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## Impaired mitochondrial energy supply coupled to increased H<sub>2</sub>O<sub>2</sub> emission under energy/redox stress leads to myocardial dysfunction during Type I diabetes

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### Abstract

In Type I diabetic (T1DM) patients, both peaks of hyperglycaemia and increased sympathetic tone probably contribute to impair systolic and diastolic function. However, how these stressors eventually alter cardiac function during T1DM is not fully understood. In the present study, we hypothesized that impaired mitochondrial energy supply and excess reactive oxygen species (ROS) emission is centrally involved in T1DM cardiac dysfunction due to metabolic/redox stress and aimed to determine the mitochondrial sites implicated in these alterations. To this end, we used isolated myocytes and mitochondria from Sham and streptozotocin (STZ)-induced T1DM guinea pigs (GPs), untreated or treated with insulin. Relative to controls, T1DM myocytes exhibited higher oxidative stress when challenged with high glucose (HG) combined with  $\beta$ -adrenergic stimulation [via isoprenaline (isoproterenol) (ISO)], leading to contraction/relaxation deficits. T1DM mitochondria had decreased respiration with complex II and IV substrates and markedly lower ADP phosphorylation rates and higher H<sub>2</sub>O<sub>2</sub> emission when challenged with oxidants to mimic the more oxidized redox milieu present in HG + ISO-treated cardiomyocytes. Since in T1DM hearts insulin-sensitivity is preserved and a glucose-to-fatty acid (FA) shift occurs, we next tested whether insulin therapy or acute palmitate (Palm) infusion prevents HG + ISO-induced cardiac dysfunction. We found that insulin rescued proper cardiac redox balance, but not mitochondrial respiration or contractile performance. Conversely, Palm restored redox balance and preserved myocyte function. Thus, stressors such as peaks of HG and adrenergic hyperactivity impair mitochondrial respiration, hampering energy supply while exacerbating ROS emission. Our study suggests that an ideal therapeutic measure to treat metabolically/redox-challenged T1DM

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#### AUTHOR CONTRIBUTION

Carlo Tocchetti, Brian Stanley, Vidhya Sivakumaran and Djahida Bedja performed experiments, analysed data and edited the manuscript before submission. Brian O'Rourke and Nazareno Paolocci analysed and interpreted data and edited the manuscript before submission. Sonia Cortassa and Miguel Aon designed and performed experiments, analysed and interpreted data and wrote the manuscript.

hearts should concomitantly correct energetic and redox abnormalities to fully maintain cardiac function.

### Keywords

adrenergic stimulation; calcium transient; contractility; diabetes; energetic transitions; glutathione; hyperglycaemia; redox balance

## INTRODUCTION

Hyperglycaemic peaks are very common in Type I diabetic (T1DM) subjects, occurring post-prandially when glycaemic control is poor. Epidemiological and cohort evidence indicate a consistent correlation of hyperglycaemia with cardiovascular risks [1–3]. Sympathetic tone is also elevated in T1DM subjects [4], probably compensating for loss of peripheral vascular tone in these patients. Hyperglycaemia and sympathetic overdrive may alter cardiac redox conditions [5,6] and endanger mitochondrial function [7,8]. In turn, perturbations of cardiac mitochondrial energetics and increased mitochondrial ROS emission, both common features in T1DM patients [1,9,10], can account for tissue redox imbalance [8,11–13] and abnormal excitation-contraction (E-C) coupling [8,14–17]. Together, these abnormalities can add up to reducing cardiac systolic and diastolic function in diabetic patients [8,12,13,18]. This diseased condition, referred as diabetic cardiomyopathy (DCM), is a major life-threatening complication that limits T1DM patients' quality of life and expectancy [3,19]. Hearts from diabetic subjects are particularly prone to excess reactive oxygen species (ROS) because sympathetic hyperactivation and hyperglycaemia are present in a large cohort of these patients [20,21]. Yet, how these stressors alter mitochondrial energy/redox assets, thus contributing to cardiac dysfunction in T1DM patients, remains to be determined. More specifically, it is not clear yet what cardiac mitochondrial sites are eventually targeted under the above-mentioned stress conditions.

Prevailing wisdom is that during T1DM, the myocardial shift from glucose to fatty acid (FA) utilization may aggravate mitochondrial dysfunction [22,23], fuelling contractile deficit. However, studies have also shown that FAs may actually benefit cardiac function in the course of metabolic syndrome. This is the case in obese (*ob/ob*) mice treated with insulin and FA [24] or *db/db* mice exposed to high glucose (HG) and  $\beta$ -adrenergic stimulation [8]. Moreover, very recently, it has been reported that in the Zucker Diabetic Fatty (ZDF) rat, HG had a significant negative impact on the contractile performance of heart trabeculae. Palmitate (Palm), but not insulin, was able to prevent this effect [12], via higher content of reduced glutathione and augmented mitochondrial ROS-scavenging capacity. Importantly, unlike T2DM, cardiac muscle retains insulin-sensitivity during T1DM, and insulin-deficient Akita hearts exhibit normal resting cardiac function with no signs of oxidative stress, despite a 2-fold increase in uncoupling protein 3, normal ATP per atom of O<sub>2</sub> (P/O) ratio and proton leak after perfusion with Palm [25]. However, whether insulin benefits function in T1DM hearts independently from changes in myocardial redox balance has not been directly tested yet.

The present study was designed to answer the following key questions that, among others, are relevant to our understanding of cardiac dysfunction in T1DM and thus to the possible therapeutic treatment of this condition: (1) what are the triggers of mitochondrial dysfunction and sites of ROS generation in T1DM in the presence of peaks of glucose and  $\beta$ -adrenergic overdrive?, (2) what is the impact of increased FA availability on mitochondrial and cardiac function in T1DM hearts under these stressing conditions?, and (3) is insulin able to reverse cardiac redox and/or E-C coupling perturbations that are likely to occur in these metabolically/redox-challenged T1DM hearts? To address these unknowns, we used a model of streptozotocin (STZ)-induced T1DM in guinea pigs (GPs) that harbours glucose levels similar to those found in human T1DM.

## MATERIALS AND METHODS

### Ethical approval

All procedures on GPs to render them diabetic were approved by the Animal Care and Use Committee of Hilltop Lab Animals, Inc. and adhere to NIH public health service guidelines. For cardiomyocyte or mitochondrial isolation, GPs were heparinized (500 units) and killed with sodium pentobarbital (180 mg/kg intraperitoneal), following the requirements of the Institutional Animal Care/Use Committee at JHU, adherent to NIH guidelines.

### Diabetic guinea pig (*Cavia porcellus*)

Diabetic GPs were generated by Hilltop Lab Animals, Inc., following our recommendations. Male GPs (200–250 g) were made diabetic by a single intraperitoneal injection of buffered STZ group (80 mg/kg in citrate buffer, pH 4.5). Age- and sex-matched animals received an equivalent volume of vehicle (citrate buffer pH 4.5; Sham). Four weeks after STZ injection, the GPs from the insulin-treated STZ group (STZ + Ins) received 1 unit/day of insulin glargine (Lantus, Aventis) subcutaneously for another 2 weeks. Since GPs eat during the day, insulin was applied in the afternoon to avoid hypoglycaemic shock due to the sustained action of insulin glargine.

Sham and STZ animals were utilized after 4 weeks of STZ administration, whereas the STZ + Ins group was treated for another 2 weeks with insulin and then analysed. Four weeks was the minimum time needed to trigger the T1DM phenotype, thus the reason for shifting 2 weeks STZ + Ins with respect to the other two groups. At the time of study termination, body mass (g  $\pm$  S.E.M.) and fasting blood glucose levels (mg/dl  $\pm$  S.E.M.; Accu-check system, Roche) were as follows: Sham: 454  $\pm$  8.6 and 153  $\pm$  3.2 ( $n=26$ ); STZ: 465  $\pm$  10 and 208  $\pm$  6.5 ( $n=24$ ); STZ + Ins: 486  $\pm$  16 and 155  $\pm$  5 ( $n=17$ ) respectively. Diabetic GPs had 36% higher levels of glucose in blood ( $P < 0.001$  with respect to controls and STZ + Ins, i.e. from ~8 to ~12 mM glucose). Blood glucose determination in duplicate was performed immediately after heart excision. No significant differences in body weight between the three groups of animals were detected.

## Myocyte and mitochondrial isolation

The isolated heart was either cannulated through the aorta allowing for retrograde perfusion and ventricular myocytes preparation as described [26] or minced and processed for mitochondrial isolation as detailed elsewhere [27,28].

