INHIBITION OF GALECTIN-3 AMELIORATES THE CONSEQUENCES OF CARDIAC LIPOTOXICITY IN A RAT MODEL OF DIET-INDUCED OBESITY

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ABSTRACT

Obesity is accompanied by metabolic alterations characterized by insulin resistance and cardiac lipotoxicity. Galectin-3 (Gal-3) induces cardiac inflammation and fibrosis in the context of obesity; however, its role in the metabolic consequences of obesity is not totally established. We have investigated the potential role of Gal-3 in the cardiac metabolic disturbances associated with obesity. In addition, we have explored whether this participation is at least partially acting on mitochondrial damage. Gal-3 inhibition in rats fed a high-fat diet (HFD) for 6 weeks with modified citrus pectin (MCP; 100 mg/kg/day) attenuated the increase in cardiac levels of total triglyceride (TG). MCP treatment also prevented the increase in cardiac protein levels of carnitine palmitoyl transferase IA, mitofusin 1 and mitochondrial complexes I and II, reactive oxygen species accumulation and decrease in those of complex V but did not affect the reduction in $^{18}$F-fluorodeoxyglucose uptake observed in HFD rats. The exposure of cardiac myoblasts (H9c2) to palmitic acid increased the rate of respiration mainly due to an increase in the proton leak, glycolysis, oxidative stress, $\beta$-oxidation and reduced mitochondrial membrane potential. Gal-3 activity inhibition was unable to affect these changes. Our findings indicate that Gal-3 inhibition attenuates some of the consequences of cardiac lipotoxicity induced by a HFD since it reduced TG and LPC levels. These reductions were accompanied by an amelioration in the mitochondrial damage observed in HFD rats, although no improvement was observed in insulin resistance. These findings increase the interest for Gal-3 as a potential new target for therapeutic intervention to prevent obesity-associated cardiac lipotoxicity and subsequent mitochondrial dysfunction.
INTRODUCTION

Obesity is a chronic disease characterized by excessive accumulation of adipose tissue and lipids forming ectopic fat deposits in different tissues, including the heart (Abdurrachim et al., 2014; French et al., 2016; Ghosh et al., 2011). This excessive accumulation of lipid in the heart—termed cardiac lipotoxicity—can trigger cellular alterations since lipids are important regulators of cardiac function through their role in membrane structure, transport, signaling and as substrate for β-oxidation for obtaining energy in the mitochondria (Cedars et al., 2009; Lim et al., 2011). Cardiac lipotoxicity not only involves an excessive accumulation of intra-myocellular triglycerides (TG) in the heart but also changes in different lipid classes, as well as in their fatty acid composition, thereby facilitating the formation of active lipid mediators which affect metabolism and cardiac function, in part by altering mitochondrial function (Bugger and Abel, 2008; Elezaby et al., 2015; Lucas et al., 2016; Wang et al., 2015).

A common additional feature of the obese heart is impaired insulin signaling, which represents an adaptation of the heart to an excess of calories that promotes the development of diabetic cardiomyopathy (Guo and Guo, 2017; Jia et al., 2016; Riehl and Abel, 2016). This condition not only alters cardiac metabolism, but also increases myocardial oxygen consumption, reduces cardiac efficiency by affecting mitochondrial function and increases oxidative stress with the mitochondria being the main source of reactive oxygen species (ROS) (Boudina et al., 2007; Elezaby et al., 2015; McMurray et al., 2016).
Galectin-3 (Gal-3) is a member of a β-galactoside binding lectin family produced in the heart and whose expression is upregulated in obesity (Martinez-Martinez et al., 2014). Its role as a central mediator of cardiovascular fibrosis and the inflammatory processes present in different pathological situations including obesity has been demonstrated (Martinez-Martinez et al., 2014; Martinez-Martinez et al., 2015b). In addition, the potential role of Gal-3 as a regulator of cardiac oxidative stress which can facilitate the development of fibrosis has been suggested since it is able to up-regulate Nox4 expression in cardiac fibroblasts (He et al., 2017). In addition, we have reported Gal-3 to be a mediator of leptin-induced ROS production in heart of obese rats (Martinez-Martinez et al., 2014; Martinez-Martinez et al., 2015b). However, information regarding the role of Gal-3 in the metabolic consequences of obesity is not well established since it has exhibited roles of being both mediator and preventer of metabolic disorders (Martinez-Martinez et al., 2016; Menini et al., 2016). Therefore, the aim of this study was to explore the potential contribution of Gal-3 in the metabolic disturbances associated with obesity. In addition, we have explored whether this participation is at least partially acting on mitochondrial damage. To address this issue, we analyzed the repercussion of a specific inhibitor of Gal-3 (Martinez-Martinez et al., 2015b), modified citrus pectin (MCP), in an animal model of diet-induced obesity and in cultured cardiomyoblasts stimulated by palmitic acid.
RESULTS

*Consequences of Gal-3 activity inhibition on body weight, cardiac function and fibrosis and blood pressure in HFD-fed rats*

Animals fed an HFD show an increase in body weight, cardiac hypertrophy and interstitial fibrosis, although no changes in cardiac function or blood pressure as compared to control rats (Martinez-Martinez et al., 2015b) after 6 weeks of HFD intake. Gal-3 expression was up-regulated in heart from HFD rats and MCP treatment reduced the increase in total cardiac collagen content without modifying either body weight or cardiac hypertrophy (Martinez-Martinez et al., 2015b). MCP did not affect any of these parameters in control animals (Martinez-Martinez et al., 2015b). Therefore, and to simplify the data, only CT, HFD and HFD+MCP will be presented in the results.

