The role of oxidative stress in the crosstalk between leptin and mineralocorticoid receptor in the cardiac fibrosis associated with obesity

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We have investigated whether mineralocorticoid receptor activation can participate in the profibrotic effects of leptin in cardiac myofibroblasts, as well as the potential mechanisms involved. The presence of eplerenone reduced the leptin-induced increase in protein levels of collagen I, transforming growth factor β, connective tissue growth factor and galectin-3 and the levels of both total and mitochondrial of superoxide anion (O₂⁻) in cardiac myofibroblasts. Likewise, the MEK/ERK inhibitor, PD98059, and the PI3/Akt inhibitor, LY294002, showed a similar pattern. Mitochondrial reactive oxygen species (ROS) scavenger (MitoTempo) attenuated the increase in body weight observed in rats fed a high fat diet (HFD). No differences were found in cardiac function or blood pressure among any group. However, the cardiac fibrosis and enhanced O₂⁻ levels observed in HFD rats were attenuated by MitoTempo, which also prevented the increased circulating leptin and aldosterone levels in HFD fed animals. This study supports a role of mineralocorticoid receptor in the cardiac fibrosis induced by leptin in the context of obesity and highlights the role of the mitochondrial ROS in this process.

Extracellular matrix (ECM) accumulation is a common response of the heart to different types of damage, including obesity. The development of cardiovascular fibrosis has been repeatedly reported in obesity in experimental and clinical studies, which is frequently accompanied by co-morbidities such as hypertension and diabetes that can favour the development of cardiac fibrosis1,4. Growing evidence indicates that myocardial fibrosis is one of the pivotal contributors to heart muscle dysfunction in obesity1,4,6. The excessive ECM deposit due to a large number of myofibroblasts, the cell mainly responsible for fibrosis, can cause an aberrant remodelling that favours functional alterations, since a reduced relaxing capability of the heart can increase its filling pressure and contribute to diastolic dysfunction.

Multiple factors have been proposed as being responsible for the increased accumulation of collagen content in the myocardium in the context of obesity, with leptin being one of these factors1,7,8. This adipokine is locally

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produced in the heart in both the epicardial fat and in the myocardium and its production is up-regulated in obese rats. In a previous study, we have shown that the cardiac levels of leptin are associated with levels of total collagen content, collagen I and transforming growth factor (TGF)-β in diet-induced obesity in rats. Leptin is also able to stimulate the synthesis of collagen I and the profibrotic mediators TGF-β, connective transforming growth factor (CTGF) and galectin-3 through the increase of oxidative stress and the activation of PI3K/Akt in cardiac myofibroblasts from adult rats. Oxidative stress is characterized by the overproduction of reactive oxygen species (ROS) with the mitochondria being the main source. Leptin seems to exert more actions at cardiac levels because elevated circulating leptin levels are associated with left ventricular hypertrophy in patients with uncomplicated obesity.

Aldosterone through binding of mineralocorticoid receptor (MR), triggers the development of cardiac fibrosis in different pathologies, and its pharmacological blockade has demonstrated reduced interstitial fibrosis in these situations. Different studies have demonstrated that aldosterone is inappropriately elevated in obesity, and MR antagonism improves left ventricle function and reduces circulating procollagen levels in patients with obesity without other comorbidities. Similarly, low doses of spironolactone showed an anti-fibrotic effect in obese rats.

Interactions among leptin and aldosterone have been previously reported in different scenarios and at different levels. Leptin raises blood pressure and induces endothelial dysfunction via aldosterone-dependent mechanisms in obese female mice. Regarding the fibrotic actions, it has been shown that leptin promotes cardiac fibrosis via MR-dependent mechanisms in control and leptin-deficient mice. These data support a link between leptin and MR, which could result in the potentiation of the myocardial fibrosis associated with obesity. However, how or at which level these interactions occur is unknown. Therefore, the aim of this study was to investigate whether MR activation can mediate the profibrotic effects of leptin in adult cardiac myofibroblasts, the main cells involved in cardiac fibrosis. In addition, we have explored the potential mechanisms involved in this process. For this purpose, we have performed in vitro and in vivo studies in adult cardiac myofibroblasts and in rats fed a high fat diet (HFD).