## Myocyte function and redox status

All experiments were carried out on freshly isolated adult myocytes. Contractility and two-photon imaging were performed using cells obtained after the same isolation batch.

## Two-photon laser-scanning fluorescence microscopy

Experiments with intact cardiomyocytes were carried out at 37 °C in a thermostatically controlled flow chamber mounted on the stage of an upright fluorescence microscope (BX61WI; Olympus) attached to a multiphoton-excited fluorescence Fluoview FV1000 MPE (Olympus) and a Deep Sea ultrafast system scanning laser (Mai Tai). Cells were loaded with the fluorescent probes (Invitrogen) for 20 min at 37 °C on the stage of the microscope, visualized with an objective 25/1.05 W MP and images were acquired as described in [8,26,29] (see also legend of Figure 1 and the Supplementary Online Data).

GSH was measured in myocytes loaded with the membrane-permeant indicator monochlorobimane (MCB) [29]. The MCB probe reports the level of GSH as the fluorescent product glutathione *S*-bimane (GSB) according to the reversible reaction,  $\text{MCB} + \text{GSH} \leftrightarrow \text{GSB}$  catalysed by GST [30]. Images were recorded with excitation at 740 nm; the red emission of MitoSOX was collected at 605 nm using a 578–630 nm band-pass filter and the blue GSB emission was recorded at 480 nm.

## Sarcomere shortening and calcium transients

Sarcomere shortening and calcium transients in isolated myocytes were studied at  $25 \pm 1$  °C. Sarcomere shortening was assessed by real-time imaging and whole-cell  $\text{Ca}^{2+}$  transients by fura 2/AM (acetoxymethyl ester) fluorescence using an inverted fluorescence microscope (Nikon TE2000) and IonOptix (Myocam) software [31].

## Mitochondrial physiological studies

Freshly isolated mitochondria from Sham, STZ or STZ + Ins hearts were monitored with spectrofluorimetry for energetic-redox responses as visualized by respiration, 90° light scattering or  $\text{H}_2\text{O}_2$  emission with Amplex Red [27]. Mitochondrial respiration ( $\dot{V}_{\text{O}_2}$ ) was

assayed with a high-throughput automated 96-well extracellular flux analyser (Seahorse Bioscience XF96) in a medium (buffer B) containing 137 mM KCl, 2 mM  $\text{KH}_2\text{PO}_4$ , 0.5 mM EGTA, 2.5 mM  $\text{MgCl}_2$  and 20 mM HEPES, at pH 7.2 and 37°C, in the presence of 0.2% FA-free BSA [8,12]. Mitochondrial protein was determined using the BCA method protein assay kit (Thermo Fisher Scientific).

NAD(P)H was monitored by exciting mitochondrial suspensions at 340 nm and collecting the emission at 450 nm, whereas  $\text{H}_2\text{O}_2$  was detected using the Amplex red kit (Invitrogen) as described in [27]. NAD(P)H and  $\text{H}_2\text{O}_2$  were monitored simultaneously with a

wavelength-scanning fluorimeter (QuantaMaster; Photon Technology International) using the aforementioned medium for measuring respiration (excluding BSA) and a multi-dye program for simultaneous online monitoring of different fluorescent probes.

The effect of palmitoyl-CoA (PCoA) on  $\dot{V}_{O_2}$  and  $H_2O_2$  emission from mitochondria in the absence or presence of  $\beta$ -oxidation was analysed in parallel under similar conditions with 96-well microplate reader using a Seahorse Bioscience XF96 analyser and a Flex Station 3 (Molecular Devices) respectively.

### Statistical analysis

Data were analysed with the software GraphPad Prism (version 6) or MicroCal Origin. Significance of the differences between treatments was evaluated with one-way or two-way ANOVA using Tukey's multiple comparison test or with Student's  $t$  test (small samples, paired Student's  $t$  test with two-tail  $P$ -values) and the results presented as mean  $\pm$  S.E.M. (95% confidence interval). The normality of the data was tested with a Kolmogorov-Smirnov test (GraphPad Prism).

## RESULTS

### High glucose and $\beta$ -adrenergic stimulation prompt redox imbalance in T1DM cardiomyocytes, an effect prevented by chronic insulin treatment or acute palmitate infusion

First, we asked whether oxidative stress occurs at baseline in myocytes from T1DM and is exacerbated by exposing these cells to HG (30 mM) and adrenergic stimulation with the  $\beta_1/\beta_2$  agonist isoprenaline (isoproterenol) (ISO, 10 nM).

Myocytes isolated from Sham, STZ or STZ + Ins GP groups were loaded with ROS or GSH sensors and imaged using two-photon laser-scanning fluorescence microscopy while superfused with normal (EG, 10 mM) or HG glucose levels, in the absence or presence of 10 nM ISO (Figure 1). Under normal glucose, NAD(P)H and GSH pools exhibited similar levels across groups (Figures 1A and 1G). Compared with baseline, un-paced cells treated with HG alone or combined with ISO did not exhibit significant change in GSH levels, except for a slight but consistent shift towards oxidation in the STZ group (Figures 1G-1I). The STZ cells displayed higher ROS levels, as detected by MitoSOX (Figures 1M-1O). Intriguingly, insulin therapy prevented the ROS increase in T1DM cardiomyocytes treated either with HG or HG + ISO (Figure 1N and 1O). Thus, myocytes from T1DM GPs exhibit similar basal redox conditions as compared with control cells; however, they become more oxidized when in the presence of HG or HG + ISO.

In T2DM hearts and myocytes, the acute infusion of Palm can preserve function after HG + ISO exposure [8] or under increased workload in heart trabeculae [12] via a maintained reduced environment. Therefore, we sought to determine whether Palm is able to reset proper cardiac redox conditions also in metabolically stressed T1DM myocytes, assessing the impact of the acute Palm infusion on ROS, GSH and NAD(P)H levels in T1DM cardiomyocytes exposed to HG and ISO. Strikingly, Palm decreased oxidative stress in all

groups and across treatments, as indexed with the ROS sensor (Figures 1P-1R). To rule out potential artefacts, extensive imaging control experiments were performed with cardiomyocytes under steady state (Supporting Figures S1-S5) and time-dependent conditions (Supplementary Figure S6).

Interestingly, Palm increased NAD(P)H significantly across treatments only in cardiomyocytes from STZ-treated GPs (Figures 1D-1F) and in low glucose, without or with insulin therapy (Figure 1D). Moreover, Palm elicited significantly higher levels of GSH across all treatments in T1DM cardiomyocytes from diabetic animals treated or not with insulin with respect to Sham controls (Figures 1J-1L). Together, these data indicate that insulin treatment of T1DM GPs or acute Palm infusion prevents redox imbalance in myocytes exposed to HG and ISO stimulation.

### **Alterations in the electron transport chain account for alterations in O<sub>2</sub> consumption by T1DM mitochondria**

Whether, and where, mitochondrial respiration is affected in T1DM hearts challenged with HG and  $\beta$ -adrenergic stimulation is unknown. Therefore, we first measured  $\dot{V}_{O_2}$  and coupling of oxidative phosphorylation (OxPhos) through the respiratory coupling ratio (RCR).  $\dot{V}_{O_2}$  and RCR were quantified with substrates of complex I, II, and IV (Figure 2). No major differences in RCR were apparent between groups in presence of substrates for complex I or IV (Figure 2A); however, with the complex II substrate succinate (Succ), divergence became evident. More specifically, a trend towards lower RCR was found in STZ mitochondria, respiring on 5 mM glutamate/malate (G/M). Importantly, with complex II and IV substrates, STZ mitochondria exhibited significantly lower  $\dot{V}_{O_2}$  in state 3 (+ADP), an alteration more prominent in STZ + Ins cells (Figures 2C and 2D). After mitochondrial uncoupling with dinitrophenol (DNP), no significant increase in  $\dot{V}_{O_2}$  was observed with Succ or TMPD. Lack of improvement of OxPhos rates by collapsing  $\Delta\Psi_m$  with an uncoupler suggests that the defect in T1DM mitochondria is not in the phosphorylation system, but rather in electron transport (Figures 2C and 2D) [32]. However, this was not the case for G/M. Indeed, an increase in  $\dot{V}_{O_2}$  above state 3 respiration was observed with DNP (Figure 2B), although the respiratory reserve capacity (i.e. the ratio of uncoupled  $\dot{V}_{O_2}$  compared with state 3 respiration) was diminished in STZ and STZ + Ins groups compared with Sham. Thus, mitochondrial dysfunction takes place in the electron transport chain as revealed with substrates of complex II and IV, and, as shown in Figures 2C and 2D, insulin therapy does not obviate this alteration.

