Gender-Dependent Alteration of Ca\textsuperscript{2+} and TNF\textalpha{} Signaling in db/db Mice, an Obesity-Linked Type 2 Diabetic Model

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Cardiovascular complications are the primary death cause in type 2 diabetes, where inflammation can play a role. We, and others, have previously shown that, in diabetic cardiomyopathy, cardiac dysfunction is associated with Ca\textsuperscript{2+} mishandling. It is possible that diabetic cardiomyopathy differently affects men and women, as the latter present higher risk to develop heart failure and a higher plasmatic level of the pro-inflammatory cytokine, tumor necrosis factor alpha (TNF\textalpha{}), than men. However, the gender-dependent regulation of Ca\textsuperscript{2+} signaling in diabetes and its relationship with TNF\textalpha{} signaling are still unclear. Here, we analyzed TNF\textalpha{} signaling pathway and its role in Ca\textsuperscript{2+} signaling dysfunction in male and female rodent models of type 2 diabetes linked to obesity (db/db mice) using confocal microscopy in freshly isolated cardiomyocytes. TNF\textalpha{} increased [Ca\textsuperscript{2+}]\textsubscript{i} transient amplitude and accelerated its decay without affecting SR Ca\textsuperscript{2+} load or Ca\textsuperscript{2+} spark frequency in cells from control mice. All TNF\textalpha{} effects on Ca\textsuperscript{2+} handling were prevented by the inhibition of the ceramidase and the phospholipase A2 (PLA2). While the plasmatic level of TNF\textalpha{} was similar in male and female db/db mice, only male db/db hearts over-expressed both TNF\textalpha{} converting enzyme (TACE) and the protective TNF\textalpha{} receptors 2 (TNF-R2). TNF\textalpha{} receptor 1 (TNF-R1) expression, involved in negative inotropic response of TNF\textalpha{}, was unchanged in both male and female db/db mice compared to controls. We found that male db/db mice cardiomyocytes presented a decrease in [Ca\textsuperscript{2+}]\textsubscript{i} transient amplitude associated to a drop of sarcoplasmic reticulum Ca\textsuperscript{2+} load, not seen in female db/db mice. Interestingly, sustained incubation with TNF\textalpha{} did not restored Ca\textsuperscript{2+} signaling alteration observed in male db/db mice but still induces an increase in Ca\textsuperscript{2+} spark frequency as seen in control littermates. In cardiomyocytes from female db/db mice, TNF\textalpha{} had no visible effects on Ca\textsuperscript{2+} handling. In conclusion, our study shows that the alteration of Ca\textsuperscript{2+} signaling and TNF\textalpha{}, seen in db/db mice, is gender specific presenting an increase in TNF\textalpha{} cardio-protective pathway in male mice.

Keywords: diabetic cardiomyopathy, TNF\textalpha{}, calcium, gender difference, db/db mice
INTRODUCTION

Cardiovascular complications, such as coronary artery diseases, hypertension, and heart failure, are leading causes of death in type 2 diabetes (Laakso, 1999; Bauters et al., 2003; Bell, 2007). Preclinical studies have shown that diabetic cardiac dysfunction, with depressed contraction and relaxation, results from dysregulation of metabolism, mitochondrial function, oxidative stress, and Ca\(^{2+}\) handling (Bugger and Abel, 2014). These knowledge result almost exclusively from male animal studies. However, in the clinical setting, the risk for developing cardiac diseases in diabetes is known to be gender specific (Gelderisi et al., 1991; Rutter et al., 2003; Toedebusch et al., 2018). Indeed, the Framingham Heart Study showed that diabetic women present a 5.1-fold increased risk to develop heart failure than non-diabetic patients, whereas in diabetic men, this risk is only multiplied by 2.4 (Gelderisi et al., 1991; Rutter et al., 2003). In addition, the hospital admission rate for cardiovascular diseases is higher in diabetic women compared to diabetic men. Yet, the gender differences in the alterations of cardiac cellular function in diabetes are unclear, notably regarding Ca\(^{2+}\) mishandling.

