic and normoglycaemic subjects, nor in the different markers of fibrosis (collagen type I and III, fibronectin 1 TGF-β) or cardiac hypertrophy (β-MHC) assessed. However, correlation and regression analysis showed that in normoglycaemic patients with aortic stenosis PPARβ/δ negatively correlated with the normalized left ventricular mass (LVMI; Figure 8).

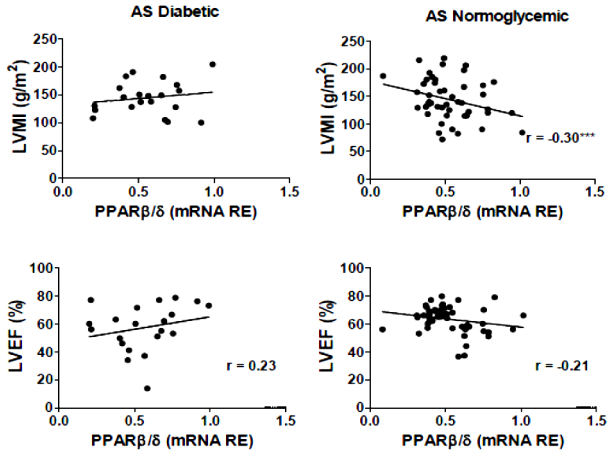

Figure 8. Relationship between the expression of PPARβ/δ and some morphofunctional echocardiographic parameters. LVMI: left ventricular mass normalized to body surface. LVEF: left ventricular ejection fraction.

During the course of this project, we also investigated the role of the deacetylase Sirt3 (sirtuin 3) in fibrosis and inflammation that occur during cardiac pathologies such as DC. We observed that mice with suppressed expression (KO) of SIRT3 showed increased cardiac fibrosis and inflammation, which was accompanied by an increase in the activity of the pro-inflammatory and pro-fibrotic transcription factor FOS/AP-1. This was confirmed in in vitro studies by overexpression of SIRT3 in neonatal rat cardiomyocytes and human AC16 cardiomyocytes (Figure 9), which showed that SIRT3 partially prevented the pro-inflammatory and pro-profibrotic response induced by the cytokine TNF-α, in a process that was dependent on the FOS/AP-1 signalling pathway. Our results also demonstrated that this effect was because SIRT3 was inhibiting FOS transcription by specific histone H3 deacetylation at K27 lysine residues within its promoter (Figure 10). Altogether, these data underscore the important role of SIRT3 in regulating the pro-fibrotic and pro-inflammatory responses in cardiac cells through the modulation of the FOS/AP-1 pathway and, since fibrosis and inflammation are crucial during the progression of DC, they suggest that SIRT3 activation might be an interesting novel therapeutic target.

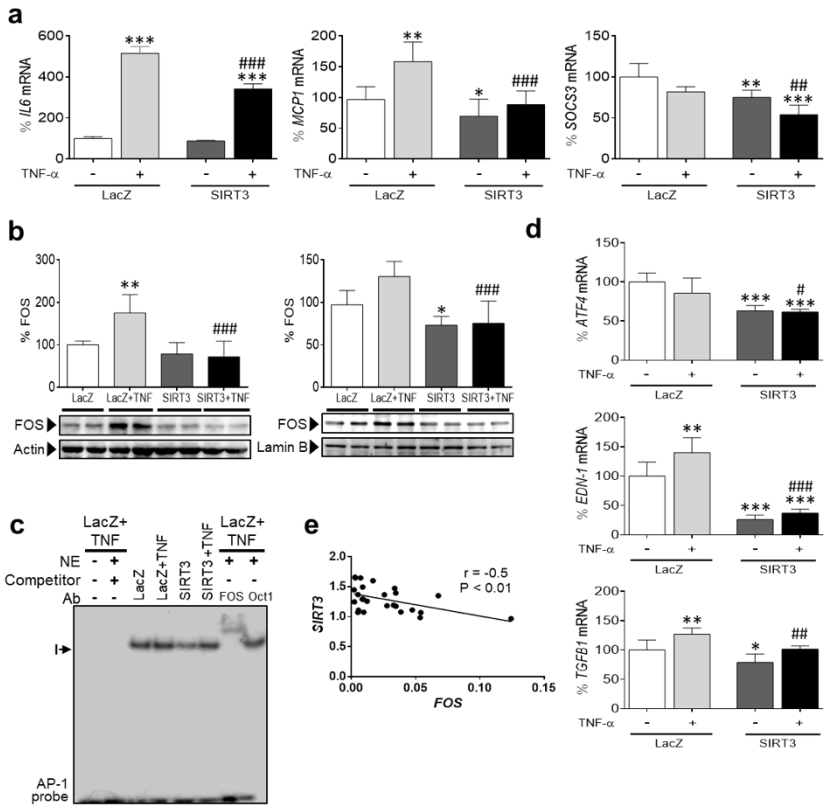

Figure 9. Gene expression analysis of different markers of inflammation (a) and fibrosis (d) in samples obtained from human cardiac AC16 cells transfected with plasmids for overexpressing Sirt3 (SIRT3) or control gene (LacZ), and in the presence or absence of the pro-inflammatory stimulus TNF-α (TNF). (b) Western-blot analysis of FOS protein levels in cytosolic and nuclear protein fractions obtained from the same samples. (c) AP-1 DNA binding activity assessed by EMSA. (e) Pearson correlation coefficient between FOS and SIRT3 gene expression in left ventricular tissue samples obtained from human patients.