### **ROS emission from T1DM heart mitochondria in the absence or presence of oxidative stress**

We next investigated whether and where increased ROS emission occurs in T1DM mitochondria and the role of PCoA, a key intermediate in Palm oxidation. First, H<sub>2</sub>O<sub>2</sub> emission rate was studied as a function of respiration state, electron transport mode and



substrate in isolated mitochondria from Sham, STZ and STZ + Ins hearts. Typically, mitochondria release different amounts of  $\text{H}_2\text{O}_2$  under forward (FET) compared with reverse electron transport (RET), hence we investigated specific rates of  $\text{H}_2\text{O}_2$  emission under both conditions [27]. Surprisingly, STZ mitochondria showed lower emission in state 4 respiration, under both FET (Figure 3A) and RET (Figure 3B). This phenomenon was even more prominent in STZ + Ins mitochondria, in keeping with insulin ability to restore baseline mitochondrial NAD(P)H to control levels (Figure 3C). With the exception of STZ mitochondria under FET, low levels of ROS emission were observed in state 3 (Figures 3A and 3B). However, differently from controls,  $\text{H}_2\text{O}_2$  emission did not decrease in state 4  $\rightarrow$  3 transition in the STZ group. Apparently, the lower  $\text{H}_2\text{O}_2$  emission levels exhibited by STZ and STZ + Ins compared with Sham mitochondria in the absence of oxidative stress was not due to differential protein expression levels of the main antioxidant systems, from both cytoplasmic and mitochondrial compartments. Indeed, the protein abundance of superoxide dismutase, glutathione peroxidase, peroxiredoxin, glutathione and thioredoxin reductases 1 and 2, catalase, thioredoxins 1 and 2 and transhydrogenase was not different in these preparations (results not shown).

Secondly, we assessed the direct impact of increased oxidative stress on mitochondrial redox function because mitochondria from T1DM cardiomyocytes operate under more oxidizing conditions when subjected to hyperglycaemia and higher energy demand (i.e. in HG + ISO, Figure 1). To mimic the pro-oxidant conditions triggered by HG + ISO in T1DM mitochondria, we imposed redox stress to mitochondria, loading them with high concentrations of MCB (50  $\mu\text{M}$ ) that compromise the scavenging capacity of the GSH pool [27] without affecting respiration [33]. When MCB-loaded T1DM mitochondria were challenged with  $\text{H}_2\text{O}_2$  (1  $\mu\text{M}$ ) and exposed to mild carbonyl cyanide 4-trifluoromethoxy-phenylhydrazone (FCCP) uncoupling (10–50 nM), higher ROS amounts were released at lower FCCP concentrations as compared with those from Sham or insulin-treated GPs (Figure 3D). The lack of ROS emission increase under oxidative stress in the STZ + Ins as compared with Sham group is related to the significantly higher mitochondrial baseline NAD(P)H levels and probably antioxidant capacity, elicited by insulin (Figure 3D). Previous work showed that, under extremely oxidizing conditions, FCCP increases ROS release due to lowered antioxidant defences [27]. Accordingly, preincubation of stressed mitochondria with FCCP further oxidized both NAD(P)H and GSH matrix pools [33].

Thirdly, we compared the impact exerted on  $\text{H}_2\text{O}_2$  emission by the intermediate of Palm oxidation, PCoA in control and T1DM mitochondria. Under FET (0.5 mM malate), in the absence of  $\beta$ -oxidation (by omitting L-carnitine), PCoA (5  $\mu\text{M}$ ) was able to decrease  $\text{H}_2\text{O}_2$  emission in Sham and STZ mitochondria in both states 4 and 3 respiration.  $\beta$ -Oxidation brought ROS emission back to control levels or even to higher values (Figure 4A). In parallel, respiration measurements performed with the same mitochondrial preparation and under the same conditions showed that PCoA was able to down-modulate ROS emission in state 4 independently from  $\beta$ -oxidation (Figure 4B) and in the absence of significant changes in respiratory flux with respect to controls. However, in state 3  $\beta$ -oxidation, a significant increase in respiratory flux concomitant with increased  $\text{H}_2\text{O}_2$  emission happened in both Sham and STZ mitochondria (Figures 4A and 4B).

Thus, from these experiments, we can conclude that heart mitochondria from diabetic GPs exhibit energetic impairment; they release less ROS but this emission persists during state 4→3 transition. Moreover, a pro-oxidant cellular environment compromises the antioxidant systems rendering T1DM mitochondria more vulnerable to oxidative stress thus releasing more ROS. When in low amounts (5  $\mu$ M), PCoA is able to decrease mitochondrial ROS release, thus suggesting a negative feedback on ROS emission exerted by this intermediate of Palm oxidation.

### **Altered mitochondrial redox assets contributes to impair the energetic behaviour of T1DM mitochondria**

Augmented oxidative stress can alter the phosphorylation capacity of mitochondria [33] limiting energy supply, particularly under situations of increased cardiac workload. However, this possibility has not been tested in T1DM hearts exposed to HG and  $\beta$ -adrenergic overdrive. To fill this gap, we quantified the time required for the mitochondria to phosphorylate added ADP. This lapse of time can be measured from the cycle orthodox-to-condensed-to-orthodox volume change associated with the state 4→3 transition [33,34] or cycling time (Figure 5A). This is a metric of the rate of ADP phosphorylation (Figures 5B-5D). We found that non-stressed T1DM mitochondria exhibit a ~2-fold lower  $V_{\max}$  for ADP phosphorylation (nmol of ADP  $\text{min}^{-1} \cdot \text{mg}^{-1}$  of protein  $\pm$  S.E.M.) (Figure 5C) as compared with Sham (Figure 5B;  $1091 \pm 98$  compared with  $2327 \pm 136$  respectively;  $n=6$ ,  $P < 0.01$ ). Insulin was able to reverse this effect (Figure 5D). Comparing the impact of stress compared with non-stress conditions, oxidants produced a more drastic decrease in  $V_{\max}$  phosphorylation rates in Sham (Figure 5B;  $569 \pm 28$  compared with  $2327 \pm 136$  respectively;  $n=6$ ,  $P < 0.001$ ) as compared with what found in T1DM mitochondria (Figure 5C;  $592 \pm 66$  compared with  $1091 \pm 98$  respectively;  $n=6$ ,  $P < 0.05$ ).

Next, we compared  $\text{H}_2\text{O}_2$  emission capacity in stressed and non-stressed mitochondria as a function of ADP addition to G/M energized mitochondria.  $\text{H}_2\text{O}_2$  emission levels were always higher in stressed than in non-stressed mitochondria across groups (Figures 5E-5G). However, one would expect that ROS levels should decrease with ADP in mitochondria harbouring a more reduced redox environment [27,28]. Intriguingly, when stressed, T1DM mitochondria exhibited increased rather than decreased levels of ROS emission as a function of ADP concentration (Figure 5F). Thus, our data demonstrate that T1DM mitochondria exposed to higher energy demand, as mimicked by ADP challenge, have a lower capacity to supply energy, an effect accompanied by exacerbated ROS emission.

### **Contractile function is altered in T1DM cardiomyocytes subjected to high glucose and $\beta$ -adrenergic stimulation**

The HG + ISO challenge unveils latent redox/energetic deficits in cardiac T1DM mitochondria and these alterations may underlie mechanical dysfunction in myocytes isolated from STZ- treated GP hearts. To directly prove this possibility and to establish whether either chronic insulin therapy or acute Palm infusion are able to obviate these eventual changes, we studied contractile function and whole  $\text{Ca}^{2+}$  transient in myocytes isolated from controls and STZ-treated GP hearts. Sham myocytes treated with HG



exhibited a smaller ISO-induced increase in contractility as compared with controls subjected to EG (Figure 6A).

This alteration became even more prominent in STZ cardiomyocytes after HG + ISO challenge (Figure 6D). ISO-evoked acceleration of myocyte relaxation was also impaired by HG, as expressed in terms of 90 % re-lengthening measurements (TR90) (Figures 6C and 6F). Parallel changes in whole-cell  $\text{Ca}^{2+}$  transients were observed (Figures 6B and 6E), accounting, at least in part, for the aforementioned myocyte mechanical dysfunction.