*Consequences of Gal-3 activity inhibition on cardiac glucose use and insulin resistance in HFD-fed rats*

Next, we addressed whether the up-regulation of Gal-3 is involved in the changes in glucose use observed in obese rats. Therefore, PET studies were performed to assess cardiac glucose use. Representative examples of PET images (Figures 1A-1B) revealed that the control group show more $^{18}$F-Fluorodeoxyglucose (FDG) uptake in the hearts compared with obese animals. The administration of MCP was unable to ameliorate myocardial $^{18}$F-FDG uptake. Similarly, MCP was unable to affect the increase in HOMA index in obese rats (Figure 1C).
As expected, obese animals show an increase in total TG content in the heart (Figure 2A) mainly due to an accumulation of those enriched with palmitic acid (16:0) and to a minor extent those enriched with stearic acid (18:0) and arachidonic acid (20:4) (Figure 2B). In fact, obese animals show an overall increase (4 fold; p<0.001) in TG enriched with saturated fatty acids, although no significant changes were observed in polyunsaturated ones (data not shown). Treatment with MCP was able to reduce this rise observed in animals fed an HFD (Figures 2A-2B). An increase in the only Ceramide (Cer) species detected (d18:1/16:0) was observed in HFD rats as compared with control animals (Figure 2C). However, a reduction in total sphingomyelins (SM) levels was observed in HFD as compared with control animals (Figure 2D). This was consequence of the decrease in the majority of the eight species detected. MCP treatment was unable to normalize these changes (Figures 2C-2D). A negative correlation was observed between total SM and Cer levels (r=-0.5274; p=0.0433). In addition, a correlation was found between Cer and total SM levels and those of $^{18}$F-FDG cardiac uptake (r= -0.668; p=0.0065 and r= 0.6220; p=0.0175; respectively). A similar correlation was found between HOMA index and Cer levels (r=0.6299; p=0.012). Given that phosphatidyl choline (PC) and lysophosphatidyl choline (LPC) are involved in various diseases and that altered serum levels of LPC was considered to be a specific metabolic trait associated with obesity, (Li et al., 2014; Tulipani et al., 2016) we focused on PC and LPC molecular species in heart from HFD rats. No significant changes were observed in total PC levels between CT and HFD rats. About 56 species of PC were detected in heart from CT rats,
and the expression pattern was very similar to that observed in the heart of the HFD group (data not shown). However, an increase in total LPC levels was found in HFD animals as compared to CT ones, (Figure 2E). This increase was mainly due to the rise in those molecular species enriched with stearic acid, LPC (18:0) and arachidonic acid LPC (20:4) (Figure 2F). The levels of the detected LPC (16:0), LPC (18:2), LPC (18:3), LPC (22:5) and LPC (22:6) were not affected by the HFD. MCP treatment was able to prevent the increase in LPC levels in both total and those enriched with stearic (18:0) or arachidonic (20:4) acids in HFD rats (Figures 2E-2F). Total LPC levels were correlated with those of TG enriched with palmitic acid (r=0.6841; p=0.0048). The increase in TG was associated with higher protein levels of the enzyme that controls the entry of long-chain fatty acyl CoA into mitochondria (CPT1A; Figure 3A). Treatment with the inhibitor of Gal-3 was able to normalize CPT1A levels in HFD rats (Figure 3A).

Consequences of Gal-3 activity inhibition on cardiac mitochondria dynamic in HFD-fed rats

Considering that there is an accumulation of fatty acids in the mitochondria of obese animals, we explored the consequences on the mitochondrial dynamic. To this end, we evaluated the levels of two proteins involved in the process of fusion and fission. As shown in Figure 3B, the protein levels of mitofusin 1 are higher in obese animals as compared with controls. MCP treatment was able to reduce these high levels. By contrast, levels of DRP1, a marker of fission, were unaffected by either obesity or Gal-3 inhibition (data not shown). In addition, obese animals showed an increase in mitochondrial oxidative stress since higher fluorescence staining was observed in the heart of the obese animals as compared with control ones in response to MitoSox (red
mitochondrial superoxide indicator; Figure 3C). A correlation was found between levels of mitochondrial ROS and those of both total TG (Table 1) and TG enriched with stearic or arachidonic acid (Table 1). We also evaluated the protein levels of the components of mitochondrial respiratory chain complexes. As shown in Figure (3D), obesity exerts a different impact on different complexes since obese animals show an increase in complex I and II and a decrease in the levels of complex V, the ATP synthase. No changes were observed in complex III and IV levels (Figure S1). MCP treatment was able to reverse these changes.

*Effect of palmitic acid on mitochondrial function in rat cardiomyoblats. Consequences of Gal-3 activity inhibition*

Taking the observed accumulation of palmitic acid into consideration, we decided to explore its effects on mitochondrial function using cultured rat cardiomyoblats. Figure S2 shows that palmitic acid was unable to affect cell viability at the doses used. Next, we performed a “mitochondrial stress test” in order to evaluate the mitochondrial bioenergetics caused by palmitic acid. The general scheme of the stress test is shown in Fig S3A. Palmitic acid caused a modest increase in the basal rate of respiration, which was dose-dependent (Figure S3A) and paralleled by a dose-dependent increase in the ECAR signal, indicating that palmitic acid is lowering the OXPHOS efficiency due to uncoupling (Figure S3B). Indeed, the proton leakage rate (Figure S3C) was significantly increased in the palmitic acid-treated cells. The presence of MCP did not alter significantly the cellular energetics induced by palmitic acid (200 µmol/L; Figures 4A-4C). Additionally, as shown in Figures S4A-S4B, the presence of palmitic acid for 24 hours was able to reduce Rhodamine 123 staining in rat cardiomyoblasts in a dose-dependent
manner, thereby reinforcing the idea that the increase in proton leak due to fatty acid uncoupling causes a reduction in the mitochondrial potential membrane. The presence of MCP did not alter the decrease in Rhodamine 123 staining caused by palmitic acid (200 µmol/L; Figure 4D). Likewise, the presence of triacsin C—the inhibitor of acyl-CoA synthase—was unable to modify the effect induced by palmitic acid (Figure S4C). In addition, palmitic acid was able to increase mitochondrial ROS production in a dose-dependent manner, as suggested by an increase in MitoSox-induced fluorescence in palmitic-treated cells relative to that of vehicle-treated cells (Figures S5A-S5B). The presence of MCP did not significantly alter the fluorescence induced by MitoSox in the cells treated with palmitic acid (200 µmol/L; Figure 4E). This was accompanied by a dose-dependent reduction in NAO staining, indicating an increase in the levels of oxidized cardiolipins (Figure S6). Finally, palmitic acid was able to increase the β-oxidation in a time-dependent manner (Figure S7). The presence of MCP did not significantly alter this effect induced by palmitic acid (Figure 4F).

