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Palmitate-induced impairment of glucose-stimulated insulin secretion precedes mitochondrial dysfunction in mouse pancreatic islets

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ABSTRACT

It has been well established that excessive levels of glucose and palmitate lower glucose-stimulated insulin secretion (GSIS) by pancreatic beta cells. This beta cell ‘glucolipotoxicity’ is possibly mediated by mitochondrial dysfunction, but involvement of bioenergetic failure in the pathological mechanism is subject of ongoing debate. We show here that increased palmitate levels impair GSIS before altering mitochondrial function. We demonstrate that GSIS defects arise from increased insulin release under basal conditions in addition to decreased insulin secretion under glucose-stimulatory conditions. Real-time respiratory analysis of intact mouse pancreatic islets reveals that mitochondrial ATP synthesis is not involved in the mechanism by which basal insulin is elevated. Equally, mitochondrial lipid oxidation and production of reactive oxygen species do not contribute to increased basal insulin secretion. Palmitate does not affect KCl-induced insulin release at a basal or stimulatory glucose level, but elevated basal insulin release is attenuated by palmitoleate and associates with increased intracellular calcium. These findings deepen our understanding of beta cell glucolipotoxicity and reveal that palmitate-induced GSIS impairment is disconnected from mitochondrial dysfunction, a notion that is important when targeting beta cells for the treatment of diabetes and when assessing islet function in human transplants.

SUMMARY STATEMENT

Mitochondrial dysfunction associates with obesity-related pancreatic beta cell failure, but the causality of this association is unclear. In this paper we show that palmitate-induced impairment of insulin secretion *precedes* mitochondrial respiratory defects in isolated mouse islets.

SHORT TITLE

Mitochondrial involvement in beta cell glucolipotoxicity

KEYWORDS

Pancreatic beta cells; glucolipotoxicity; mitochondria; oxidative phosphorylation; Type 2 diabetes; obesity

ABBREVIATIONS

DAPI, 4',6-diamidino-2-phenylindole; Fura-2AM, Fura-2-acetoxymethyl; FBS, fetal bovine serum; GSIS, glucose-stimulated insulin secretion; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; K_{ATP} channel, ATP-sensitive potassium channel; MnTMPyP, Mn(III)tetrakis(1-methyl-4-pyridyl) porphyrin pentachloride; NEFA, non-esterified fatty acids; OCR, oxygen consumption rate; ROS, reactive oxygen species; UCP2, uncoupling protein-2

INTRODUCTION

Type 2 diabetes affects over 500 million people worldwide [1] and it is generally accepted that obesity is a major risk factor in the development of this metabolic disease [2]. The obese state is characterised by high NEFA levels, palmitate in particular, that contribute to pancreatic beta cell dysfunction and are thus partly responsible for the chronic hyperglycaemia that defines diabetes [3-5]. When blood glucose levels rise, healthy beta cells increase their oxidative glucose catabolism, which leads to a rise in the cytoplasmic ATP/ADP ratio and, consequently, to closure of K_{ATP} channels, depolarisation of the plasma membrane potential, opening of voltage-gated Ca^{2+} channels, influx of Ca^{2+} and the eventual exocytosis of insulin-containing granules [6]. Mitochondria are essential for this GSIS as they couple the oxidation of glucose-derived reducing equivalents to the synthesis of ATP and thus provide a key signal in the canonical GSIS pathway. Mitochondria also generate ROS that have been implicated in insulin secretion [7-9] and produce other non-canonical signals that amplify GSIS [10].

In line with studies on human and mouse pancreatic islets [11,12], we have recently reported that palmitate exposure in the presence of a glucose surplus dampens GSIS in INS-1E insulinoma cells [13,14]. This GSIS impairment coincides with mitochondrial defects in oxidative phosphorylation [13,14]. Moreover, we have shown that palmitate-induced mitochondrial superoxide formation underlies NEFA-provoked INS-1E cell loss [15]. This cell loss can be prevented by palmitoleate, the monounsaturated equivalent of palmitate [15]. Mitochondria thus associate with several aspects of beta cell damage that is caused by 'glucolipotoxicity'. More generally, mitochondrial function has been identified as promising predictor of functional islet integrity, and 'bioenergetic health' indeed correlates well with positive clinical outcomes in islet transplantation trials [16]. Dependable predictions of islet functionality would clearly benefit transplantation protocols, which is why we set out to further explore the reliability of mitochondrial function as potential biomarker of islet health.