We next asked whether chronic insulin treatment can prevent some, if not all, of these functional alterations. Cardiomyocytes isolated from insulin-treated diabetic GPs still displayed markedly reduced contraction and relaxation after HG + ISO (Figures 6G and 6I). Conversely, Palm preserved both, either in the absence (Figures 6D and 6F) or the presence of insulin therapy (Figures 6G and 6I). Moreover, Palm was able to rescue the  $\text{Ca}^{2+}$  transient amplitude (Figures 6E and 6H). Thus, HG and  $\beta$ -adrenergic stimulation markedly reduce T1DM myocyte function, an effect that Palm is able to largely prevent. In contrast, chronic supplementation of insulin fails to afford these beneficial effects, despite its ability to rescue cardiac redox balance.

## DISCUSSION

Old and new evidence shows that peaks of glucose and concomitant adrenergic hyperactivity may alter cardiac function in T1DM hearts, but the mechanisms accounting for these functional deficits remain largely unknown. In the present study, we report that energetic and redox-based stresses unveil marked differences in mitochondrial function between T1DM myocytes and their respective non-diabetic controls. The redox environment found in the former at baseline becomes even more oxidized when these cells are exposed to HG + ISO and this event prompts alterations in myocyte contraction and relaxation. When challenged with oxidizing agents, T1DM mitochondria exhibit a lower energetic response to ADP and impaired respiration in the presence of complex II and IV substrates, probably accounting to a large extent for myocyte mechanical deficits. Chronic insulin therapy restores T1DM myocyte/mitochondrial redox balance, decreasing mitochondrial ROS emission, but it fails to forestall loss-of-function in T1DM myocyte challenged with HG + ISO. Conversely, acute Palm superfusion succeeds in preventing both the build-up of a more oxidized cardiac environment and changes in  $\text{Ca}^{2+}$  handling, thus maintaining function in T1DM myocytes even in the presence of HG/ISO challenge.

### Mitochondrial ROS emission, altered ATP/ADP cycling and myocyte mechanical dysfunction in T1DM hearts

Hyperglycaemia plays a central role in the pathogenesis of DCM, acting as an initial trigger for several adaptive and maladaptive processes [35]. Altered  $\text{Ca}^{2+}$  handling, along with cytosolic and mitochondrial redox balance and perturbed energy production from these organelles, is a major determinant [8,9,12,36]. An altered pro/anti-oxidant balance is chiefly associated with the onset/progression and pathological consequences of diabetes, providing a mechanistic link between mitochondrial dysfunction and hyperglycaemia [37].

Our contribution provides additional novel insights into the mechanism underlying excessive ROS emission in T1DM mitochondria and it explains how this ROS burst leads to cardiac energetic deficit and eventually to myocyte dysfunction. In fact, in the present study, we show that NAD(P)H oxidation is increased in T1DM mitochondria and demonstrate that they exhibit greater H<sub>2</sub>O<sub>2</sub> emission following mild uncoupling, in keeping with the redox-optimized ROS balance (R-ORB) hypothesis [27]. If the mitochondrial environment is more oxidized, further oxidative challenge will increase rather than decrease ROS emission [33]. To mimic this *in vivo* higher oxidative stress, we stressed isolated mitochondria with exogenous H<sub>2</sub>O<sub>2</sub>. The presence of oxidizing conditions in T1DM mitochondria decreased ADP phosphorylation rates and impaired the transition to increased energy demand because higher rather than lower ROS emission occurred when ADP was increased (Figure 5F) [8,28,33]. The opposite would be true in the absence of oxidative stress. Indeed, the relationship between respiration and ROS is altered by redox imbalance, resulting in decreased mitochondrial energetic performance and higher levels of ROS emission [33]. The present findings show that, unlike Sham controls, non-stressed and stressed T1DM mitochondria exhibit more similar energetic responses.

Diastolic dysfunction is regarded as an early sign, being detected in up to 75% of asymptomatic diabetic patients [3,38], and a propeller for systolic deficit in DCM. Two important interrelated aspects of Ca<sup>2+</sup> handling participate in the onset of diastolic dysfunction, i.e. the rate of Ca<sup>2+</sup> reuptake into SR following release (which regulates the speed of myocyte relaxation) and the levels of resting or end-diastolic Ca<sup>2+</sup> (which determines the extent of cell relaxation or diastolic tension) [39]. Mitochondria may influence diastolic Ca<sup>2+</sup> levels through diverse mechanisms, including uptake via the Ca<sup>2+</sup> uniporter [40]. Because mitochondrial Ca<sup>2+</sup> concentrations are in the range that allows modulation of the Ca<sup>2+</sup>-sensitive dehydrogenase activation [41], lower levels of Ca<sup>2+</sup> in DCM may adversely affect tricarboxylic acid (TCA) cycle function, contributing to impaired ATP generation in the heart. The present findings showing that T1DM myocytes exposed to HG + ISO are no longer able to fully contract and relax tie together impaired Ca<sup>2+</sup> handling, altered myocyte mechanical function and diminished ATP synthesis at the onset of DCM [15,19]. They help to explain how changes in mitochondrial redox assets, i.e. the ability of keeping ROS emission at low levels despite increased work demand, influence mitochondrial energy generation. The higher oxidative burden elicited by mitochondria in challenged T1DM hearts is in agreement and may to further explain the potential arrhythmogenic substrate already documented in these hearts [42]. Indeed, diabetic GPs show a higher vulnerability to triggering of arrhythmias when GSH is oxidized by diamide treatment [42], supporting that heterogeneity in the action potential duration may underlie the higher propensity to arrhythmias exhibited by the T1DM heart.

### **Insulin therapy does not fully rescue function in stressed T1DM cardiomyocytes**

Another important finding of the present study is that chronic insulin treatment decreased oxidative stress in cardiac myocytes (Figure 1), improving mitochondrial redox balance (Figure 3) and their energetic response to ADP in presence of respiratory substrates of complex I (Figure 5). Remarkably, mitochondria from insulin-treated STZ GPs subjected to oxidant stress were able to phosphorylate ADP at the same rate as non-stressed ones (Figure

5D), exhibiting similar behaviour to controls (Figure 5B). However, insulin treatment did not rescue mechanical function (Figure 6) and mitochondrial respiration from substrates of respiratory complexes II and IV (Figure 2). On the one hand, these results suggest that the respiratory deficit in complex II and IV did not hinder electron flow in the presence of substrates from complex I in mitochondria from the STZ + Ins group. On the other, they suggest that a cytoplasmic mechanism triggered by insulin that contributes to mechanical function is lacking in the isolated cardiac myocyte. This possibility could be associated with insulin signalling mediated by physiological levels of ROS supplied from sources other than mitochondria such as NADPH oxidase [43,44]. For example, contractile function in the heart is regulated via stimulation of  $\beta$ -adrenergic receptors (ARs) and an adverse cross-talk between insulin and the adrenergic stimulation signalling pathway was recently shown [45]. Insulin was able to directly impair the adrenergic signalling pathway for contractile function via an insulin receptor (IR) and  $\beta_2$ -adrenergic receptor ( $\beta_2$ AR) signalling complex in animal hearts, providing a plausible mechanism for damage of myocardial inotropic reserve in T2DM subjects [45].

Overall, our results are in agreement and extend reported data showing alteration of redox status concomitantly with the appearance of diabetic symptoms (e.g. insulin resistance, augmented levels of glycosylated haemoglobin, HbA<sub>1c</sub>) [36]. Clinical evidence also shows that the positive or negative effects of insulin on diabetic patients or experimental animal models depend upon the organ analysed and the conditions of administration [46]. Our findings also suggest that insulin therapy is able to have a long-lasting influence on the *in vivo* redox status of mitochondria from T1DM GPs, but is unable to overcome the impairment of respiratory complexes elicited by the diabetic condition. The latter hindered the recovery of contractile function in cardiac myocytes from insulin-treated T1DM GPs, a capacity that may be dependent on insulin signalling [43].