**DISCUSSION**

The role of Gal-3 as a central mediator of cardiovascular fibrosis and the inflammatory processes present in different pathological situations has been amply demonstrated (Calvier et al., 2015; Calvier et al., 2013; Martinez-Martinez et al., 2015a; Martinez-Martinez et al., 2014; Martinez-Martinez et al., 2015b). We herein report for the first time that Gal-3 can modulate some of the metabolic consequences of obesity since it is demonstrated that the inhibitor of Gal-3 activity, MCP, reduced cardiac lipotoxicity and ameliorated the mitochondrial damage observed in the heart of obese rats.
The present data show a significant increase in TG levels in the heart of normotensive obese animals, confirming previous clinical and experimental studies (Kroon et al., 2017; Shimabukuro et al., 2013). This increase was mainly consequence of a rise in the levels of TG enriched with the saturated (16:0, 18:0) and the polyunsaturated 20:4 fatty acids. We have likewise found an increase in LPC levels, mainly consequence of those enriched with 20:4 and 18:0. A similar increase has been reported in circulating LPC levels in obese patients and experimental models of obesity (Eisinger et al., 2014; Li et al., 2014; Tonks et al., 2016). It should be noted, however, that reductions have been reported in other studies, which may be the result of varying LPC fatty acid composition (Li et al., 2014; Tulipani et al., 2016; Wahl et al., 2012) and suggests a more complex effect of obesity in this lipid class. In contrast, PC profiles were not affected in HFD rats. Therefore, these data support the idea that cardiac lipotoxicity involved not only variations in lipid classes but also differences in their fatty acid composition.

Neither TG nor LPC levels seem to be major determinant of the altered cardiac glucose use observed in HFD animals since no correlation was found amongst these parameters. In addition, MCP treatment was able to normalize both cardiac TG and LPC levels without altering the abnormal 18F-FDG cardiac uptake. These data confirm previous observations in which no link was found between either TG or LPC circulating levels and insulin resistance in the context of obesity (Coen and Goodpaster, 2012; Del Bas et al., 2016; Eisinger et al., 2014; Tulipani et al., 2016). However, the reduced cardiac SM levels observed in HFD rats could participate in the cardiac insulin resistance observed in these animals since a direct correlation between levels of SM and 18F-FDG uptake was observed, confirming previous data (Denimal et al., 2016; Tonks et al., 2016).
agreement with this observation, we have found that cardiac SM levels were independent predictors of GLUT4 cardiac levels in HFD (Marin-Royo et al., 2017). In addition, and considering the role of Cer in the pathogenesis of diabetes (Galadari et al., 2013), our data support the participation of this lipid class, whose levels were increased in HFD and associated with cardiac levels of $^{18}$F-FDG uptake. Cer levels result from both the novo synthesis as well as the hydrolysis of SM, suggesting a link between both lipids; in fact, a negative correlation was found between them. A variety of potential mechanisms—oxidative stress, changes in mitochondrial function and endoplasmic reticulum stress—can be underlying these effects (Fucho et al., 2017; Petersen and Shulman, 2017; Yazici and Sezer, 2017).

Our study shows an increase in mitochondrial oxidative stress in the heart of normotensive obese animals, which was accompanied by some mitochondrial alterations: an increase in CPT1A, mitofusin 1, and respiratory chain complex I and II and as well a reduction of complex V. These alterations suggest that changes are happening not only in the mitochondrial machinery content but also in mitochondrial morphology, as well. This is in agreement with the concept that mitochondrial dysfunction has been suggested as one mechanism which participates in the cardiac damage associated with obesity, as mitochondria plays a central role in the energy production essential to maintaining cardiac activity (Mercer et al., 2010; Wang et al., 2015). The fact that MCP treatment reduced oxidative stress and normalized the levels of CPT1A, mitofusin 1, and respiratory chain complexes further supports this role. In fact, connections among oxidative stress, lipotoxicity and mitochondrial dysfunction has been suggested (Mercer et al., 2010; Schulze et al., 2016; Wang et al., 2015). Supporting this concept, we have found a correlation between TG and LPC cardiac levels and those of mitochondrial ROS.
in MCP-treated and untreated HFD rats. In addition, we have observed that palmitic acid, the most elevated fatty acid in the cardiac energetic reservoir (TG) of HFD rats, was able to stimulate mitochondrial ROS production in H9c2 cells, confirming previous observation (Miller et al., 2005).