Over recent years, extracellular flux (XF) measurement has become fairly routine for probing real-time bioenergetics of cultured cells [17] and the approach has been adopted to measure islet respiration [18]. In this study, we used XF technology to quantify effects of palmitate exposure on the bioenergetics of intact isolated mouse islets. We show that GSIS defects emerge without any sign of mitochondrial respiratory dysfunction after 24-h palmitate exposure. Palmitate lowers the glucose-sensitivity of mitochondrial respiration after 48-h exposure, whilst leaving basal oxygen consumption unaffected. Concomitant GSIS impairment mirrors this palmitate-induced respiratory defect, but, importantly, is caused to a large extent by elevated *basal* insulin secretion. A similar palmitate effect on basal insulin release is seen in INS-1E cells, where it is accompanied by a rise in intracellular calcium and where it is independent of palmitate-induced mitochondrial superoxide, unrelated to GSIS amplification and attenuated by palmitoleate. We conclude that mitochondrial dysfunction does not trigger palmitate-induced GSIS impairment under the glucolipotoxic conditions we applied, and that the observed bioenergetic changes are mostly coincidental, not predictive.

EXPERIMENTAL

Animals

C57BL/6 mice (Jackson Laboratory) were housed in air conditioned rooms maintained at 26 ± 2 °C with a 12-h light and dark cycle. Animal protocols were approved by Plymouth University and Helmholtz Zentrum München in accordance with directive 2010/63/EU on the protection of animals used for scientific purposes.

Islets

Islets were isolated according to [19] from randomly selected 12-18 week old female or male mice by direct liberase injection into the common bile duct. Islets were incubated overnight under a

humidified carbogen atmosphere at 37 °C in Connaught Medical Research Laboratories (CMRL) medium containing 5.5 mmol/l glucose, 15 % (v/v) FBS, 50 units/ml penicillin, 50 µg/ml streptomycin, 500 µmol/l 2-mercaptoethanol, 17.8 mmol/l NaHCO₃, and 2 mmol/l GlutaMAX. After this recovery period, islets were hand-picked at random into sterile Petri dishes containing 10 ml serum-free CMRL with 11 mmol/l glucose, and then incubated ± palmitate for 24 or 48 h at 37 °C under carbogen. Palmitate was administered in conjugation to BSA as described previously [13] to yield an estimated free concentration of 40 nmol/l. Control islets were exposed to BSA alone.

Cells

INS-1E cells were maintained in RPMI medium according to [20]. Cells were seeded on multi-well plates at 6×10^4 cells/well and, at 70–80% confluence, exposed to BSA-conjugated palmitate and/or palmitoleate, or to BSA alone for 24 h in serum-free RPMI containing 4 or 11 mmol/l glucose ± 0.2 mmol/l etomoxir.

GSIS

Islets were hand-picked into v-shape-bottomed 96-well plates (7 islets/well) containing 100 µl/well pre-warmed (37 °C) low-glucose Krebs-Ringer Bicarbonate (KRB) medium comprising 5.5 mmol/l glucose (i.e., fasting level in C57BL/6 mice [21]), 119 mmol/l NaCl, 4.7 mmol/l KCl, 2.5 mmol/l CaCl₂, 1.2 mmol/l KH₂PO₄, 1.2 mmol/l MgCl₂, 30.7 mmol/l NaHCO₃, 10 mmol/l HEPES (pH 7.4), 2 mmol/l GlutaMAX and 0.2 % (w/v) BSA. Islets were incubated at 37 °C under carbogen for 1 h, washed into fresh KRB and then incubated again for 1 h. Next, supernatant was collected and replaced with KRB containing 28 mmol/l glucose (i.e., a typical level to stimulate insulin release in mouse islets [19]) and after another 1-h incubation, supernatant was collected again. Supernatants were centrifuged at 10,000g and then assayed for insulin by ELISA (Mercodia, Uppsala, Sweden). Secreted insulin was normalised to islet DNA content as determined from PicoGreen fluorescence using Quant-iT™ PicoGreen® dsDNA Assay Kit (P11496, Life Technologies, UK).