### Fatty acids and myocardial performance in T1DM stressed hearts

An increase in myocardial FA uptake and oxidation has been described in humans with both T1DM and T2DM and in animal models [19,23,47]. FAs contribute 60–70% of myocardial ATP at physiological levels and it is very plausible that T1DM hearts/myocytes rely more heavily on energy derived from FA catabolism [22]. The three times higher than glucose energetic budget provided by Palm is up to 2/3 in the form of reducing equivalents derived from  $\beta$ -oxidation in mitochondria. Cellular ROS levels were markedly reduced after Palm supplementation (Supplementary Figures S2, S3 and S6), an event associated with a significant rise in intracellular GSH (Figure 1). Likewise, in cardiomyocytes from T2DM *db/db* mice [8] or heart trabeculae from ZDF rats [12], it was shown that Palm determines a transition from oxidized to reduced cellular redox status increasing intracellular GSH, preventing the increase in ROS levels. These effects were accompanied by the rescue of the mitochondrial thioredoxin reductase 2 (TrxR2)/thioredoxin 2 (Trx2)/peroxiredoxin 3 (Prx3) pathway [8], a major controller of H<sub>2</sub>O<sub>2</sub> emission from mitochondria [28,48]. Similarly, in heart trabeculae from the T2DM ZDF rat subjected to workload, HG + Palm, but not insulin, exerted a positive effect on contractile work the opposite was observed in lean trabeculae [12]. Probably, this difference is due to protective mechanisms that operate in diabetic but not normal hearts [1].

Moreover, very recent metabolomics data combined with computational modelling [49] showed that Palm elicits the activation of the pentose phosphate pathway, thus improving myocardial redox balance via increased NADPH supply and GSH levels in Langendorff-perfused hearts from mice.

Palm is able to effectively restore myocardial redox balance in HG + ISO-stressed T1DM hearts (Figure 1). In the present study, we further investigated the impact of PCoA in this phenomenon. In the absence of  $\beta$ -oxidation, low PCoA concentration significantly decreased ROS emission in isolated mitochondria under states 4 and 3 respiration (Figure 4). Importantly, this effect happened without significant changes in respiratory flux in state 4 with or without PCoA oxidation. Ongoing studies with isolated mitochondria show that, at low concentrations and in the absence of  $\beta$ -oxidation (i.e. no L-carnitine added), PCoA is not consumed by mitochondria, inhibits complex I and can gradually decrease or abolish ROS emission under FET or RET, in the latter case similar to rotenone (Miguel A. Aon and Sonia Cortassa, unpublished work). Thus, low concentrations of intermediary metabolites from Palm catabolism may down-modulate ROS emission from mitochondria independently of  $\beta$ -oxidation. This evidence suggests the existence of a built-in negative-feedback mechanism controlling mitochondrial ROS emission that could be involved in the improvement of the redox status of cardiac myocytes by Palm.

A complementary explanation of the salutary action of Palm under energy/redox stress may involve mechanisms of storage/utilization in lipid droplets (LDs). In the diabetes scenario, lipid excess packaging into LDs may represent an adaptive response to fulfilling energy supply without hindering mitochondrial or cellular redox status while keeping a low concentration of lipotoxic intermediates [1,50].

### Limitations of the present study

The Palm salutary action described in the present study and in earlier studies [8,12] was evident under conditions of acute energy/redox stress. Additional studies are needed to determine the outcome of long supplementation of Palm or, for comparison, of other FAs in T1DM animals, in presence of increased cardiac workload (e.g. exercise). Moreover, in the present study, we aimed at determining the differential effects of insulin on cardiomyocyte/mitochondria function after hormone therapy *in vivo*. Whether the results and conclusions of the present work will hold in response to further treatment with insulin *in vitro* warrants additional separate investigation.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

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**Abbreviations:**

<b>AR</b>	adrenergic receptor
<b>DCM</b>	diabetic cardiomyopathy
<b>DNP</b>	dinitrophenol
<b>E-C</b>	excitation-contraction
<b>FA</b>	fatty acid
<b>FET</b>	forward electron transport
<b>G/M</b>	glutamate/malate
<b>GP</b>	guinea pig
<b>GSB</b>	glutathione S-bimane
<b>HG</b>	high glucose
<b>Ins</b>	insulin-treated
<b>ISO</b>	isoprenaline (isoproterenol)
<b>LD</b>	lipid droplet
<b>MCB</b>	monochlorobimane
<b>OxPhos</b>	oxidative phosphorylation
<b>Palm</b>	palmitate
<b>PCoA</b>	palmitoyl-CoA
<b>RET</b>	reverse electron transport
<b>ROS</b>	reactive oxygen species
<b>STZ</b>	streptozotocin
<b>Succ</b>	succinate
<b>T1DM</b>	Type I diabetic
<b>ZDF</b>	Zucker Diabetic Fatty

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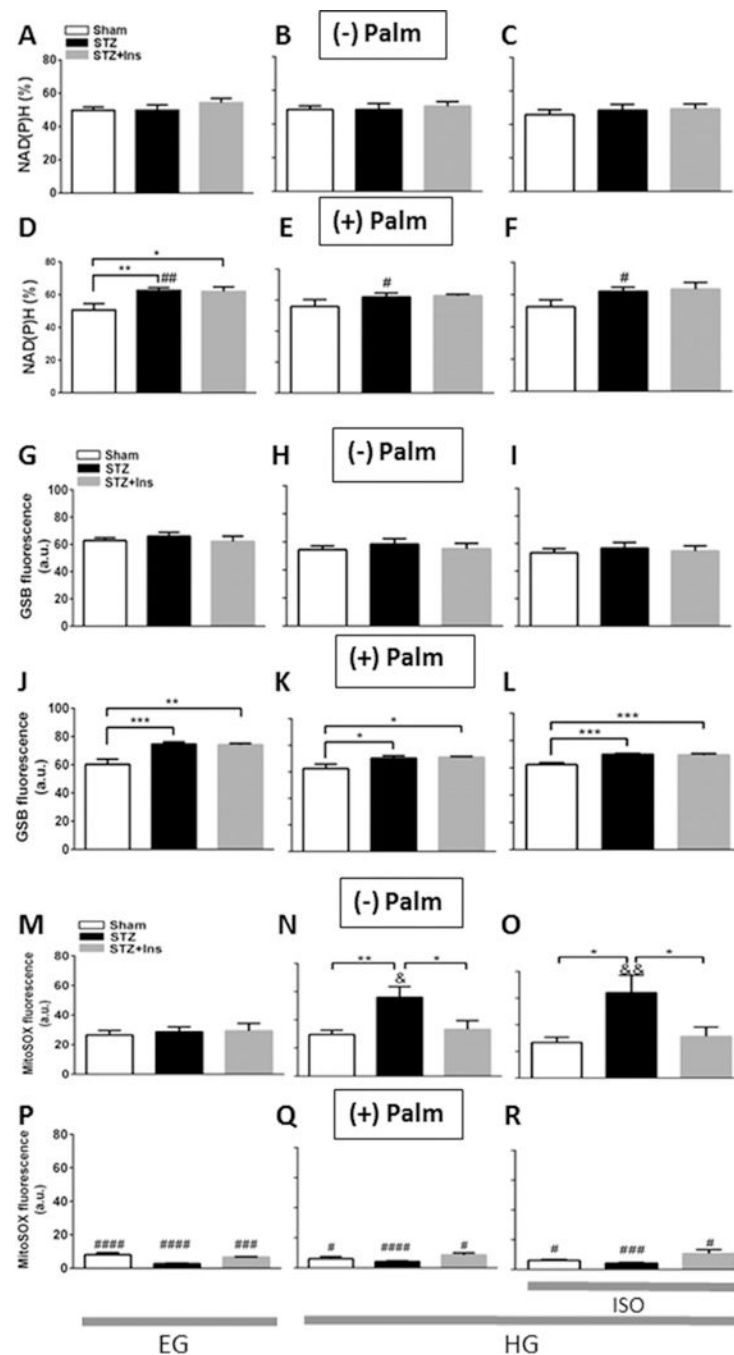