Ca\(^{2+}\) regulates contraction through the excitation-contraction coupling in cardiomyocytes. For each heartbeat, sarcolemmal L type Ca\(^{2+}\) channels open during the action potential, leading to Ca\(^{2+}\) influx that activates Ca\(^{2+}\) release from the ryanodine receptors (RyR) located at the sarcoplasmic reticulum (SR). This release of Ca\(^{2+}\) by the RyR (visualized as a [Ca\(^{2+}\)\], transient) activates contractile myofibrils to generate cardiomyocyte contraction. After the contraction, the Ca\(^{2+}\) is re-uptaken into the SR by the SERCA pump and extruded outside the cardiomyocytes mainly by the Na\(^+\)/Ca\(^{2+}\) exchanger, resulting in cardiomyocyte relaxation. We and others have shown that, in animal models of type 2 diabetes linked to obesity, contractile dysfunction is associated with a decrease in the Ca\(^{2+}\) transient amplitude. This lower Ca\(^{2+}\) transient amplitude is associated to reduced L-type Ca\(^{2+}\) current density combined with downregulation of RyR expression (Belke et al., 2004; Pereira et al., 2006b, 2014). We found that these alterations may be different in male and female db/db mice (Pereira et al., 2014); however, the mechanisms remain unclear.

Clinical and preclinical studies pointed out an increase in plasmatic level of TNF\(\alpha\), in type 2 diabetes, notably in women (Yamakawa et al., 1995; Pereira et al., 2006a; Preciado-Puga et al., 2014). TNF\(\alpha\) is an inflammatory cytokine commonly associated to infectious and non-infectious cardiomyopathy, such as viral myocarditis, congestive heart failure, and myocardial infarction. The level of TNF\(\alpha\) seems correlated to the development of cardiac dysfunction (Feldman et al., 2000; Blum and Miller, 2001), and its over-expression leads to cardiac hypertrophy, fibrosis, arrhythmia, and dysfunction (Kubota et al., 1997; Kadokami et al., 2000; London et al., 2003). Yet, whether TNF\(\alpha\) is a cause or a consequence of cardiac dysfunction is still under debate. The biological response of TNF\(\alpha\) is mediated through two receptors, the TNF\(\alpha\) receptor 1 (TNF-R1) and TNF\(\alpha\) receptor 2 (TNF-R2). TNF-R1 activation is responsible for a cardiac negative inotropic response, whereas TNF-R2 mediates cardiac positive inotropic response (Meldrum, 1998). At the cellular level, TNF\(\alpha\) regulates contraction either by direct regulation of Ca\(^{2+}\) signaling in acute condition or via iNOS activation in sustained conditions (Fernandez-Velasco et al., 2007). Still, whether TNF\(\alpha\) activation positively or negatively alters the Ca\(^{2+}\) transient is quite controversial, and studies found either a decrease, an increase, or no effect on Ca\(^{2+}\) transient. Those discrepancies seem to depend on the animal model, the concentration of TNF\(\alpha\) used, and the incubation time (Yokoyama et al., 1993; Goldhaber et al., 1996; Bick et al., 1997; Sugishita et al., 1999; Li et al., 2003; Zhang et al., 2005; Duncan et al., 2010; Greensmith and Nirmalan, 2013). In addition, whether the regulation of TNF\(\alpha\) signaling in type 2 diabetic cardiomyopathy linked to obesity is gender specific remains unknown.

Considering all these controversial findings surrounding TNF\(\alpha\) regulation of Ca\(^{2+}\) handling, we first studied the effect of TNF\(\alpha\) on Ca\(^{2+}\) signaling in WT mice. Then, using the db/db mice, an animal model of type 2 diabetes with insulin resistance linked to obesity, we found that both Ca\(^{2+}\) and TNF\(\alpha\) signaling underwent distinct alterations in male compared to female. Here, we found that male db/db mice presented a depressed Ca\(^{2+}\) transient associated with a lower SR Ca\(^{2+}\) load, not seen in female db/db mice. More interestingly, in male db/db, cardiomyocytes seem to put in place a protective mechanism to counteract those alterations by increasing the expression of cardio-protective TNF-R2 signaling pathway.

MATERIALS AND METHODS

Cell Isolation

Experiments were carried out according to the ethical principles of the French Ministry of Agriculture and the European Parliament on the protection of animals. Ventricular adult cardiomyocytes were isolated from 8 weeks old male C56Bl6 mice, male and female 15 weeks old db/db (Janvier), and their control littermates (db/+). Mice were euthanized by intraperitoneal injection of sodium pentobarbital (100 mg/kg). Cardiac ventricular myocyte isolation was performed by standard enzymatic methods (collagenase type II, Worthington) using the Langendorff perfusion as previously described (Pereira et al., 2006b, 2007, 2012; Leroy et al., 2011; Ruiz-Hurtado et al., 2015). After isolation, cells were kept in 1 mM [Ca\(^{2+}\)] for an hour prior experiments. Only rod-shaped cells and quiescent cells when unstimulated and excitable were used for the Ca\(^{2+}\) experiments.