INS-1E cells seeded and palmitate-exposed on 96-well culture plates were washed and starved for 1 h at 37 °C under air in low-glucose Krebs-Ringer Hepes (KRH) medium comprising 135 mmol/l NaCl, 3.6 mmol/l KCl, 10 mmol/l Hepes (pH 7.4), 0.5 mmol MgCl₂, 1.5 mmol/l CaCl₂, 0.5 mmol/l NaH₂PO₄, 2 mmol/l GlutaMAX, 2.5 mmol/l glucose and 0.2% (w/v) BSA. Medium was then replaced with fresh KRH and cells were incubated for 30 min on a shaking plate incubator (Labnet International, Oakham, UK) at 100 rpm. Next, supernatants were collected on ice and replaced with KRH containing 28 mmol/l glucose. In separate experiments, supernatants were replaced with KRH containing 50 mM KCl and 0.25 mmol/l diazoxide against a background of 2.5 or 28 mmol/l glucose. After another 30-min incubation, supernatants were collected on ice. Supernatants were centrifuged at 10,000g to pellet any detached cells and were then assayed for insulin by ELISA. Secreted insulin was normalised to cell density determined from DAPI fluorescence [15].

Mitochondrial respiration

Islets were hand-picked into XF24 islet capture plates (Seahorse Bioscience, North Billerica, MA) at 20-30 islets/well in 500 µl/well DMEM containing 5.5 mmol/l glucose and 2 mmol/l GlutaMAX. Islets were contained by inserting pre-wetted capture discs into the wells [18]. Following a 1-h incubation at 37 °C under air, plates were transferred to an XF24 analyzer. OCRs were measured at 37 °C in the absence and the cumulative presence of 28 mmol/l glucose, 10 µg/ml oligomycin and a mix of 2 µmol/l rotenone and 2 µmol/l antimycin A to determine, respectively, the glucose-sensitivity of cellular respiration, coupling efficiency of oxidative phosphorylation, and non-mitochondrial cellular oxygen uptake [13]. Basal respiration was calculated as the average of rate measurements 1-3 (see Figs 1G and 2G), glucose-stimulated respiration as measurement 10, oligomycin-insensitive respiration as the average of measurements 16-17, and non-mitochondrial respiration as the average of measurements 23-24. Non-mitochondrial OCRs were subtracted from other respiratory rates to calculate mitochondrial respiration. INS-1E cells were prepared for respiratory analysis as reported before [13].

Reactive oxygen species

Mitochondrial ROS levels were determined in INS-1E cells by monitoring MitoSOX oxidation as described before [13] \pm 20 μ mol/l MnTMPyP (Cayman Chemical, Cambridge Bioscience, UK).

Calcium

Intracellular Ca^{2+} was quantified in INS-1E cells using Fura-2AM (Molecular Probes, Invitrogen). Cells seeded and palmitate-exposed on 96-well plates were incubated in glucose-free KRH for 1 h and then loaded with 1 μ mol/l Fura-2AM for 40 min at 37 °C under air in the dark. Loaded cells were washed twice in glucose-free KRH and left in the dark for 20 min after which fluorescent products were measured in a multimode plate reader (PHERAstar FS, BMG Labtech). Total well fluorescence was detected in multi-chromatic well mode at $\lambda_{\text{ex/em}} = 380/510$ nm and $\lambda_{\text{ex/em}} = 340/510$ nm for the ion-free and ion-bound indicator, respectively. The plate reader's gain was set to 1500 and 1900 for 380/510 nm and 340/510 nm, respectively, and focal height was adjusted to 5.0 mm. Fluorescence was measured at 3-sec intervals for 2.5 min and was calibrated by sequential injection of 5 μ mol/l 4-bromo A-23187 (Life Technologies, UK) and 20 mmol/l EGTA to saturate and scavenge intracellular Ca^{2+} , respectively. $[\text{Ca}^{2+}]$ was calculated from the 340/380 fluorescence ratio [22].

Statistical analysis

Significance of mean differences was tested by unpaired Student's t-test (Figs 1-3, 7) or 2-way ANOVA (Figs 4-6) using GraphPad Prism Version 6.0 for Mac OS X (GraphPad software, San Diego, CA, USA). Data are presented as means \pm SEM.