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### CLINICAL PERSPECTIVES

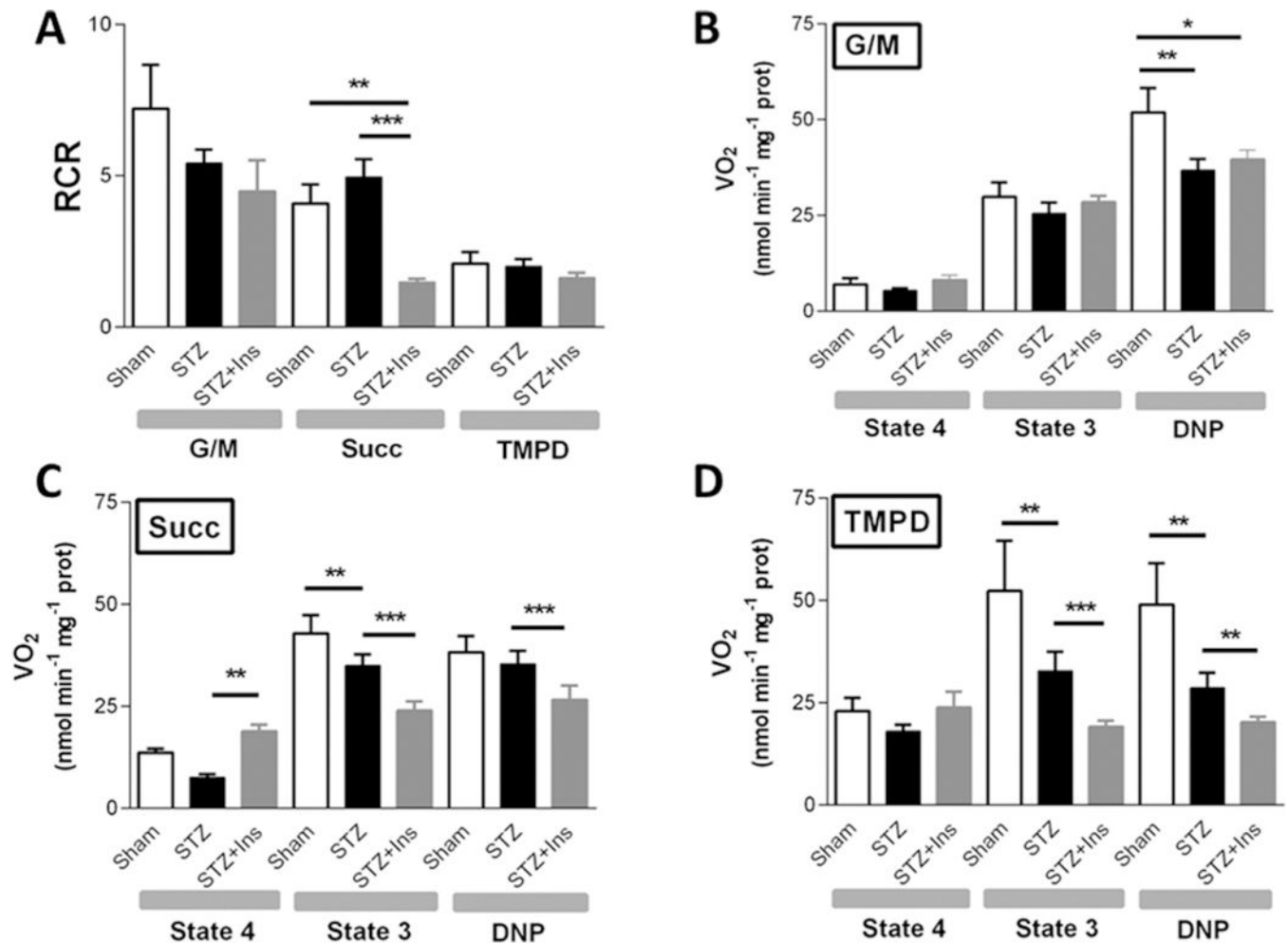
- In chronic diseases such as diabetes, mitochondria function under oxidizing conditions, thus chiefly contributing to cardiac dysfunction development. Our study adds to this scenario that lower and slower energetic output and higher ROS emission is present in mitochondria of T1DM hearts when cardiac work demand increases and more oxidizing conditions are in place.
- These findings highlight myocardial redox imbalance and mitochondrial dysfunction as potential therapeutic targets in diabetic patients particularly prone to excessive ROS emission due to fluctuating blood glucose levels and increased sympathetic tone.
- They suggest that tackling both mitochondrial energetic deficit and redox alterations at the same time should provide a better therapeutic approach to DCM.



**Figure 1. Imaging of Sham, STZ and STZ + Ins cardiomyocyte redox status under normal or HG without or with ISO and Palm**

Freshly isolated GP cardiomyocytes loaded with 2  $\mu$ M MitoSOX (M-R) were imaged simultaneously for NAD(P)H autofluorescence with two-photon microscopy (A-F). GSB was imaged in myocytes loaded with 50  $\mu$ M MCB (G-L) [8,29] (see also the Materials and methods section). Baseline imaging of cells was done with Tyrode's buffer, pH 7.5, containing 1 mM  $\text{Ca}^{2+}$  and 10 mM glucose (EG) followed by the same solution with 30 mM glucose (HG), in the absence or in the presence of 10 nM ISO (ISO). The same protocol was

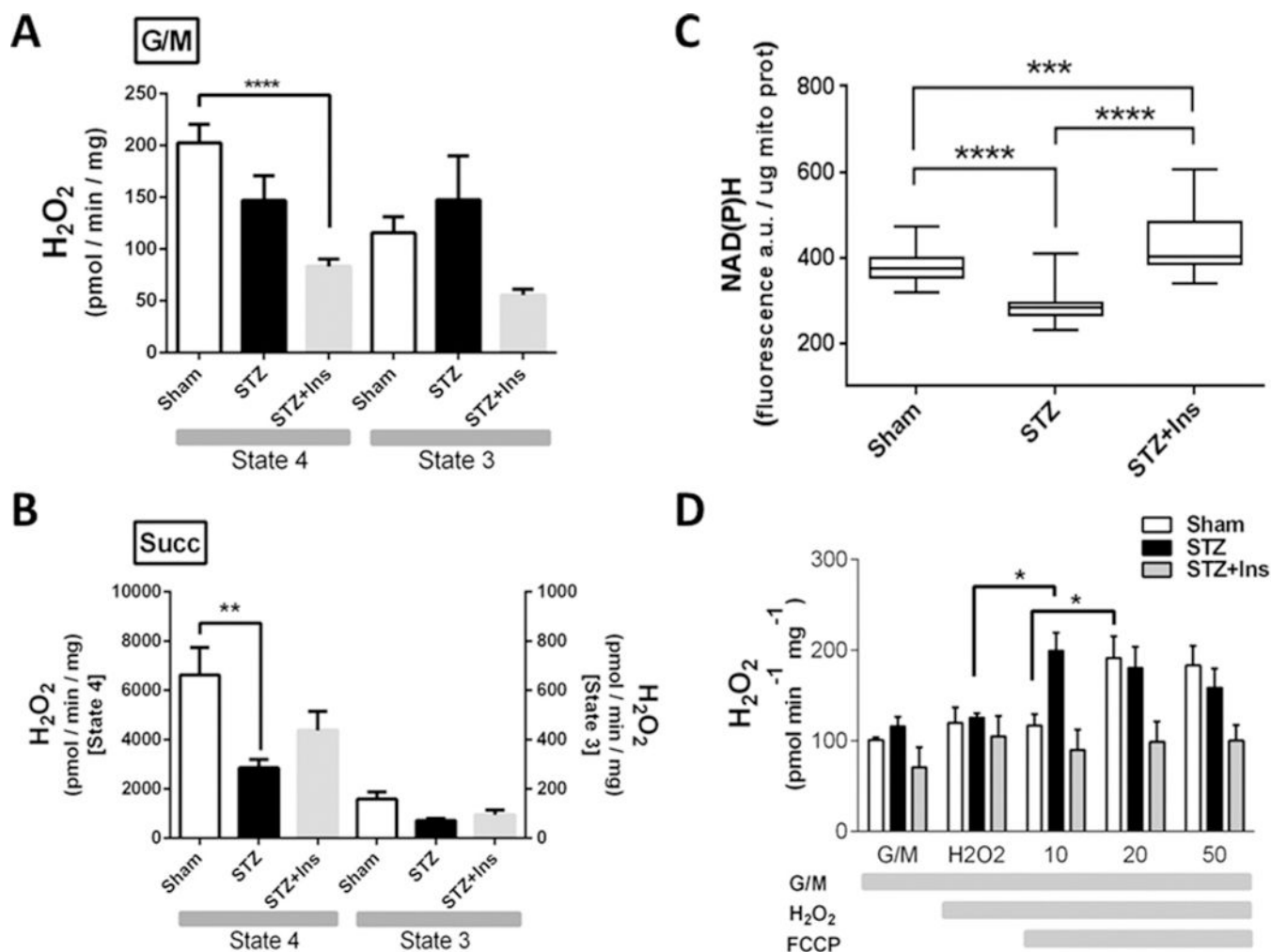
repeated in the presence of 0.4 mM Palm for Sham, STZ and STZ + Ins groups. Palm was bound to 10% albumin FA-free (4:1 molar ratio Palm/albumin) and prepared as described in [8] (see also the Supplementary Online Data). Cardiomyocytes in EG were perfused for 30 min or 3 min with HG without/with ISO respectively. After the incubation time, triplicate images were taken every 2 min from the same microscopic field during 10 min, and this process was repeated for a total of three fields, adding to at least six to ten cardiomyocytes in each experiment. This procedure enabled us to ascertain the steady state of ROS/GSH/NAD(P)H fluorescent signals while obtaining triplicate fluorescence measurements, the average of which was used for each cell. All observations were paired, i.e. performed in the same cells at all treatments and for all imaging experiments. Depicted are the results from four experiments (four hearts) with  $n=40-60$  for each treatment and fluorescent probe after pooling the four data sets. The statistical significance of the differences between groups (Sham/STZ/STZ + Ins) was calculated by one-way ANOVA (OWA) within (e.g. EG) and across (EG/HG/HG + ISO) treatments and by two-way ANOVA (TWA) within treatments but  $\pm$  Palm (e.g. HG, Sham/STZ/STZ + Ins, + or - Palm). Statistical significance within and across treatments is denoted \* and & respectively, for OWA and # for TWA; \*, #, &  $P < 0.05$ ; \*\*, ##, &&  $P < 0.01$ ; \*\*\*, ###  $P < 0.001$ ; \*\*\*\*, ####  $P < 0.0001$ .