An increase in ROS can be consequence of either an increase in oxidative metabolism or a reduction in antioxidant capacity (Cheng et al., 2017; Vakifahmetoglu-Norberg et al., 2017). Apart from the major contributors to mitochondrial ROS production, complex I and complex III, several oxidoreductases located in mitochondrial membrane may produce superoxide at significant rates during oxidation of fatty acids (Andreyev et al., 2015; Brand, 2010). This oxidant environment can disturb mitochondrial membrane phospholipids, including cardiolipins, as was evident by the significant reduction in NAO fluorescence. The peroxidized cardiolipin generated changes in the physico-chemical properties of the mitochondrial membrane, which could be altering mitochondrial bioenergetics since cardiolipins play a central role in normal function and structure of the inner mitochondrial membrane (Birk et al., 2014; Paradies et al., 2014). In fact, this could explain the observed increase in mitofusin 1, which may suggest an increase in mitofusion, a process that represents an adaptive pro-survival response against stress (Tondera et al., 2009).

The increase in the β-oxidation induced by palmitic acid in H9c2 cells could be a compensatory mechanism for the reduced oxidative phosphorylation, which is suggested by the increase in proton leak. This process may occur in the heart of obese animals since the decreased ATP synthase levels observed in these animals was accompanied by an increase in CPT1A involved in the mitochondrial uptake of fatty acids, an essential step for the β-oxidation in the mitochondria. However, the
compensatory increase in glycolysis induced by palmitic acid in H9c2 in order to maintain ATP levels adequate for meeting energy cell demands through anaerobic ATP production could be limited in obese animals, since glucose uptake by the heart is reduced. We have found that palmitic acid was able to reduce mitochondrial membrane potential independently of its transformation in acyl-CoA since the presence of an inhibitor of the enzyme involved in this step, triacsin C, had no effect. Indeed, it has been reported that fatty acids have a protonophoric activity likely mediated by the adenine nucleotide translocator (ANT) (Ash and Merry, 2011).

The data show that the inhibitor of Gal-3 activity is able to prevent some of the changes observed in the cardiac lipid profile in obese animals and thus supports a role of Gal-3 in cardiac lipotoxicity. In fact, this was able to reduce the excessive accumulation of TG and LPC. The potential mechanism through which Gal-3 is able to participate in cardiac lipotoxicity is unclear, although its ability to produce an oxidant environment, which can finally affect mitochondrial activity, could be suggested. Supporting this concept is the observation that Gal-3 co-localized with ATP synthase in the inner membrane of mitochondria and has an inhibitory effect on ATP synthase in human colon cancer cells (Kim et al., 2008). Interestingly, MCP do not protect against palmitic acid-induced mitochondrial dysfunction on H9c2 cells in vitro, thus suggesting that the beneficial effect induced by MCP in obese animals is consequence of Gal-3 inhibition and not a consequence of other actions of this drug, since no production of Gal-3 was induced by palmitic acid in the cells (data not shown).
By contrast, MCP was unable to affect glucose homeostasis. In fact, MCP did not modify SM and Cer levels, which are related to insulin resistance, further supporting the lack of effect of Gal-3 in glucose homeostasis in our model. By contrast, a recent study showed that in mice, Gal-3 administration is associated with insulin resistance and Gal-3 pharmacologic and genetic loss function caused glucose intolerance in HFD animals for 8 weeks (Li et al., 2016). However, this role has not been supported by previous data, which showed that knockout Gal-3 mice fed a HDF for 12 or 18 weeks presented dysregulated glucose metabolism (Pang et al., 2013; Pejnovic et al., 2013). Therefore, the specific role of Gal-3 as a player in metabolic disorders needs further studies.

In summary, the understanding of the mechanisms underlying the damage associated with cardiac lipotoxicity in the context of obesity is critical. These data suggest a role of Gal-3 in this damage, draw a more complex scenario for the actions of Gal-3 actions, which is overexpressed in the heart in obesity, and seem to modulate some of the consequences of cardiac lipotoxicity. However, further work on the demonstration of its specific role in mitochondrial function would help clarify the underlying mechanisms, which would not only help in their understanding but also suggest new approaches in the management of obesity-related heart damage.

**MATERIAL AND METHODS**

*Animal model*

Male Wistar rats of 150 g (Harlan Ibérica, Barcelona, Spain) were fed either a high-fat diet (HFD, 35% fat; Harlan Teklad number, TD.03307, MN; n=16) or a standard diet (3.5% fat; Harlan Teklad number, TD.2014, MN; n=16) for 6 weeks. For the same period the
Gal-3 activity inhibitor, MCP (100 mg/kg per day), was supplied to half of each group in the drinking water. The Animal Care and Use Committee of Universidad Complutense de Madrid approved all experimental procedures according to the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63/UE.

**In vivo PET–CT imaging for studying \(^{18}\text{F}^{-}\text{Fluorodeoxyglucose heart uptake}\)**

Myocardial metabolic activity was evaluated by means of a small-animal dedicated dual scanner (Albira PET/CT scanner, Bruker NMI, Valencia Spain). One week before the end of the evolution period, animals were fasted for 18 hours and they were i.p. injected with \(^{18}\text{F}^{-}\text{Flurodeoxyglucose (FDG; 12.99±0.04MBq in 0.2ml of 0.9\% NaCl; Instituto Tecnológico PET, Madrid, Spain). Twenty min later, rats underwent PET and computed tomography (CT) acquisitions under isoflurane anesthesia. The acquired tomographic images were then reconstructed using the maximum likelihood expectation maximization and filtered back projection algorithms for the PET and CT images, respectively. In order to account for the different rat weights and \[^{18}\text{F}\] injected FDG doses, we calculated the standardized uptake value (SUV). The semi-quantitative SUV measurement is the most widely used in both small animal and human \[^{18}\text{F}\] FDG PET studies (Byrnes et al., 2014; Deleye et al., 2014). The software used was the PMOD 3.6 software (PMOD Technologies Ltd., Zurich, Switzerland).