RESULTS

Palmitate effects on insulin secretion precede mitochondrial dysfunction

Consistent with the literature [11,12,23], 24-hr palmitate exposure attenuates GSIS in isolated mouse islets (Fig. 1A). This GSIS impairment is accounted for by statistically non-significant changes in absolute insulin release, i.e., by a marginal secretory increase at 5.5 mmol/l glucose (Fig. 1B) and a somewhat larger decrease at 28 mmol/l (Fig. 1C). Importantly, palmitate has not altered islet respiration at this point in any way (Fig. 1G), which is reflected by unchanged absolute mitochondrial OCRs at 5.5 and 28 mmol/l glucose (Figs 1E and 1F, respectively). The islets' respiratory response to glucose (~2.5-fold increase in mitochondrial oxygen uptake) thus remains unaffected after 24-h palmitate exposure (Fig. 1D). After 48-h, both palmitate stimulation of insulin release at 5.5 mmol/l glucose (Fig. 2B) and inhibition at 28 mmol/l (Fig. 2C) reach statistical significance. Consequently, GSIS impairment is more prominent after 48-h (Fig. 2A) than after 24-h (Fig. 1A). Following prolonged exposure, palmitate has also lowered the islets' respiratory response to glucose (Fig. 2G), which is reflected by a relatively low glucose sensitivity of mitochondrial respiration (Fig. 2D). The statistically significant effect of palmitate on this internally normalized respiratory parameter is exclusively owing to a non-significant inhibition of the absolute mitochondrial OCR at 28 mmol/l glucose (Fig. 2F). Oxygen uptake at 5.5 mmol/l glucose remains unaffected by palmitate, even after 48-h exposure (Fig. 2E). This unchanged oxygen consumption is noteworthy as palmitate insensitivity of basal mitochondrial respiration appears at odds with the palmitate-induced stimulation of basal insulin release (Fig. 2B). Any differences in absolute mitochondrial oxygen consumption between incubation times should be interpreted with caution as different islet preparations were used for the respective experiments.

Glucose-stimulated oxygen consumption is lowered by 70 % \pm 3.6 % in control mouse islets when the ATP synthase is inhibited with oligomycin (Figs. 1G and 2G, closed symbols). Such inhibition demonstrates that roughly two-thirds of *total* islet respiration is used to drive ATP synthesis whilst the other one-third is associated with either mitochondrial proton leak or non-mitochondrial oxygen consumption. When corrected for non-mitochondrial respiration, it transpires that the proportion of

mitochondrial respiratory activity used to make ATP (coupling efficiency of oxidative phosphorylation) is not changed by palmitate, irrespective of exposure time (Figs 1H and 2H).

We have recently reported that palmitate impairs GSIS in INS-1E cells [13,14]. In line with our ‘48-h’ islet data (Fig. 2), palmitate-induced GSIS annulment in INS-1E cells (Fig. 3A) appears the combined result of augmented insulin release at basal glucose (Fig. 3B) and decreased release at 28 mmol/l glucose (Fig. 3C). Consequently, it seems that glucose attenuates rather than stimulates insulin secretion in palmitate-exposed cells, albeit non-significantly (*cf.* Fig. 5A). Concomitantly, palmitate dampens the glucose sensitivity of INS-1E mitochondrial respiration (Fig. 3D) by lowering absolute glucose-stimulated mitochondrial oxygen consumption (Fig. 3F). Importantly, however, palmitate leaves basal mitochondrial respiration unchanged (Fig. 3E). Palmitate-induced GSIS defects in INS-1E cells are thus disconnected, at least partly, from coinciding mitochondrial defects in oxidative phosphorylation. The cell data are consistent with our islet observations despite differences in exposure time and dose, which likely arise from differential palmitate accessibility.

Palmitate stimulation of basal insulin secretion

Palmitate-induced mitochondrial superoxide – Because mitochondrial ROS have been implicated in insulin secretion [7] and because palmitate increases mitochondrial superoxide in INS-1E cells [15], we assessed the effect of ROS scavenging on palmitate stimulation of basal insulin release (Fig. 4). In line with our recent findings [15], 24-h exposure of INS-1E cells to palmitate at high glucose increases the rate at which these cells oxidise MitoSOX (Fig. 4A), a mitochondria-targeted superoxide probe. MitoSOX oxidation in palmitate-exposed cells is lowered significantly by 20 $\mu\text{mol/l}$ MnTMPyP and is unaffected by this cell-permeant antioxidant [24] in control cells (Fig. 4A). Interestingly, MnTMPyP does not prevent palmitate stimulation of basal insulin secretion, but tends to amplify it a little (Fig. 4B). Although palmitate-induced mitochondrial superoxide is not annulled entirely by MnTMPyP (Fig. 4A), the comparably low superoxide level reached is below the threshold at which damage to INS-1E cells appears [14,15]. These data suggest that palmitate stimulation of basal insulin release is not mediated by increased mitochondrial superoxide. Note that palmitate does not increase cytoplasmic superoxide levels under the applied conditions [15].