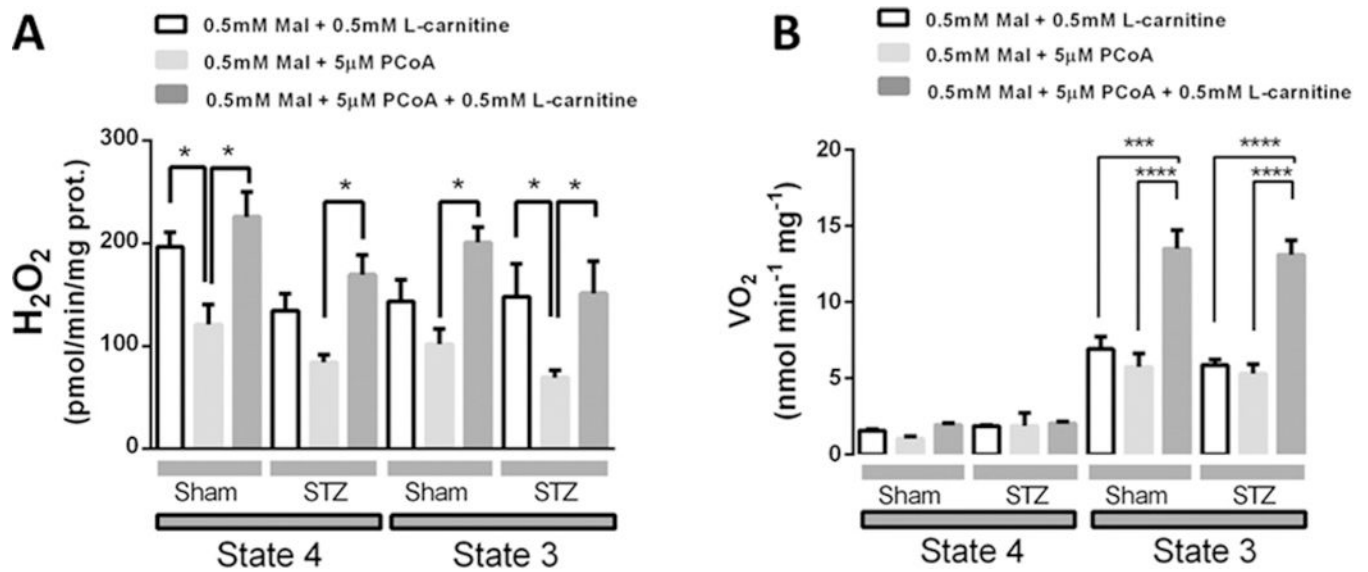
**Figure 2. Energetic behaviour of isolated heart mitochondria from Sham, STZ and STZ + Ins GPs**

Respiration in freshly isolated heart mitochondria from the three groups of GPs were analysed with a Seahorse Bioscience XF96 analyser as described in the Materials and methods section. (A–D)  $\dot{V}_{O_2}$  was assayed under states 4 and 3 as described in [8,12] with substrates from (B) complex I (5mM G/M), (C) complex II (5mM Succ and 1  $\mu$ M rotenone) and (D) complex IV [0.5 mM TMPD (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine) and 3 mM sodium ascorbate]. State 3 was induced with 1 mM ADP in all cases. (A) RCR determined as the ratio of state 3/state 4.  $n=10-16$  replicates from three experiments/hearts.





**Figure 3. Redox and ROS status of mitochondria in the absence or presence of oxidative stress** Heart mitochondria from Sham, STZ and STZ + Ins groups were isolated and assayed as in the legend of Figure 2 and the Materials and methods section. **(A and B)** Specific rates of H<sub>2</sub>O<sub>2</sub> emission from mitochondria (100–150  $\mu$ g of mitochondrial protein) in states 4 and 3 respiration under **(A)** FET or **(B)** RET with Amplex Red [ARed] (see [8,12] and the Materials and methods section). The conditions utilized to measure H<sub>2</sub>O<sub>2</sub> emission were the same as those employed for measuring respiration with the exception of RET where rotenone was omitted (see the Materials and methods section and the Figure 2 legend). **(C)** Basal levels of NADH fluorescence normalized with respect to protein from isolated mitochondria. **(D)** Isolated mitochondria were subjected to redox stress understate 4 respiration, as described in [27,33] (see the Results section ‘ROS emission from T1DM heart mitochondria in the absence or presence of oxidative stress’). The numbers 10, 20 and 50 in the x-axis correspond to nanomolar FCCP. The data shown in **(A–D)** correspond to three experiments/hearts with  $n=10$  (**A and B**),  $n=34$  (**C**) and  $n=6$  (**D**). Differences in ROS emission from state 3 respiration (**A and B**) were not significant. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ .

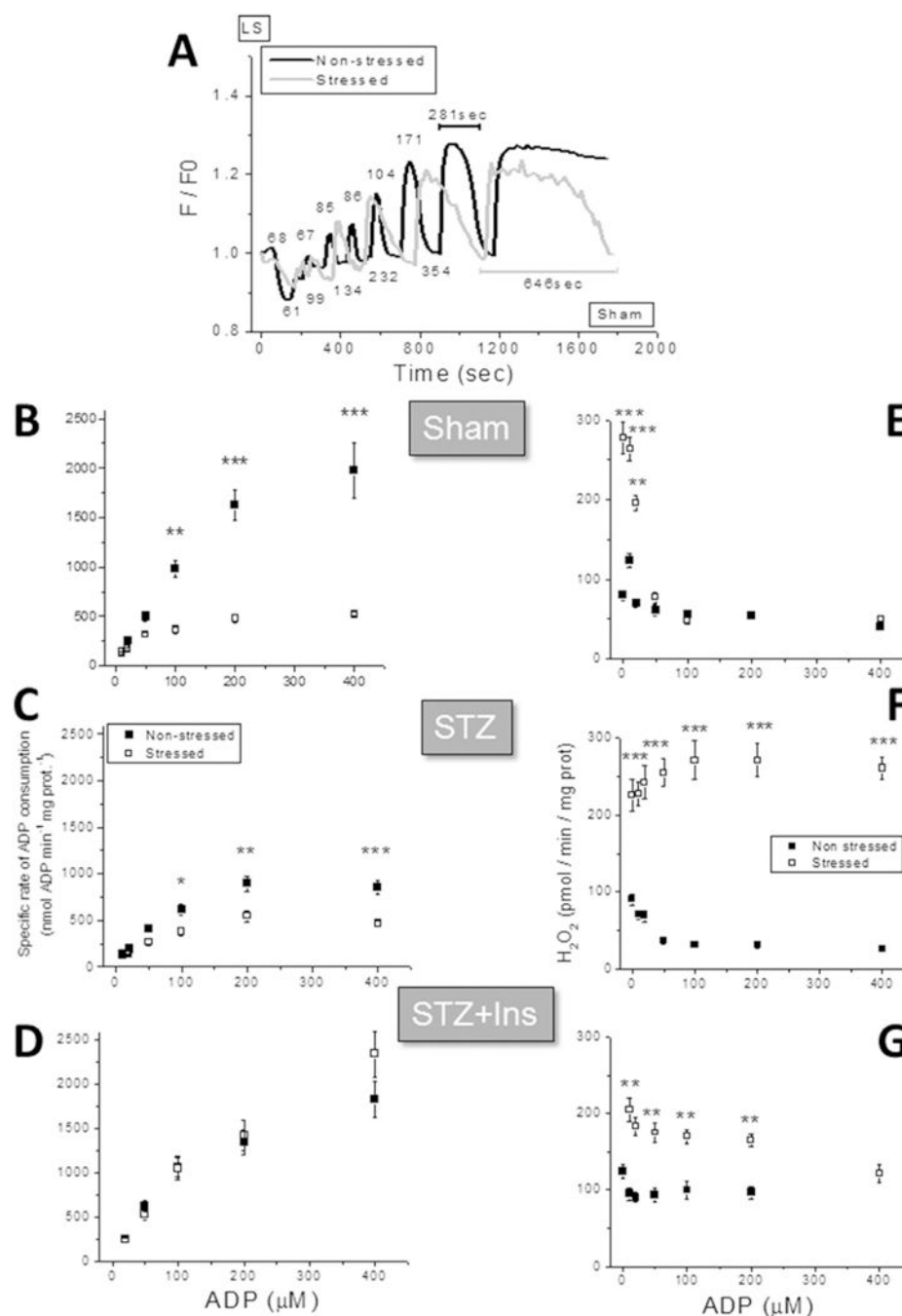


**Figure 4. ROS emission and respiration from Sham and STZ mitochondria without or with PCoA**

Heart mitochondria from Sham and STZ groups were isolated and assayed in parallel for H<sub>2</sub>O<sub>2</sub> emission or for  $\dot{V}_{O_2}$  as described in the Materials and Methods section ‘Mitochondrial

physiological studies’. (A) Specific rates of H<sub>2</sub>O<sub>2</sub> emission from mitochondria (10 μg of mitochondrial protein) were determined in states 4 and 3 respiration under FET in the presence of 0.5 mM malate (Mal) without or with 5 μM PCoA and in the absence (omitting L-carnitine) or presence of β-oxidation (adding 0.5 mM L-carnitine), as indicated. (B)  $\dot{V}_{O_2}$

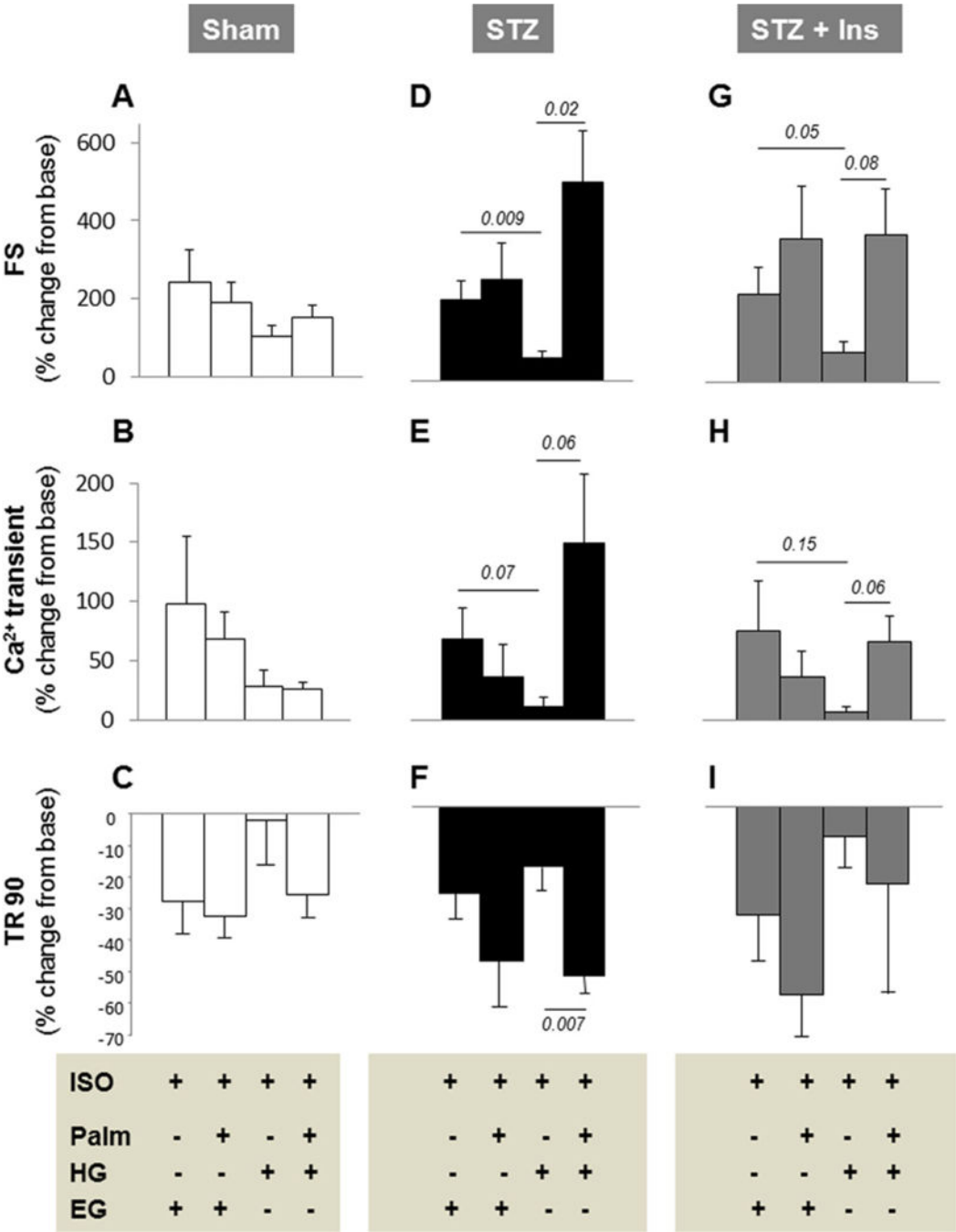
was assayed in states 4 and 3 (10 μg of mitochondrial protein) as in the legend of Figure 2 under the same substrate conditions described in (A). *n*=12 replicates from three experiments/hearts in each Sham or STZ group. \**P*<0.05; \*\**P*<0.01; \*\*\**P*<0.001; \*\*\*\**P*<0.0001.



**Figure 5. Mitochondrial energetic and ROS responses as a function of ADP in the absence or presence of oxidative stress**

Freshly Isolated mitochondria from Sham (A,B and E), STZ (C and F) and STZ + Ins (D and G) hearts were monitored by spectrofluorimetry for 90° light scattering (LS; A-D) [27] or H<sub>2</sub>O<sub>2</sub> emission with ARed (E-G). G/M (5/5 mM) energized heart mitochondria [100–150 μg of mitochondrial protein] were challenged with consecutive addition of increasing ADP concentrations. The stress protocol consisted of loading the mitochondria with 50 μM MCB and then further challenging them with 1 μM H<sub>2</sub>O<sub>2</sub> when assayed in the fluorimeter [33].

(A) Representative LS responses (low LS, state 4 relaxed mitochondria; high LS, state 3 condensed mitochondria) in non-stressed (black trace) or stressed (grey trace) mitochondria. Numbers indicate the cycling time in seconds. (B-D) From the cycling times determined in (A) and the amount of ADP in each addition we could calculate the rate of ADP phosphorylation assuming complete utilization. Depicted are the specific rates of ADP phosphorylation as a function of ADP concentration from non-stressed (black squares) and stressed (white squares) mitochondria. (E-G) Specific rate of H<sub>2</sub>O<sub>2</sub> emission as a function of ADP in non-stressed (black squares) and stressed (white squares) mitochondria. Results in duplicate from three independent mitochondrial isolations (three hearts); \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .



**Figure 6. Fractional shortening,  $Ca^{2+}$  transient and rate of sarcomere re-lengthening in Sham, STZ and STZ + Ins cardiomyocytes**  
Cardiomyocytes were isolated from hearts of the three GP groups as in [26] and analysed at room temperature for percentage fractional shortening (FS; **A**, **D** and **G**),  $Ca^{2+}$  transient amplitude (**B**, **E** and **H**) and TR90, time to 90% sarcomere re-lengthening (**C**, **F** and **I**), in parallel to the imaging studies (Figure 1). EG (10 mM glucose) and HG (30 mM glucose) and HG were utilized, without or with 10 nM ISO, in the absence or presence of pre-incubation with 0.4 mM Palm.  $n=20-30$  from four to six hearts.