Abbreviations: ATK, arachidonyl trifluoromethyl ketone; TNF\(\alpha\), tumor necrosis factor alpha; TNF-R1, TNF\(\alpha\) receptor 1; TNF-R2, TNF\(\alpha\) receptor 2; KO, knock-out; NO, nitric oxide; NOE, n-octylethanolamine; oil, oil immersion; PKA, protein kinase A; PLA2, phospholipase A2; RyR, cardiac ryanodine receptor; SR, sarcoplasmic reticulum; SERCA, sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase; TACE, TNF\(\alpha\) converting enzyme.
Measurements of Plasmatic TNFα

TNFα determination by ELISA Soluble TNFα concentration was determined in plasma samples from mice using commercial ELISA test (BIOTRAK, Amersham Life Science, Sweden).

Confocal Microscopy

Ca²⁺ handling was recorded in freshly isolated ventricular adult cardiomyocytes loaded with the fluorescent Ca²⁺ dye, the Fluo-3 acetoxyethyl ester (Fluo-3 AM, Molecular Probes) at 5 µM diluted in a mixture of DMSO-pluronic acid 20%. A line scan across the longitudinal axis of the myocyte was performed to measure cardiomyocyte shortening. Cardiomyocyte shortening corresponds to the difference between cardiomyocyte length at rest and cardiomyocyte length during contraction (during electrical stimulation), as previously described (Fernandez-Velasco et al., 2009). Ca²⁺ transient, Ca²⁺ sparks, and SR Ca²⁺ load were recorded using confocal microscopy (Meta Zeiss LSM 510, objective wi. 63×, n.a. 1.2) in line scan mode (1.54 ms) along the longitudinal axis of the cell. Ca²⁺ transients were evoked by field stimulation (1 Hz) applied through two parallel platinum electrodes. Spontaneous Ca²⁺ sparks were recorded in quiescent cells after Ca²⁺ transient recording. Ca²⁺ transient decay time corresponds to the kinetic of the relaxation phase due to the re-uptake of Ca²⁺ into the SR by the SERCA pump as well as the extrusion of Ca²⁺ by the Na⁺/Ca²⁺ exchanger. Ca²⁺ transient decay time is calculated using a monoeponential function to fit the Ca²⁺ transient decline phase. SR Ca²⁺ load was assessed by rapid caffeine application (10 mM) after 1 min pacing to reach the steady state. Parameters were studied with or without TNFα (1 h to 1 h 30 min) supplemented or not with a ceramidase inhibitor n-oleoyethanolamine (NOE, 5 µM) and a phospholipase A2 (PLA2) inhibitor (ATK, 10 µM) (Sigma-Aldrich). Fluo-3 AM was excited with an Argon laser (λex = 488 nm), and emission was collected at wavelengths >505 nm. Image analysis was performed using homemade routines in interactive data language (IDL).

Western-Blot Analysis

Adult ventricular homogenates were quickly frozen in liquid nitrogen and then placed in Tris solution (50 mmol/L, pH = 7.4) containing proteases and phosphatase inhibitors (10 µg/ml leupeptin, 10 µg/ml trypsin inhibitor, 2 µg/ml aprotinin, and 5 µM okadaic acid). Homogenization was performed on ice using a Poltron. Homogenate was centrifuged at 18,925 g for 10 min at 4°C. Proteins were resuspended in Laemmli (5%) sample buffer, boiled (90°C for 5–10 min), and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gels. After separation, proteins were transferred to polyvinylidene fluoride membranes (Amersham Biosciences), and non-specific binding sites were blocked overnight at 4°C in 5% dried milk and Tris-buffer saline (TBS, pH = 7.4) and 0.01% Tween 20. Membranes were incubated overnight (at 4°C) for the rabbit polyclonal anti-TACE (1:300; Proscience) and the rabbit polyclonal anti-TNFβ2 (H-202) (1:250; Santa Cruz), at room temperature for 1 h 30 min for the rabbit polyclonal anti-TNFRI (H-271) (1:500; Santa Cruz). A secondary horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham Biosciences) was used in combination with an enhanced chemiluminescence detection system (SuperSignal West Pico Chemiluminescent Substrate, Pierce) to visualize the primary antibodies. Band densities were determined with a laser-scanning densitometer (HP-3970) and Quantity One software (BioRad SA). Protein loading was controlled by probing all Western blots with anti-GADPH antibody (1:4,000) (Ambion).