**P*<0.05, ***P*<0.01 and ****P*<0.001 vs. LacZ; #*P*<0.05, ##*P*<0.01 and ###*P*<0.001 vs. LacZ +TNF-α.

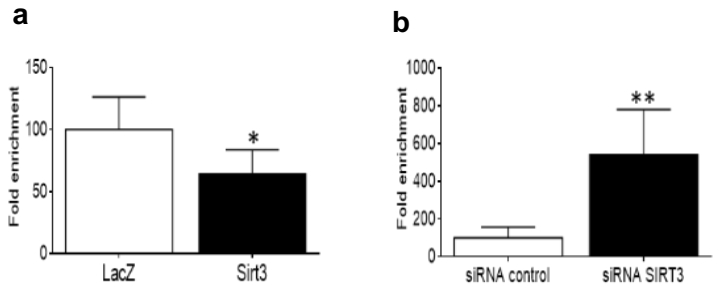

Figure 10. Chromatin immune-precipitation (ChIP) assays were performed to determine the levels of lysine K27 acetylation in histone H3 at the promoter of FOS gene in (a) AC16 human cardiac cells transfected with plasmids to overexpress Sirt3 (SIRT3) or the corresponding control gene (LacZ), or (b) Sirt3 or control siRNA to silence the expression of this gene *in vitro*.

**P*<0.05, ***P*<0.01 and ****P*<0.001 vs. LacZ (a) or Control siRNA (b).

3. Relevance of the project and future implications

Activation of the PPARβ/δ nuclear receptor has been proposed as a new therapeutic target for the treatment of several heart diseases. However, the molecular mechanisms by which its activation might be useful to treat these pathologies remain to be elucidated. The results obtained thanks to this project funded by La Marató de TV3 demonstrate that the activation of PPARβ/δ improves diabetic cardiomyopathy in animal models, mostly due to the inhibition of inflammation and fibrosis pathways. Likewise, data obtained by the Santander Group have enabled us to get further insight into the possible relationship between PPARβ/δ and the mechanisms responsible for the development of diabetic cardiomyopathy in patients suffering from this disease. Overall, these results confirm that PPARβ/δ is a potential target for treating diabetic cardiomyopathy, a disease for which there is still no specific pharmacological treatment. In fact, there are currently no PPARβ/δ agonist drugs approved for commercialization, although elafibranor, a dual PPARα and PPARβ/δ agonist, is expected to be marketed soon for non-alcoholic steatohepatitis (NASH) treatment. Once this drug is marketed, it will be easier to study its beneficial effects on other therapeutic indications such as diabetic cardiomyopathy. It should also be mentioned that NASH is characterized by the presence of inflammation and fibrosis similarly to what occurs in diabetic cardiomyopathy. This suggests that if elafibranor improves liver disease by downregulating inflammation and fibrosis, the activation of PPARβ/δ in the heart could also improve diabetic cardiomyopathy in humans by reducing these processes, as we have already demonstrated in mice. Obviously, clinical trials will be required to confirm this possibility but we believe that this project has established the molecular mechanisms of the possible positive effects of PPARβ/δ activators in the treatment of diabetic cardiomyopathy.

4. Resulting scientific outcomes

Faculty of Pharmacy and Food Sciences, University of Barcelona, Barcelona.

Publications

Rodríguez-Calvo et al. Small heterodimer partner (SHP) contributes to insulin resistance in cardiomyocytes. *Biochim. Biophys. Acta*. 2017, 1862: 541-551.

Palomer et al. Emerging Actors in Diabetic Cardiomyopathy: Heartbreaker Biomarkers or Therapeutic Targets? *Trends Pharmacol Sci.* 2018, 39(5): 452-467.

Palomer et al. PPARβ/δ: A key therapeutic target in metabolic disorders. *Int J. Mol. Sci*. 2018, 19: 913-926.

Palomer et al. SIRT3-mediated inhibition of FOS through histone H3 deacetylation prevents cardiac fibrosis and inflammation. *Signal Transduct. Target. Ther*. In press, DOI: 10.1038/s41392-020-0114-1.

Presentations at scientific conferences

Xavier Palomer et al. "Targeting PPARbeta/delta to prevent diabetic cardiomyopathy progression in mice". CIBERDEM Meeting. MAY 23-25, 2018. Hotel Campus de la Universitat Autònoma de Barcelona, Cerdanyola del Vallès.

Valdecilla University Hospital, Santander

Selected publications

Merino et al. Experimental modelling of cardiac pressure overload hypertrophy: Modified technique for precise, reproducible, safe and easy aortic arch banding/debanding in mice. *Sci Rep*. 2018, 16; 8(1): 3167. doi: 10.1038/s41598-018-21548-x.

Cañes et al. Neuron-derived orphan receptor-1 modulates cardiac gene expression and exacerbates angiotensin II-induced cardiac hypertrophy. *Clinical Science*. 2020, 134: 359-377.

Selected presentations at scientific conferences

Expósito et al. Role of cytokines of the TGF beta family in the atrial structural remodelling underlying atrial fibrillation in aortic stenosis patients. Frontiers in Cardiovascular Biology. Fifth Congress of the European Society of Cardiology Council on Basic Cardiovascular Science. Póster. *Cardiovascular Research*. Supplements 2018;114: S34. Vienna, Austria.

Expósito et al. Desregulación de la expresión de las citocinas de la superfamilia TGF-β y remodelado patológico pro-fibrótico auricular en pacientes con estenosis aórtica que desarrollan fibrilación auricular. SEC 2018 - Congreso de las Enfermedades Cardiovasculares. Póster. *Rev Esp Cardiol*. 2018, 71 Supl 1: 572. 25-27 October, 2018. Seville.

Expósito et al. Relación entre el remodelado estructural auricular y el desarrollo de fibrilación auricular postoperatoria. SEC 2018 - Congreso de las Enfermedades Cardiovasculares. Póster. *Rev Esp Cardiol*. 2018:71 Supl 1: 738. 25-27 October, 2018. Seville.