For the metabolic activity quantification, the procedure used was as follows: the CT image of the heart from each animal was co-registered to its corresponding PET image. Then a three-dimensional region of interest (ROI) was drawn on the fused PET/CT image to measure the metabolic activity of the whole left ventricle. These steps were carried out with PMOD 3.0 software (PMOD Technologies Ltd., Zurich, Switzerland). SUV was
obtained as an index of regional metabolic activity. The SUV parameter was calculated as a ratio of the ROI radioactivity concentration (kBq/ml) measured by the scanner and the administered dose (kBq) was decay-corrected at the time of the injection, divided by the body weight (g).

**Isolation of cardiac mitochondria**

Cardiac mitochondria were isolated as reported (Doerrier et al., 2016). Frozen hearts were placed and washed in cold homogenization medium containing 0.075mol/L sucrose, 1mmol/L EDTA, 10mmol/L Tris–HCl, pH 7.4. Briefly, heart tissue was homogenized (1:10 w/v) at 800rpm in a homogenizer (T 10 basic Ultra-turrax, Ika-Werke; Germany). The homogenates were centrifuged at 1,300g for 5min at 4ºC to remove nuclei and debris. Supernatants were separated and centrifuged at 12,000g for 10min at 4º C. The resulting pellets were suspended in homogenization medium and centrifuged twice at 14,400g for 3min at 4º C to wash the mitochondrial fraction. Mitochondrial pellets were stored at −80º C until use. Protein concentration was determined by the Bradford method.

**Western blot**

Mitochondrial proteins were separated by SDS-PAGE on polyacrylamide gels and transferred to Hybond-c Extra nitrocellulose membranes (Hybond-P; Amersham Biosciences, Piscataway, NJ). Membranes were probed with primary antibody for mitochondrial complex (Mitoprofile Total OXPHOS –CI subunit NDUFB8; CII-30kDa, CIII-Core protein 2; CIV subunit I and CV alpha subunit– Abcam, Cambridge; dilution 1/1000), carnitine palmitoyl transferase I (CPT1A, Abcam, Cambridge; dilution 1/1000), dynamin-1-like protein(DRP1, Abcam, Cambridge; dilution 1/1000), mitofusin 1 (MFN1, Abcam,
Cambridge; dilution 1/1000) and porin (Abcam, Cambridge; dilution 1/1000) as a mitochondrial protein loading control. Signals were detected using the ECL system (Amersham Pharmacia Biotech). Results are expressed as an n-fold increase over the values of the control group in densitometric arbitrary units.

**Measurement of mitochondrial reactive oxygen species (ROS) production**

For detection of mitochondrial ion $\text{O}_2^-$ production, cardiac sections (6 µm) were incubated with MitoSox™ (red mitochondrial superoxide indicator; 5µmol/L) for 10 minutes at 37º C. MitoSox™ Red reagent is a live-cell permeant probe and is rapidly and selectively targeted to the mitochondria. Once in the mitochondria, MitoSox™ Red reagent is oxidized by superoxide and exhibits red fluorescence. MitoSox™ Red reagent is readily oxidized by superoxide but not by other ROS- or reactive nitrogen species (RNS)–generating systems. The oxidation product becomes highly fluorescent upon binding to nucleic acids. Fluorescent signals were viewed by fluorescent laser scanning microscope (40X objective in Leica DMI 3000 microscope).

Quantitative analysis of $\text{O}_2^-$ production was performed with image analyser (LEICA Q550 IWB). Three sections per animal were quantified and averaged for each experimental condition. The mean fluorescence densities in the target region were analyzed. Results are expressed as an n-fold increase over the values of the control group in arbitrary units.
**Lipidomic analysis**

Methanol:chloroform (1:2) cardiac extracts were evaporated to dryness and the pellet resuspended in 250 µL of Acetone:2-propanol:Ethanol (3:4:3) and used for triglyceride (TG) measurement. The other aliquot was evaporated to dryness and the pellet resuspended in 200 µL Methanol:water (9:1) and used for phospholipid (PPLs) measurement. Extracts were kept at -80ºC until analysis.

TG compounds were eluted at a flow rate of 0.4 mL/min using a gradient as follows: initial, 100% A; 3 min, 100% A; 6 min, 98% A; 8 min, 98% A; 9.5 min, 95% A; 11 min, 95% A; 16 min, 100% A, and this was kept isocratic for 2 min to recover initial pressure before next injection. Solvents A and B were methanol:acetonitrile:isopropanol (Met:ACN:IPr 30:30:40, v/v/v) and ACN:IPr (3:7, v/v), respectively, both 0.1% NH₄OH (25%). In order to avoid the carry-over, which was calculated to be 12% for unsaturated TG and 5% for saturated TG, methanol was injected and an entire chromatographic run was performed, with an additional one after five samples were injected. An extract volume of 7.5 µL was injected. Mass spectrometric analysis was performed in positive mode (ESI+) using parameters as follows: capillary, 0.8 kV; sampling cone, 15 V; source temperature, 90º C; desolvation temperature, 280º C; cone gas, 40 L/h; and desolvation gas, 700 L/h. Data were acquired with the software Mass Lynx at a rate of 5 scans/s within the range 0-18 min, and m/z 100-1200 Da for the low-energy function and m/z 100-900 Da for the high-energy function (MS² method, trap collision energy 30 V). LC and MS methods were optimized using the commercial standards TG (18:2/18:2/18:2)
and TG (16:0/16:0/16:0). These standards were also used to draw calibration curves for quantification.