Statistical Analysis

Results were expressed as mean ± SEM. Significance between two groups was determined using unpaired Student’s t test or non-parametric Mann-Whitney test. Data involving more than two groups were analyzed using either one-way ANOVA or two-way ANOVA as appropriate. We used GraphPad Prism 7 (GraphPad) for statistical comparison. Differences with values of p<0.05 were considered significant.

RESULTS

Sustained TNFα Exposure Increases Ca²⁺-Induced Ca²⁺ Release

TNFα-mediated Ca²⁺ signaling regulation is quite controversial, which is probably due to protocol differences. Therefore, we first studied, in our experimental settings, the effect of sustained activation (1–1 h 30 min) of TNFα on Ca²⁺ handling parameters such as Ca²⁺ transient, Ca²⁺ spark frequency, and SR Ca²⁺ load (Figure 1). In our hands, 10 and 50 ng/ml TNFα treatment significantly increased Ca²⁺ transient amplitude (F/F₀ of 3.1 ± 0.3 for 10 ng/ml, 3.5 ± 0.3 for 50 ng/ml vs. 2.5 ± 0.14 for baseline, p < 0.05). Moreover, TNFα significantly accelerated the Ca²⁺ re-uptake into the SR as shown by the faster SR Ca²⁺ transient decay time (Figures 1A,B) (~29% faster for 10 ng/ml and ~25% for 50 ng/ml, p < 0.01). This acceleration of Ca²⁺ re-uptake did not modify SR Ca²⁺ load (Figure 1D) and did not affect Ca²⁺ spark frequency (Figures 1E,F) at any concentration studied. However, 100 ng/ml of TNFα had no effects on either Ca²⁺ transient amplitude, Ca²⁺ spark frequency, or SR Ca²⁺ load. However, 100 ng/ml of TNFα still accelerated the Ca²⁺ transient decay (Figure 1C). These results clearly show that sustained TNFα activation mediates an increase in systolic Ca²⁺ release. Altogether, our results lean toward the idea of a positive inotropic effect.

PLA2 and Ceramidase Mediate TNFα Regulation of Ca²⁺ Signaling

Previous work has suggested that TNFα response is mediated by the sphingosine signaling pathway (Hofmann et al., 2003). To investigate the signaling pathway involved in TNFα regulation of Ca²⁺ signaling, we used a ceramidase inhibitor
(5 μM NOE) and a PLA2 inhibitor (10 μM ATK). NOE fully prevented the increase of Ca^{2+} transient amplitude and the faster Ca^{2+} transient decay time induced by 10 ng/ml of TNFα (Figure 2C). NOE had no significant effects on either the Ca^{2+} spark frequency nor the SR Ca^{2+} load (Figures 2D–F). Similarly, the phospholipase A2 inhibitor blunted all TNFα-mediated effects on the Ca^{2+} transient and the Ca^{2+} transient decay time (Figures 2B,C). As for NOE, ATK had no effect on SR Ca^{2+} load (Figure 2D). However, ATK, contrarily to NOE, did significantly reduce basal Ca^{2+} spark frequency. Altogether, those results suggest that TNFα alters Ca^{2+} signaling via the activation of the ceramidase and phospholipase A2 signaling pathway.

Gender Differences in Upstream TNFα Signaling Pathway in Obesity-Linked Type 2 Diabetic Mice (db/db)

Since plasmatic TNFα level is significantly elevated in type 2 diabetic patients, we first measured the plasmatic level of TNFα in male and female db/db mice. At 15 weeks old, db/db mice develop a type 2 diabetes linked to obesity with associated cardiomyopathy (Pereira et al., 2006b). Surprisingly, neither male nor female db/db mice presented an increase in their plasmatic level of TNFα compared to control (Figure 3A). Then, we measured the expression of key proteins involved in the TNFα signaling pathway, such as type 1 and type 2 TNFα receptors and the TNFα conversion enzyme TACE in both male and female db/db
mice. Interestingly, TACE expression was significantly higher in male \( db/db \) mice compared to controls, whereas no change was detectable in the female group (Figure 3B). Moreover, while TNF-R1 receptor expression was unchanged in both \( db/db \) groups (Figure 3C), TNF-R2 in the \( db/db \) male group was significantly increased (Figure 3D). These results clearly suggest that in male \( db/db \) mice hearts, the TNF-R2, known to mediate a cardio-protective pathway, is over-expressed, probably to protect the heart from diabetic-induced stress.