**Methods**

Detailed methods are available in the online-only Data Supplement.

**Cell culture conditions.** Cardiac fibroblasts were isolated from the heart of adult male Wistar rats and used between passages 4 and 5. All assays in the present study were done at a temperature of 37 °C, 95% sterile air and 5% CO2 in a saturation humidified incubator. Cells were treated with leptin (100 ng/mL, BioVendor, Germany) between passages 4 and 5. All assays in the present study were done at a temperature of 37 °C, 95% sterile air and 5% CO2 in a saturation humidified incubator. Cells were treated with leptin (100 ng/mL, BioVendor, Germany) for 24 h in the presence or absence of the MR antagonist (eplerenone 10−6 mol/L; Sigma; St Louis, MO, USA), and in the presence or absence of the inhibitors of either PI3K or MEK pathways, LY294002 (20 × 10−6 mol/L) and PD98059 (25 × 10−6 mol/L), respectively.

**Animals.** Male Wistar rats of 150 g (Harlan Ibérica, Barcelona, Spain) were fed either a high-fat diet (HFD), 35% fat; Harlan Teklad #TD.03307, Haslett, MI, USA; n = 16) or a standard diet (3.5% fat; Harlan Teklad #TD.2014; Haslett, MI, USA; n = 16) for 6 weeks. Half of the animals of each group received either the mitochondrial antioxidant MitoTempo (0.7 mg Kg−1 day−1 Sigma, Louis, MO, USA) i.p. or vehicle (saline) from the third week on. The dose used of MitoTempo was chosen from previous publication. Animal weight was controlled every week. Food and water intake were administered at libitum and determined throughout the experimental period. Blood and heart were collected at the end of the experiment. The Animal Care and Use Committee of Universidad Complutense de Madrid and Dirección General de Medio Ambiente, Comunidad de Madrid (PROEX 242/15) approved all experimental procedures according to the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63/UE.

**Statistical analysis.** Data are expressed as mean ± SEM. Normality of distributions was verified by means of the Kolmogorov–Smirnov test. Pearson correlation analysis was used to examine association among different variables. Data were analyzed using a one-way analysis of variance, followed by a Newman–Keuls to assess specific differences among groups or conditions or unpaired Student’s t-test as corresponding using GraphPad Software Inc. (San Diego, CA, USA). The predetermined significance level was p < 0.05.

**Results**

**Mineralocorticoid receptor blockade reduces the ECM production and oxidative stress induced by leptin.** As shown in Fig. 1a, eplerenone was able to reduce the collagen I production induced by leptin in cardiac myofibroblasts. Similarly, eplerenone was able to prevent the production of the profibrotic mediators involved in the collagen production induced by this adipokine: TGF-β, CTGF and galectin-3 (Fig. 1a).

Leptin increased mitochondrial superoxide anion (O2−) production in both a dose- (data not shown) and a time-dependent manner (Fig. 1b) as well as total O2− (Fig. 1c) in cardiac myofibroblast. Eplerenone reduced oxidative stress in cardiac myofibroblast by reducing total (Fig. 1c) and mitochondrial O2− levels (Fig. 1d). Eplerenone alone was unable to affect any of the studied parameters (data not shown).

Likewise, leptin induced nitrosative stress in cardiac myofibroblasts, which was also diminished by the presence of eplerenone. As shown in Fig. S1a and b, cardiac myofibroblasts treated with leptin had detectable levels of intracellular nitric oxide (NO), which increased in a time-dependent manner. Untreated cells were mostly negative for the presence of NO. The presence of eplerenone abolished leptin-evoked NO production (Fig. S1c).