PPLs compounds were eluted at a flow rate of 0.35 mL/min using a gradient as follows: initial, 100% A; 1 min, 100% A; 2.5 min, 20% A; 4 min, 20% A; 5.5 min, 0.1% A; 8.0 min, 0.1% A; 10 min, 100% A, and this was kept isocratic for 2 min to recover initial pressure before the following injection. Solvents were (A) methanol:water:formic acid (Met:H2O:FA 50:50:0.5, v/v/v) and (B) Met:ACN:FA (59:40:0.5, v/v/v), both with 5 mmol/L ammonium formate. Methanol was injected every five samples and an entire chromatographic run was performed in order to clean the system for possible carry-over (< 1%). An extract volume of 7.5 µL was injected. Mass spectrometer parameters were fitted as follows: capillary, 0.9 kV; sampling cone, 18 V; source temperature, 90º C; desolvation temperature, 320º C; cone gas, 45 L/h; and desolvation gas, 900 L/h. Data were acquired with the software MassLynx at a rate of 5 scans/s within the range 0-12 min and 100-1200 Da m/z for the low-energy function, and 50-900 Da m/z for the high-energy function (MS² method, trap collision energy 30 V), with ionization in positive mode (ESI+) for detection of diacylphosphatidylcholines (PCs), ceramides (Cer) and sphingomyelins (SM). External commercial standards PC (10:0/10:0) were used for method optimization and quantification.

Up to three different chromatograms were manually checked for mass spectral peak identification where possible. Within each chromatographic point, m/z values with an intensity >= 700 were checked for this in order to afford a defined chromatographic peak (Extracted Ion Chromatogram, EIC); if positive, the elemental composition tool was then used to determine all the possible chemical compositions (CₐHₘOₚNₛPᵣSᵣ) that were
compatible with the isotopic distribution (M, M+1, M+2 and M+3 peaks) of a given m/z value.

Using LipidMaps, Metlin, CheBI, LipidBank, and KEGG databases, a particular elemental composition was searched for possible known compounds. Where possible, acyl chains were aimed at being identified by data from the high-energy function (fragmentation). To assess the specific location of each acyl chain at the positions sn-1 or sn-2 of the glycerol backbone is not possible using this methodology; thus, the most current structure is indicated. The chromatographic peak area from the EIC of every m/z value detected, whether or not having been identified, was quantified using the QuanLynx application.

Chromatograms and mass spectra of all samples were processed with a MarkerLynx method in order to search for differential features (retention time m/z) amongst sample groups. Five injections of methanol were used as blanks to determine features prone to rejection. Only features that appeared in 66% of the samples were accepted. Sets of about 1650 features for data in negative mode and about 2109 features for data in positive mode were detected using the MarkerLynx application. They were checked manually to remove all the features that were present in the blanks. The array resulting from this process, which is comprised of samples and features as independent variables, and the feature signal intensity as dependent variable was submitted to multivariate statistical analysis using the Extended Statistics application that is available with the instrument software; this application is licensed from part of the statistical software SIMCA+ from Umetrics Ltd. (Sweden).
Cell culture

H9c2, rat cardiomyoblast cell line (Merck, Darmstadt, Germany) were maintained in medium DMEM (Merck, Darmstadt, Germany) supplemented with 25 mmol/L glucose, 1 mmol/L pyruvate and 2 mmol/L L-glutamine. Cells were cultured according to the manufacturer’s instructions and were used until passages 20-22. Cells were stimulated with 100, 200 or 300 μmol/L of palmitate-BSA conjugated in 10% FFA-free BSA for 24 hours for the different analysis in order to choose the dose appropriate for performing the experiments. The dose of 200 μmol/L was finally used in all analyses in the presence or absence of MPC (0.01%), which was added before incubation with the palmitic acid.

Measurements of cellular respiration and estimation of the rate of glycolysis

An XF24-3 extracellular flux analyzer (Seahorse Biosciences, North Billerica, MA) was used for determining the bioenergetic profile of the H9c2 cardiac myoblasts. 40x10^3 cells were seeded per well in Seahorse XF24 plates and stimulated for 24 hours with palmitate-BSA conjugated in 10% FFA-free BSA in the presence or absence of MCP. For the XF24-3 assays, DMEM growth media was replaced by unbuffered DMEM supplemented with 5.5 mmol/L glucose, 1 mmol/L pyruvate and 10 mmol/L L-glutamine, stimuli were re-added and cells incubated at 37 °C in a CO₂-free incubator for 1 h. Subsequently, the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), a proxy for lactate production, were recorded to assess the mitochondrial respiratory activity and glycolytic activity, respectively. After four measurements under basal conditions, cells were treated sequentially with 1 μmol/L oligomycin, 0.6 μmol/L carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP) and 0.4 μmol/L FCCP with three consecutive determinations under each condition that were averaged during
data evaluation. At the end of the run, 1 μmol/L rotenone and 1 μmol/L antimycin A were added to determine the mitochondria-independent oxygen consumption and the value subtracted from all OCR measurements. ATP turnover was estimated from the difference between the basal and the oligomycin-inhibited respiration, and maximum respiratory capacity was the rate in the presence of the uncoupler FCCP. Protein concentration in each well was determined using the BCA method and results were normalized according to protein content. Experiments were repeated four times with similar results.

**Viability assay**

H9c2 cell proliferation was evaluated by using the Promega kit (Madison, WI, USA), Cell Titer 96® Aqueous One Solution Cell Proliferation Assay, according to the manufacturer’s recommendations. Briefly, cells were seeded in 96-well plates and serum starved for 24 h. Cells were then stimulated with 100, 200 or 300 μmol/L of palmitate-BSA or 20% of fetal bovine serum (FBS). After 24 h of incubation, formazan product formation was assayed by recording the absorbance at 490 nm in a 96-well plate reader (OD value). Formazan is measured as an assessment of the number of metabolically active cells and expressed in percentages relative to unstimulated cells.