**Gender Differences in Obesity-Linked Type 2 Diabetic (db/db) \( \text{Ca}^{2+} \) Mishandling**

In \( db/db \) mice, cardiac dysfunction has been associated with a decrease in SR \( \text{Ca}^{2+} \) transient amplitude and SR \( \text{Ca}^{2+} \) load (Belke et al., 2004; Pereira et al., 2006b, 2014). Here, we confirmed, in isolated cardiac myocytes from male \( db/db \) mice, that \( \text{Ca}^{2+} \) transient amplitude is significantly decreased (Figures 4A,B). This drop in \( \text{Ca}^{2+} \) transient amplitude (~51% lower than control, \( p < 0.01 \)) is correlated with a drop in SR \( \text{Ca}^{2+} \) load (Figure 4D) (~51% lower than control, \( p < 0.01 \)), which could explain the smaller (although not significant) cardiac cell shortening (Figure 4C). In our experimental conditions, \( \text{Ca}^{2+} \) spark frequency does not seem to be altered in \( db/db \) compared to control (\( db/+ \)) (\( p = \text{N.S.} \)) (Figures 4E,F). In female \( db/db \) mice, the \( \text{Ca}^{2+} \) handling was similar in \( db/db \) compared to their control littermates (Figure 5). Indeed, all parameters such as \( \text{Ca}^{2+} \) transient amplitude (Figure 5A), \( \text{Ca}^{2+} \) spark frequency (Figure 5C), SR \( \text{Ca}^{2+} \) load (Figure 5D), and cell shortening (Figure 5B) were not significantly modified in freshly isolated cardiomyocytes in female \( db/db \) compared to control.

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**Figure 2** | TNFα regulates \( \text{Ca}^{2+} \) signaling via the sphingosine pathway. (A) Representative \( \text{Ca}^{2+} \) transient examples obtained at baseline, at 10 ng/ml TNFα ± ceramide inhibitor (NOE) or phospholipase A2 inhibitor (ATK). (B) Percentage of effect on \( \text{Ca}^{2+} \) transient amplitude of TNFα treatment ± NOE or ATK (\( n = 20 \) for 10 ng/ml TNFα, \( n = 10 \) for TNFα+NOE, and \( n = 10 \) for TNFα+ATK). (C) \( \text{Ca}^{2+} \) transient decay time (in ms) in same groups (respectively, \( n = 19 \), \( n = 10 \), and \( n = 10 \)). (D) Mean of SR \( \text{Ca}^{2+} \) load in the same groups (respectively, \( n = 11 \), \( n = 10 \), and \( n = 9 \)). (E) Examples \( \text{Ca}^{2+} \) spark frequency recording in freshly isolated cardiomyocytes at baseline, at TNFα ± NOE or ATK. (F) Mean of CaSpF obtained in conditions (respectively, \( n = 20 \), \( n = 10 \), and \( n = 10 \)). *\( p < 0.05 \), **\( p < 0.01 \).
Delgado et al. Gender and TNFα in db/db Mice

| A | B |
| --- | --- |
| ![Image](https://example.com/image1.png) | ![Image](https://example.com/image2.png) |

**FIGURE 3** | Gender-dependent alteration of the TNFα signaling pathway in type 2 diabetic mice (db/db). (A) Plasmatic level of TNFα obtained in male and female control and db/db heart tissue homogenates (male control: n = 5, male db/db: n = 7; female control and db/db: n = 8). (B) Representative Western-blot example of TACE (98 kDa) and the percent of relative density normalized by GAPDH signal from male and female control (n = 15, n = 17) and db/db (n = 14, n = 17) heart tissue homogenates. (C) Representative Western-blot example of TNF-R1 (55 kDa) expression and percentage of relative density normalized by GAPDH signal in male and female control (n = 16, n = 18) and db/db (n = 16, n = 18) heart tissue homogenates. (D) Representative Western-blot example of TNF-R2 (75 kDa) expression and percentage of relative density normalized by GAPDH signal in male and female control (n = 13, n = 13) and db/db (n = 13, n = 13) heart tissue homogenates. *p < 0.05.

Therefore, there are gender differences in Ca²⁺ mishandling and the underlying mechanisms in type 2 diabetes.