We also investigated whether leptin stimulation affected mitochondrial membrane potential. Leptin treatment for up to 24 h did not induce a substantial decline in Rd123 fluorescence nor in JC-1 fluorescence, indicating that...
Involvement of Akt and ERK pathways in profibrotic and prooxidant effects of leptin. Leptin was able to stimulate the phosphorylation of both Akt and ERK1/2 in cardiac myofibroblasts, reaching the maximum level at 15 minutes (Fig. 2a and b). This activation shows a biphasic pattern because it was also observed 24 hours after exposure to leptin (Fig. S3). Taking into consideration that PI3K/Akt and MAPK/ERK pathways could mediate the phosphorylation of the signal transducer and activator of transcription 3 (STAT3) which regulates genes involved in ECM, we evaluated this possibility. As shown in Fig. S4a, leptin was able to stimulate the phosphorylation of STAT3 in cardiac myofibroblasts, reaching the maximum level at 30 minutes (Fig. S4a). STAT3 phosphorylation was also stimulated by leptin at 24 hours and this effect was blocked by the presence of both pathway inhibitors (LY294002 and PD98059; Fig. S4B).

Next, we determined the role of these signaling mediators in profibrotic and prooxidant effects of leptin in cardiac myofibroblasts measured in cells pretreated with the selective inhibitors of these pathways PD98059 and LY294002, respectively. Both inhibitors reduced the production of collagen type I induced by leptin (Fig. 2c), as well as that of the mediators involved in this production TGF-β, CTGF and galectin-3 (Fig. 2c). Moreover, the presence of PD98059 and LY294002 was able to reduce the production of total ROS (Fig. 2d and e).
Effect of leptin in activation of EGFR. In order to determine if the activation of the receptor EGFR is involved in the effects of leptin in cardiac myofibroblasts, we examined the EGFR phosphorylation status at Tyr845 and Tyr1176 in response to 100 ng/ml of leptin at different times. Data of Fig. 3a and b show that leptin does not produce a significant effect in EGFR activation.

Interactions between mineralocorticoid receptor and leptin in the proliferation of cardiac myofibroblasts. In order to evaluate whether this interaction between MR and leptin not only involved ECM production but other actions, we explored if leptin is able to stimulate the proliferation of cardiac myofibroblasts in the presence or absence of aldosterone. Leptin alone or in the presence of aldosterone was unable to stimulate the proliferation of these cells (Fig. S5).

Mitochondrial ROS have a role in cardiac fibrosis and leptin and aldosterone levels in diet-induced obese rats. Taking into consideration that oxidative stress participates in the fibrotic effect induced by leptin and eplerenone reduced the oxidative stress induced by leptin, we evaluated whether oxidative stress is also involved in the possible interaction between MR and leptin observed in vivo. We also explored the effect of the administration of the mitochondrial ROS scavenger MitoTempo in rats fed an HFD. Body weight gain was expectedly significantly higher in rats fed an HFD as compared with rats fed a standard diet. This increase was smaller in animals treated with MitoTempo (Table 1). Similarly, MitoTempo reduced the increase in the body weight in obese animals (Table 1). It should be noted that the changes in body weight triggered by MitoTempo in HFD-fed rats were not consequence of a reduction in food intake (data not shown). Neither diet nor MitoTempo were able to modify systolic or diastolic function (Table 1). In addition, no differences in blood pressure levels were observed among any group along the study. Cardiac interstitial fibrosis was higher in obese animals than in

Figure 2. Effect of leptin on Akt and MEK pathways and impact of inhibition of Akt and MEK pathways on profibrotic protein factors and ROS levels in cardiac myofibroblasts. Protein levels of (a) pAkt/Akt and (b) pERK 1/2/ERK1/2 stimulated by leptin (100 ng/mL) for indicated time intervals. Cardiac myofibroblasts stimulated for 24 hours with leptin (100 ng/mL) in the presence or absence of the inhibitors of either MEK (PD98059; PD; 25 × 10⁻⁶ mol/L) or Akt (LY294002; LY; 20 × 10⁻⁶ mol/L) pathways for 24 hours. (c) Protein levels of collagen type I, TGF-β, CTGF and galectin-3. (d) Representative microphotographs in cells labeled with DHE and (e) Quantification of total superoxide anions and. Bar graphs represent the mean ± SD of 3–4 assays in arbitrary units normalized to β-actin. *p < 0.05; ***p < 0.001 vs. control. ††p < 0.01; †††p < 0.001 vs. leptin.
controls (Fig. 4a and b). These levels were correlated with those of leptin ($r = 0.8003$, $p < 0.001$) and aldosterone ($r = 0.6630$, $p < 0.01$). The increase in cardiac fibrosis observed in HFD was prevented by the administration of MitoTempo. This antioxidant also prevented the altered cardiac $O_2\text{-}\text{r}$ production in heart observed in HFD animals (Fig. 4c and d). In fact, cardiac ROS and fibrosis levels correlated with each other ($r = 0.6630$, $p < 0.001$).