**Fatty acid oxidation Assay**

H9c2 myoblast were seeded on six-well plates, grown until semi-confluence and serum starved overnight. Therefore, cells were incubated with $^{3}$H-Palmitate (0.25 μCi/ml) for 1 h at 37º C, and washed three times with 0.5% BSA-PBS to remove any unincorporated and surface-bound fatty acid. Subsequently, cells were pretreated with 5 μmol/L of triacsin C for 30 min at 37ºC and stimulated with 200 μmol/L of palmitate-BSA. After 6 h
of incubation at 37º C, DMEM-medium was removed from the plates and total cellular lipids were extracted according to the method of Bligh and Dyer (Bligh and Dyer, 1959). The incorporation of 3H-Palmitate into total lipids as well as the radioactivity present in the aqueous phase corresponding to 3H-soluble metabolites (taken as measure of fatty acid β oxidation) were assayed for radioactivity by liquid scintillation counting.

**Measurement of mitochondrial superoxide anion production and mitochondrial inner transmembrane potential detection**

For detection of mitochondrial ion O₂⁻ production, H9c2 myoblasts were stimulated at 37º C with the indicated doses of palmitate-BSA for 24 h. Afterwards, cells were washed and loaded with 5 μmol/L of MitoSOX™ Red for 30 minutes, at 37º C. Fluorescent signal was analyzed by recording FL2 fluorescence in a Gallius™ flow cytometrer (Beckman Coulter).

To evaluate mitochondrial transmembrane potential (ΔΨm), H9c2 myoblasts were incubated with 4μmol/L of Rhodamine 123 for 15 min at 37º C. Stained cells were washed with serum free medium and stimulated with the indicated doses with palmitate-BSA for 24 h at 37º C. After treatment, cells were washed with PBS and changes in fluorescence were monitored using flow cytometry analysis. In some experiments, before incubation with 200 µmol/L of palmitate-BSA, H9c2 cells were pretreated for 30 min with 3 µmol/L of Triacsin C, an inhibitor of long fatty acid acyl-CoA synthetase (Sigma; St Louis, MO, USA) or with the indicated dose of MCP. Experiments were repeated at least three times. Cells were also visualized on a Leica TCS SP5X confocal microscope with a ×40 objective.
Measurement of mitochondrial cardiolipin with NonylAcridine Orange (NAO)

10-N-nonyl-Acridin Orange (NAO, Molecular Probes, Inc.) is a fluorochrome that binds to intact mitochondrial cardiolipin and it is independent of the mitochondrial membrane potential over a physiologically relevant range (Maftah et al., 1989; Petit et al., 1992). Decreases in the fluorescence of NAO in cells have been reported to reflect the peroxidation of intracellular cardiolipin because the dye loses its affinity for peroxidised cardiolipin (Nomura et al., 2000).

H9c2 cells were treated with 100, 200 or 300 µmol/L of palmitic acid/BSA for 24 h at 37°C. Afterwards, cells were stained with 5µmol/L of NAO (NAO; Invitrogen, Carlsbad, CA, USA) for 15 min at 37°C in dark. Cells were then washed with PBS and NAO fluorescence intensity was analyzed by recording FL1 fluorescence in a GalliusTM flow cytometer (Beckman Coulter). Cells were also visualized on a Leica TCS SP5X confocal microscope with a ×40 objective. The cells were excited using 488 nm and emission of NAO was measured beyond 585 nm. Nuclei of cells were co-stained with DAPI as a counter stain. Experiments were repeated at least three times.

Statistical analysis

Continuous variables are expressed as mean± SEM. Normality of distributions was verified by means of the Kolmogorov–Smirnov test. One-way ANOVA was used and followed by Tukey test. Either Pearson or Spearman correlation analysis was used to examine association among different variables according to whether they are normally distributed. A value of P<0.05 was used as the cutoff value for defining statistical
significance. Data analysis was performed using the statistical program SPSS version 22.0 (SPSS Inc, Chicago, IL).

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CONFLICT OF INTEREST

The authors declare no conflict of interest


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**Figure 1. Impact of Gal-3 inhibition on cardiac $^{18}$F-FDG-uptake and HOMA Index in obese rats.** (A) Representative photographs of $^{18}$F-Fluorodeoxyglucose (FDG) positron emission tomography/computed tomography scan of the heart from rats fed a standard diet (CT) or a high fat diet (HFD) treated with vehicle or with the inhibitor of Gal-3 activity (Modified citrus pectin; MCP; 100 mg/Kg/day) in coronal, sagittal and trans-axial views scaled to SUV. (B) Quantification in SUV units. (C) HOMA index of the different experimental groups. Bar graphs represent the mean ± SEM of 6-8 animals. * p<0.05; **p<0.01 control group.
Figure 2. Effects of Gal-3 inhibition on lipid species in heart from control and obese rats. Cardiac levels of (A) total triglycerides (TG), (B) main TG species, (C) ceramide (Cer), (D) total sphingomyelins (SM), (E) total lyso phosphatidylcholine (LPC) and (F) main LPC species of rats fed a standard diet (CT) or a high fat diet (HFD) treated with vehicle or with the inhibitor of Gal-3 activity (Modified citrus pectin; MCP; 100 mg/Kg/day) Bar graphs represent the mean ± SEM of 6-8 animals.* p<0.05; **p<0.01; *** p<0.001 vs. control group. †p<0.05; ††p<0.01; †††p<0.001 vs. HFD group.
Figure 3. Impact of Gal-3 inhibition on proteins and superoxide anion production in heart from control and obese rats. Heart from rats fed a standard diet (CT) or a high fat diet (HFD) treated with vehicle or with the inhibitor of Gal-3 activity (Modified citrus pectin; MCP; 100 mg/Kg/day) were analyzed. Protein expression of (A) carnitine palmitoyl transferase IA (CPT1A), (B) mitofusin 1 (MFN1), (D) for mitochondrial complexes I (subunit NDUFB8), II (30kDa) and V (alpha subunit) are presented. (C) Representative microphotographs (magnification x40) of cardiac sections labeled with MitoSox and quantification of superoxide anions in heart. Bar graphs represent the mean ± SEM of 6-8 animals normalized to porin. Scale bar 50 µm. * p<0.05; **p<0.01 vs. control group. †p<0.05; ††p<0.01 vs. HFD group.
Figure 4. Effects of Gal-3 inhibition on mitochondrial function, glycolysis, membrane potential, ROS production and β-oxidation in palmitic acid treated H9c2 cells. (A) Basal respiration expressed as oxygen consumption rate (OCR), (B) Basal glycolysis expressed as extracellular acidification rate (ECAR), (C) proton leak respiration expressed as OCR, (D) Quantification of flow cytometry analysis of mitochondrial membrane potential in cells stained with Rhodamine 123 expressed as mean fluorescence intensity (MFI), (E) Quantification of flow cytometry analysis of mitochondrial superoxide anions in cells labeled with MitoSox expressed as MFI, (F) Quantification β-oxidation in cardiac myoblasts treated for 24 hours with palmitic acid (200 µmol/L) in the presence of absence inhibitor of Gal-3 activity (Modified citrus pectin; MCP;0.01%). Bar graphs represent the mean ± SEM of 4 assays. **p<0.01; ***p<0.001 vs. vehicle treated cells (CT).
Figure S1. Impact of Gal-3 inhibition on protein in heart from control and obese rats.