**DISCUSSION**

We have previously shown that cardiac dysfunction in type 2 diabetes is associated with cardiomyocyte Ca²⁺ mishandling, resulting from a decrease in the Ca²⁺ channels involved in the Ca²⁺-induced Ca²⁺ release process (RyR and L-Type Ca²⁺ channels) (Belke et al., 2004; Pereira et al., 2006b). Although TNFα is elevated in diabetic patient and animal model of diabetes (Yamakawa et al., 1995; Pereira et al., 2006a; Preciado-Puga et al., 2014), little was known about its role in cellular alteration, notably regarding the Ca²⁺ signaling pathway and gender specificity in animal model of diabetes linked to obesity. Here, we found a gender-specific alteration of Ca²⁺ and TNFα signaling in db/db mice, a common model of type 2 diabetes linked to obesity. Indeed, we found that male db/db mice, not

Conclusion, we found a gender-specific alteration of Ca²⁺ handling in db/db mice, with lower SR Ca²⁺ release associated to a drop in SR Ca²⁺ load in male, not seen in female.

**Gender Differences of TNFα-Mediated Effect in Type 2 Diabetic (db/db)**

Next, we compared TNFα regulation of Ca²⁺ signaling between male and female db/db mice. In male db/db mice, 10 ng/ml TNFα did not alter Ca²⁺ transient amplitude, cell shortening, nor SR Ca²⁺ load (Figures 4A–C, F). However, 10 ng/ml of TNFα similarly increased Ca²⁺ spark frequency in both control (~3.29 fold, p < 0.05) and db/db (1.5 fold, p = 0.06) (Figure 4D). In female control, the higher Ca²⁺ transient amplitude and cell shortening did not reach significance. Both female db/db and control had unchanged Ca²⁺ spark frequency. Those results suggest that, in 15 weeks old female db/db, the excitation-contraction coupling is unchanged compared to female control. Moreover, TNFα fails to show the effects found in male db/db (Figure 4D).
female, presented the previously described Ca\(^{2+}\) mishandling with lower systolic Ca\(^{2+}\) release and SR Ca\(^{2+}\) load. More interestingly, we found that male and female db/db mice expressed differently TNF-R2, with an increased expression in male db/db mice that might reflect the activation of the TNF\(\alpha\) cardio-protective TNF-R2-dependent pathway, not seen in female db/db mice.

**Cardiac Positive Inotropic Effect of TNF\(\alpha\)**

Discrepancies regarding the TNF\(\alpha\) regulation of Ca\(^{2+}\) signaling are quite important in the literature with reported positive or negative ionotropic effect. For instance, in cat cardiomyocytes, short time exposure of TNF\(\alpha\) reduced Ca\(^{2+}\) transient amplitude in response to a disruption of Ca\(^{2+}\) influx via L type Ca\(^{2+}\) channels leading to cellular shortening, supporting, then, a negative ionotropic effect of TNF\(\alpha\) (Yokoyama et al., 1993). This negative inotropic effect of TNF\(\alpha\) has been also described, in rabbit and guinea pigs, with TNF\(\alpha\)-induced impaired cellular shortening cardiomyocytes mediated by NO dependent but Ca\(^{2+}\) independent (Goldhaber et al., 1996; Sugishita et al., 1999). However, various studies performed in rodents have shown that TNF\(\alpha\) can lead to inotropic positive effects (Bick et al., 1997; Greensmith and Nirmalan, 2013). Here, we found that TNF\(\alpha\) treatments (10 and 50 ng/ml) induced a time and concentration-dependent effect leading to a significant increase in Ca\(^{2+}\) transient amplitude between 1 h and 1 h 30 min suggesting a positive inotropic effect. Our results are in concordance with Bick et al. study (Bick et al., 1997), who have found that TNF\(\alpha\) incubation increases Ca\(^{2+}\) transient and cellular contraction in neo-natal cardiomyocytes. In adult rat cardiomyocytes treated with 50 ng/ml of TNF\(\alpha\) (Greensmith and Nirmalan, 2013), Ca\(^{2+}\) transient amplitude and cellular shortening were also increased (Greensmith and Nirmalan, 2013). The absence of effect observed under 100 ng/ml of TNF\(\alpha\) might be explained by its bimodal effect, as previously described in cardiomyocytes, depending on exposure time or dose (Amadou et al., 2002; Shanmugam et al., 2016). Then, 100 ng/ml TNF\(\alpha\)
or higher doses, and with prolonged exposure, is expected to induce negative inotropic effects on Ca\textsuperscript{2+} handling.