Leptin levels were expectedly higher in obese animals than in controls, which were reduced in those animals treated with MitoTempo (Fig. 4e). Aldosterone plasma levels show a similar pattern to those observed with leptin (Fig. 4f). In fact, a correlation was observed between both leptin and aldosterone levels ($r = 0.6634$, $p < 0.01$). In addition, both leptin ($r = 0.6227$; $p < 0.05$) and aldosterone levels ($r = 0.7861$, $p < 0.001$) were correlated with those of cardiac ROS. The administration of MitoTempo did not modify any of the evaluated parameters in control animals (data not shown).

**Discussion**

The purpose of this study was to investigate the involvement of MR in the ECM production induced by leptin in cardiac myofibroblasts, which participates in the cardiac fibrosis associated with obesity. We herein report that the MR antagonist eplerenone prevents the increase in collagen I synthesis induced by this adipokine. Eplerenone also reduced oxidative stress, TGF-β, CTGF and galectin-3 levels which are involved in the ECM production induced by leptin. These results might suggest that the cardiac fibrotic effect of leptin could involve MR activation through oxidative stress-dependent pathway which activates the down-stream mediators by the activation of PI3K/Akt and MAPK/ERK pathways. Likewise, the in vivo data also show that the administration of a mitochondrial ROS scavenger in rats fed an HFD reduced cardiac fibrosis, as well as leptin and aldosterone plasma levels, supporting that there is an association among fibrosis, oxidative stress, leptin, and MR in obese normotensive rats. These data could help to understand the complex scenario involved in the development of cardiac fibrosis in the context of obesity.

The present data suggests an interaction between leptin and MR, which seems to be relevant in the development of cardiac fibrosis in the context of obesity in which both play a significant role. This affirmation is based on two facts: First, the presence in the incubation media of eplerenone reduced the increase in collagen type I induced by leptin in cardiac myofibroblasts, the main cells involved in cardiac fibrosis. Second,
Eplerenone prevented the production of the end effectors of leptin-induced ECM production (TGF-β, CTGF or galectin-3) in these cells. In addition, a correlation between aldosterone levels and total cardiac collagen was observed in normotensive animals fed an HFD in the presence of high leptin levels. Moreover, the reduction of cardiac fibrosis induced by the mitochondrial ROS scavenger MitoTempo in animals fed an HFD was accompanied by a reduction in both leptin and aldosterone plasma levels. We have previously reported the role of oxidative stress in the fibrotic effect induced by leptin in cardiac and vascular fibrosis. Therefore, the data suggest that leptin through ROS production can facilitate MR activation, which can mediate ECM production induced by this adipokine. In fact, this interaction seems to be more complex because eplerenone was able to reduce oxidative stress induced by leptin independently of its origin or nature (total ROS, nitro-oxidative species or mitochondrial ROS) in cardiac myofibroblasts, supporting the notion that there exists a vicious circle between oxidative stress and MR.