Heart from rats fed a standard diet (CT) or a high fat diet (HFD) treated with vehicle or with the inhibitor of Gal-3 activity (Modified citrus pectin; MCP; 100 mg/Kg/day) were analyzed. Protein expression of subunits 2 and 1 from, respectively, mitochondrial complexes III and IV are presented. Bar graphs represent the mean ± SEM of 6-8 animals normalized to porin.
Figure S2. Effects of palmitic acid on the viability in H9c2 cells. Cardiac myoblasts were stimulated with palmitic acid (100-300 µmol/L) or 20 % of fetal bovine serum (FBS) for 24 hours. Viability was determined by an MTT assay. Data are expressed as percent of unstimulated cells. Values are mean±SEM of three assays. ***p<0.001 vs. vehicle treated cells.
Figure S3. Effects of palmitic acid on mitochondrial function and glycolysis in H9c2 cells. (A) Representative mitochondrial respiratory profile from a XF Mitochondrial Stress Test. Oxygen consumption rate (OCR) was measured under basal conditions followed by the sequential addition (vertical lines) of oligomycin (O; 1 µmol/L); FCCP carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (0.6 µmol/L), FCCP (0.4 µmol/L); R+A, 1 µM rotenone plus 1 µmol/L antimycin A as indicated. H9c2 myoblasts were treated for 24 hours with palmitic acid (PA; 200 µmol/L) in the presence of absence inhibitor of Gal-3 activity (Modified citrus pectin; MCP; 0.01%). Each data point represents an OCR measurement as mean ± SEM (n = 5). (B) Basal respiration expressed as oxygen consumption rate (OCR), (C) Basal glycolysis expressed as extracellular acidification rate (ECAR), (D) proton leak respiration expressed as OCR in cardiac myoblasts treated for 24 hours with palmitic acid (PA;100-300 µmol/L). Bar graphs represent the mean ± SEM of 4 assays. *p<0.05 vehicle treated cells (CT).
Figure S4. Effects of palmitic acid on mitochondrial membrane potential in H9c2 cells.

(A) Quantification of flow cytometry analysis of mitochondrial membrane potential in cardiac myoblasts stained with Rhodamine 123 treated for 24 hours with palmitic acid (PA; 100-300 µmol/L) and expressed as mean fluorescence intensity (MFI). (B) Representative histogram, untreated cells (solid curves) were compared with stimulated cells (open curves). (C) Flow cytometry analysis of mitochondrial membrane potential in cardiac myoblasts stained with Rhodamine 123 treated for 24 hours with palmitic acid (200 µmol/L) in the presence of absence of the inhibitor of long fatty acyl CoA synthetase, Triasin C (3 µmol/L) and expressed a MFI. Bar graphs represent the mean ± SEM of 3 assays. ***p<0.001 vehicle treated cell.
Figure S5. Effects of palmitic acid on ROS production in H9c2 cells. (A) Quantification of flow cytometry analysis of mitochondrial superoxide anions in cardiac myoblasts labeled with MitoSox and treated for 24 hours with palmitic acid (PA; 100-300 µmol/L) and expressed as mean fluorescence intensity (MFI), (B) Representative histogram, untreated cells (solid curves) were compared with stimulated cells (open curves), (C) Representative microphotographs showing H9c2 cells labeled with MitoSox. Nuclei of cells were co-stained with DAPI. Bar graphs represent the mean ± SEM of 3 assays. ** p<0.01; ***p<0.001 vehicle treated cell.
Figure S6. Effects of palmitic acid on cardiolipin oxidation in H9c2 cells. (A) Quantification of flow cytometry analysis of cardiolipin oxidation levels in cardiac myoblasts stained with 10-Nony acridine orange treated for 24 hours with palmitic acid (PA; 100-300 µmol/L) and expressed as mean fluorescence intensity (MFI). (B) Representative microphotographs showing H9c2 cells labeled with 10-Nony acridine orange. Nuclei of cells were co-stained with DAPI. Representative microphotographies. Bar graphs represent the mean ± SEM of 3 assays. ** p<0.01; *** p<0.001 vehicle treated cell.
Figure S7. Effects of palmitic acid on β-oxidation in H9c2 cells. Quantification of β-oxidation in cardiac myoblasts treated for 4 or 6 hours with palmitic acid (PA; 200 µmol/L) and expressed as palmitic acid hydrosoluble derived metabolites. Bar graphs represent the mean ± SEM of 3 assays. * p<0.05 vehicle treated cell.