**In Mice Cardiomyocytes, TNFα Regulates Ca\textsuperscript{2+} Signaling via the Sphingosine and PLA2 Pathways**

Previous studies have shown that TNFα produces myocardial effects (negative or positive inotropic effect) through different mechanisms such as PLA2 or sphingosine signaling pathway (Murray and Freeman, 1996; Oral et al., 1997; Liu and McHowat, 1998). Here, we found that exposure of TNFα (1 h to 1 h 30 min) mediates Ca\textsuperscript{2+} transient increase via the activation of both ceramidase (sphingosine precursor) and PLA2 (for arachidonic acid production). Sphingosine is commonly associated to short-term (within minutes) negative inotropic effect of TNFα (Oral et al., 1997). However, other studies have shown that ceramide enhanced SR Ca\textsuperscript{2+} release and SR Ca\textsuperscript{2+} re-uptake in adult ventricular myocytes (Liu and Kennedy, 2003). Those results are in line with our prevention of TNFα-mediated elevation of systolic Ca\textsuperscript{2+} release and Ca\textsuperscript{2+} transient decay time in cardiomyocytes treated with the ceramidase inhibitor NOE (Figures 2B,C). Moreover, inhibition of the PLA2 prevented TNFα-mediated increase in Ca\textsuperscript{2+} transient amplitude and SR Ca\textsuperscript{2+} transient decay time, suggesting that TNFα induces Ca\textsuperscript{2+} mishandling via PLA2-mediated phosphorylation of RyR. Indeed, 10 ng/ml of TNFα has been shown to increase Ca\textsuperscript{2+} transient amplitude as a result of PLA-2 mediated RyR PKA phosphorylation at serine 2,808 in wild-type mice of RASSF1A knock out (Mohamed et al., 2014). This PKA-dependent mediated effect of PLA-2/arachidonic acid on the RyR phosphorylation state perfectly explains why we observed a dramatic drop of Ca\textsuperscript{2+} spark frequency under the inhibition of the PLA-2 (Figure 2F). In addition, TNFα also accelerates SR Ca\textsuperscript{2+} re-uptake reflecting an increase in SERCA pump activity as seen under PKA phosphorylation of phospholamban supporting the TNFα/PLA-2/PKA pathway. This mechanism is confirmed by the restoration of the TNFα-mediated acceleration Ca\textsuperscript{2+} transient decay time under ATK, the PLA-2 inhibitor (Figures 2B,C).

**Gender-Dependent Ca\textsuperscript{2+} Mishandling in db/db Mice, an Obesity-Linked Type 2 Diabetic Model**

Type 2 diabetes is the most common form of diabetes. In western countries, 80% of type 2 diabetic patients have developed a diabetes linked to obesity resulting in severe glucose intolerance...
compared to lean type 2 diabetic patients (Schaffer and Mozaffari, 1996). Our study was performed in db/db mice, a model that recapitulates, in that sense, the human pathology. Indeed, the leptin receptor mutation of db/db mice impairs the satiety feeling and leads to obesity around 4–5 weeks of age, which is followed by diabetic state with hyperglycemia and insulin resistance (Coleman, 1978). In type 2 diabetes linked to obesity, cardiac dysfunction has been associated to Ca²⁺ mishandling and structural remodeling (Belke et al., 2004; Pereira et al., 2006b; Falcao-Pires and Leite-Moreira, 2012). Indeed, overall, animal models of type 2 diabetes present a reduced Na⁺/Ca²⁺ exchanger activity, and depressed Ca²⁺ transient linked to downregulation of Ca²⁺ channels, RyRs, and reduced SERCA activity (Nerticadan et al., 2001; Zhong et al., 2001; Abe et al., 2002; Belke et al., 2004; Pereira et al., 2006b; Boudina and Abel, 2010). Here, our results show that those effects are recapitulated in male db/db mice (Figure 4), but not in female db/db mice. However, the gender-specific regulation in Ca²⁺ handling and/or β-adrenergic response has been previously described (Parks et al., 2014). Supporting this idea, we found that basal Ca²⁺ transient amplitude is lower in female control compared to male control cardiomyocytes. Although Parks et al. (2014) have shown that Ca²⁺ current, diastolic Ca²⁺, and SR Ca²⁺ load were similar between control male and female, basal cAMP level was lower in control female compared to control male due to higher PDE4B expression in female. These results are in line with our previous work showing that db/db female mice have reduced phosphorylation of the RyR, which reduce Ca²⁺ spark frequency and could explain the preserve SR Ca²⁺ load and Ca²⁺ transient seen in female db/db compared to db/db male. Our results are paradoxical compared to the higher risk to develop heart failure for type 2 diabetic women compared to diabetic men. This discrepancy could be explain as follow: the decrease in [Ca²⁺], transient in male db/db mice could be protective at long term, maybe by preventing Ca²⁺ toxic effects such as apoptosis or preserve ATP content by limiting the ATP expense in pumping Ca²⁺ (Javorková et al., 2010; Parks et al., 2014). Future studies will be needed to confirm this hypothesis.