GSIS amplification – Next, we explored possible involvement of non-canonical GSIS amplifying pathways [10] in the stimulatory palmitate effect. Evidence for GSIS amplification typically comes from a glucose-dependent augmentation of KCl-provoked insulin release in the presence of K_{ATP} channel opener diazoxide [25], a phenomenon that has indeed been reported in INS-1E cells [20]. At 2.5 mmol/l glucose and 0.25 mmol/l diazoxide, 50 mmol/l KCl stimulates insulin release by our control cells almost 4.5-fold – this stimulation is not increased further at 28 mmol/l glucose (Fig. 5A). Absolute KCl-induced insulin secretion, either at 2.5 or 28 mmol/l glucose, is not affected significantly by palmitate, and is indeed similar to the secretory rate provoked by palmitate alone (Fig. 5A). Diazoxide alone does not significantly affect basal insulin release, but it almost fully annuls insulin secretion at high glucose ($P < 0.001$) and tends to lower palmitate stimulation of basal insulin secretion (Fig. 5A). These data suggest a lack of GSIS amplification under our experimental conditions, which renders it unlikely that GSIS amplifying pathways are responsible for palmitate stimulation of basal insulin release.

GSIS potentiation, lipid oxidation and palmitoleate protection – Acutely, palmitate has no effect on insulin release at a basal glucose level, but augments insulin secretion at stimulatory glucose and thus potentiates GSIS [26,27]. It is unlikely, however, that a potentiation-like mechanism underlies palmitate stimulation of basal insulin release after 24 h, as the effect is independent of the glucose level during exposure – stimulation occurs both against a 4 and 11 mmol/l glucose background (Fig. 5B). Furthermore, inhibiting mitochondrial beta oxidation with etomoxir does not significantly affect this stimulation, at 4 or 11 mmol/l glucose (Fig. 5B), ruling out mechanistic involvement of palmitate oxidation. In line with the comparably harmless effects of monounsaturated NEFAs on beta cell behaviour [28] palmitoleate does not increase basal insulin secretion and indeed attenuates the palmitate-induced stimulation (Fig. 5C).

Calcium and insulin content – Ca^{2+} was measured in intact INS-1E cells with Fura-2AM, which was calibrated in individual experiments by sequential addition of the ionophore 4-bromo A23187 and EGTA (Fig 6A). Cells grown in fully supplemented RPMI contain $95 \text{ nmol/l} \pm 20 \text{ nmol/l}$ Ca^{2+} (Fig 6B), a level that is typical for insulinoma cells [29] and that appears somewhat increased (to $160 \pm 12 \text{ nmol/l}$) in serum-depleted, BSA-exposed control cells (Fig. 6B) without notable effect on insulin secretion (data not shown). Importantly, palmitate causes a further rise in Ca^{2+} to $210 \pm 15 \text{ nmol/l}$ (Fig 6B). This Ca^{2+} increase suggests that palmitate stimulation of basal insulin secretion is linked to exocytotic events [30] and is not simply owing to insulin leakage from dead or damaged cells [16]. The insulin content of INS-1E cells is not affected significantly by palmitate, although the average level tends to be increased (Fig 7A). In mouse islets, on the other hand, 48-h palmitate exposure more than halves insulin content when levels are normalised to DNA (Fig 7B).

DISCUSSION

Real-time functional analysis of cellular bioenergetics has become relatively common over the last decade or so [17], but the application of XF technology to probe mitochondrial respiratory activity of isolated pancreatic islets has, to our knowledge, been limited to little more than a handful of studies [9,18,27,31-33]. The results reported in this paper highlight the strength of functional islet respiratory measurements as they provide novel insight in mitochondrial engagement with pancreatic beta cell glucolipotoxicity (Fig. 8). Our findings reveal that palmitate-induced GSIS impairment is disconnected from coincident mitochondrial dysfunction. Furthermore, the data substantiate published palmitate effects on basal insulin release and provide further clues as to the molecular nature of such effects.