It is necessary to mention that we cannot exclude the possibility that the reduction in body weight gain, which was not consequence of a reduction in food intake, elicited by MitoTempo in HFD animals could also be partially responsible for the reduction in cardiac fibrosis and in leptin and aldosterone levels observed in the treated group. A similar finding has been reported with another mitochondrial ROS scavenger (MitoQ) in obese rats. However, MitoQ was able to reduce cardiac fibrosis in a model of cardiac toxicity in rats without body weight change or reduced Akt activation in rat cardiac myocyte. Total antioxidant capacity in the diet has been found to be inversely related to central adiposity, metabolic and oxidative stress bio-markers, and risk for cardiovascular diseases. Therefore, it could be proposed that the observed effect on body weight could also be a consequence of the antioxidant capacity of MitoTempo. Given that changes in food intake were not observed further investigation focusing on lipid absorption, metabolism and/or accumulation are required to understand the underlying mechanisms involved in the in the sliming effect.
Consistent with previous studies in different conditions\textsuperscript{23,29–34}, leptin stimulates PI3K/Akt, STAT-3 and MAPK/ERK signaling mediators in cardiac myofibroblasts that seem to be involved in the ECM production in cardiac myofibroblasts caused by this adipokine. This affirmation is based on the fact that the presence of their inhibitors blocks not only the synthesis of collagen I but also the downstream mediators (TGF-β, CTGF and galectin-3) induced by leptin. Therefore, taking into consideration that phosphorylation of PI3K/Akt and MAPK/ERK is involved in the fibrotic effects of both leptin and mineralocorticoid receptor activation\textsuperscript{35–37}, these data suggest that PI3K/Akt and MAPK/ERK can act as common signaling mediators for both factors, regulating downstream events which lead to the production of end effectors, including TGF-β, CTGF and galectin-3, and which finally lead to the synthesis of ECM in cardiac myofibroblasts. The possible interaction between leptin and MR signaling pathways leading to PI3K/Akt and MAPK/ERK activation may occur at several different stages and their understanding warrants a more detailed analysis.

The EGFR family and its ligands serve as a switchboard for the regulation of multiple cellular processes, including fibrosis. EGFR transactivation in cardiac cells has been observed following stimulation with leptin after MR activation, and its action has been implicated in cardiac remodelling\textsuperscript{38–41}. EGFR transactivation is also involved in ROS generation, induced by high concentrations of glucose in rat cardiomyocytes\textsuperscript{42} and supporting that transactivation of EGFR plays a central role in mediating cardiac damage. However, our data does not support the phosphorylation of EGFR as a signaling which engages in leptin-mineralocorticoid receptor crosstalk in ECM production in adult cardiac myofibroblasts.

In summary, our data show that the cardiac fibrotic effect of leptin could involve the MR activation through oxidative stress-dependent pathway which activates the PI3K/Akt and MAPK/ERK pathways and consequently the production of end effectors, including TGF-β, CTGF and galectin-3, which are mainly responsible for the final synthesis of ECM in cardiac fibroblasts. These findings will aid in understanding the crosstalk among these factors in the production of ECM, which plays a role in the development of cardiac remodelling in the context of obesity and support the relevance of mitochondrial oxidative stress in this process. Therefore, the study highlights the complexity of the molecular mechanisms involved in the development of myocardial fibrosis associated with obesity.

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Acknowledgements
We thank Avelina Hidalgo, Roberto Cañadas, Blanca Martínez, and Virginia Peinado for their technical help and Anthony DeMarco for his help in editing the article. This work was supported by Instituto de Salud Carlos III–Fondo Europeo de Desarrollo Regional (FEDER) (PI15/01060; RD12/0042/0024 and RD12/0042/0033, CIBERCV; Miguel Servet contract CP13/00221) a way to build Europe. Ministerio de Economía y Competitividad (SAF2012-34460 and SAF2016-81063), the FPI Program del Gobierno de Castilla y León (co-funded by FSE) and COST ADMIRE network (BM1301).

Author Contributions
J.G.T. performed experiments and data analysis and helped to write the manuscript, G.M.R. performed data analysis and helped to write the manuscript, E.M.M. performed experiments and data analysis and helped to write the manuscript, R.M. performed experiments and data analysis, M.M. performed experiments and data analysis and helped to write the manuscript, N.L.A. contributed to discussion and the writing of the manuscript, R.J.L. performed experiments and data analysis, I.G. performed experiments and data analysis, M.L. performed experiments and data analysis and helped to write the manuscript, J.A.S.R. contributed to discussion and the writing of the manuscript, M.G.A. performed experiments and data analysis, M.S. contributed to discussion and the writing of the manuscript, M.L.N. designed the study, performed experiments and data analysis and wrote the manuscript, V.C. designed the study, performed experiments and data analysis and wrote the manuscript.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-017-17103-9.

Competing Interests: The authors declare that they have no competing interests.

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