Gender Dependent Alteration of Molecular TNFα Signaling Pathway in db/db

To our knowledge, plasmatic TNFα level parallels the degree of cardiac dysfunction in diabetic patients. In the db/db mice, we did not observe any changes in the plasmatic level of TNFα compared to control. Even though circulating TNFα is unchanged, male db/db mice present an increase in TACE expression suggesting a paracrine elevation of TNFα in the heart. Surprisingly, despite cardiomyocyte treatment with 10 ng/ml of TNFα, a concentration within the in vivo range measured under stress and injury (Bitterman et al., 1991), TNFα did not induce an increase in Ca²⁺ transient amplitude or decay time in db/db, as seen in C57Bl6 mice (Figures 4B,C). One explanation could be that in db/db control littermate strain background (C57BlKS/J strain), TNFα is not as effective as in C57Bl6 strain. Indeed, genetic background, such as between C57Bl6/J and C57Bl6/N, has been shown to influence cardiac phenotype and propensity to develop cardiomyopathies (Tian et al., 2011; Simon et al., 2013). This could also explain the ineffective response of TNFα in female control and db/db mice (Figure 5). Although TNFα activation has been linked with oxidative stress, no gender-specific difference in cardiomyocytes redox state at baseline or during pathology has been observed (Ren, 2007; Bell et al., 2015). Another possibility could be that in male db/db, the dramatically reduced SR Ca²⁺ load would prevent the high Ca²⁺ systolic release induced by TNFα probably due to the phosphorylation of the RyR via the activation of PLA2. Indeed, we found in the presence of TNFα an increase in Ca²⁺ spark frequency in both db/+ and db/db mice reflecting an elevated diastolic RyR opening resulting from RyR phosphorylation by PKA previously described in male db/db (Pereira et al., 2014). Interestingly, in male db/db mice, the TNF-R2 was overexpressed, which is known to exert cardio-protective effects via the activation of NF-kB (Burchfield et al., 2010). Indeed, in liver, TNFα inhibits PDE3 expression elevating cAMP level and PKA activation (Ke et al., 2015). This activation of PKA could explain, in cardiomyocytes, the elevation of Ca²⁺ spark frequency in male db/+ cardiomyocytes treated with TNFα (Figure 4F). Moreover, TNF-R2 is known to be involved in positive cardiac inotropic effect (Defer et al., 2007). As a result, [Ca²⁺] overload was prevented and Ca²⁺ transient increased leading to an increase in inotropic response. The over-expressed TNF-R2 in a male db/db appears as an attempt to counteract the already present Ca²⁺ mishandling to protect from cardiac dysfunction. Indeed, prolonged activation of the TNF-R2 pathway in the db/db male cardiomyocytes could then activate phosphorylation of excitation-contraction coupling key proteins, such as phospholamban, to restore Ca²⁺ transient and cardiomyocytes contraction.

In conclusion, we found for the first time that both Ca²⁺ and TNFα signaling are altered only in male type 2 diabetic mice, whereas female does not seem to be affected. Although this study has several limitations in the interpretation such as non-comparable hormonal state between female db/db mice and diabetic women, lower effect of TNFα in db/+ than C57BL6 control, we still clearly show that male db/db mice develop Ca²⁺ mishandling leading to impaired contraction already at a young age, while woman seemed to be protected. Moreover, we found that male db/db mice put into place a protective mechanism to counteract those negative effects by over-expressing TNF-R2 cardio-protective signaling pathway.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The study was carried out in accordance to the ethical principles of the French Ministry of Agriculture and the European Parliament on the protection of animals. The protocol was
approved by the French Ministry of Agriculture and Bioethical Committee of the CSIC following recommendation of the Spanish Animal Care and the European Parliament on the protection of animals.

AUTHOR CONTRIBUTIONS

CD and AG conceived and designed the project, supervised the data acquisition and participated in analysis. LP and GR performed most of the experiments and analyses. LP interpreted the data and wrote the first draft of the manuscript. MS participated in the figure preparation. All authors have edited the manuscript.

FUNDING

This work has been funded by Acuerdos Bilaterales España-Francia (CSIC-INSERM) grant no. 2005FR0020 and partially by SAF2017-84777R from the Spanish Ministerio de Industria, Economía y Competitividad (to CD) and partially by CP15/00129 and PI17/01093 (to GR), and the ANR-11-1DEX-0003-02 to LP as members of the Laboratory of Excellence LERMIT.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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