Mitochondrial involvement in beta cell glucolipotoxicity

It is well established that prolonged exposure of pancreatic beta cells to elevated levels of glucose and NEFAs is detrimental to the function and viability of these cells [34]. Possible molecular defects that underlie glucolipotoxic beta cell death and secretory dysfunction are in abundance, but the relative importance of the multifarious fatty-acid-induced cellular defects remains unclear, as indeed do their causal relations. Glucolipotoxicity likely influences the bioenergetics of beta cells since UCP2 has been reported to mediate palmitate-induced insulin secretory defects in mice and isolated islets [35,36]. A proposed protective role against oxidative stress [37], on the other hand, would predict that UCP2 activity ameliorates glucolipotoxic damage instead. Studying INS-1E cells, we have shown that palmitate triggers ROS generation and oxidative phosphorylation defects, functional mitochondrial changes that coincide with cell loss and attenuated GSIS [13]. In our hands, UCP2 is not involved with mitochondrial superoxide-mediated cell loss [15] or with the mitochondrial respiratory defects that correlate with GSIS impairment [14]. The INS-1E cell respiratory data shown in Fig. 3 are consistent with our previous reports [13,14] in so much that the deleterious palmitate effect on the *normalised* mitochondrial respiratory response to glucose - i.e., an intrinsic mitochondrial defect (Fig. 3D) - is echoed by a similar negative effect of palmitate on the normalised response of insulin secretion to glucose (Fig. 3A). In mouse islets exposed to palmitate for 48 h, the situation is the same: impaired GSIS (Fig. 2A) correlates with a decreased normalised glucose respiratory response (Fig. 2D). Dissection of these internally normalised parameters into their absolute components, however, sheds important new light on these apparent relations between mitochondrial respiratory dysfunction and impaired GSIS. Both in islets and cells, significant increases in basal insulin release (Figs 2B and 3B, respectively) contribute to palmitate-impaired GSIS, whilst basal mitochondrial respiration is unaffected by palmitate in both systems (Figs 2E and 3E, respectively). Disconnection between palmitate effects on mitochondrial respiration and insulin secretion is further evident when islets are exposed to palmitate for only 24 h after which GSIS impairment (Fig 1A) emerges without any sign of mitochondrial respiratory dysfunction (Figs 1D-H). Similarly, 48-h exposure to palmitoleate, the monounsaturated, less harmful equivalent of palmitate, impairs islet GSIS without change in glucose-stimulated mitochondrial respiration (data not shown). In INS-1E cells, palmitoleate prevents a significant

increase in basal insulin release by palmitate (Fig. 5C) but does not affect absolute basal mitochondrial respiration, either with or without palmitate [13].

Mechanism by which palmitate increases basal insulin release

Stimulatory effects of palmitate on basal insulin secretion (Figs 2B and 3B) are well documented in cells [38] and in rodent [11,39] and human [12] islets. Importantly, individuals suffering from Type 2 diabetes also exhibit elevated basal insulin release [40]. Although the phenomenon is thus not new by any means, its molecular nature has not been established firmly. Our results render a metabolic origin of the palmitate effect unlikely as stimulation of basal insulin release is not caused by boosted mitochondrial ATP production (Figs 1-3), increased fatty acid oxidation (Fig. 5B), heightened superoxide formation (Fig 4), or by GSIS amplification/potentialisation (Fig 5). As palmitate increases basal Ca^{2+} (Fig 6), stimulated insulin release is not owing to non-specific leakage from damaged or dying cells, but more likely to altered electrical activity of beta cells. Indeed, palmitate effects on K_{ATP} and voltage-gated Ca^{2+} channels have been reported [23,30,39]. Diazoxide tends to partially reverse palmitate stimulation of basal insulin secretion (Fig 5A), which would be consistent with a direct action on K_{ATP} channels given the lack of palmitate effect on oxidative phosphorylation at basal glucose (Fig 3E). An additional direct action on Ca^{2+} channels cannot be excluded at present.

Mitochondrial activity as biomarker

In light of islet cell transplantation as promising treatment for Type 1 diabetes patients, the need for reliable assays ascertaining functional islet integrity prior to transplantation is increasing [16]. Assays that evaluate mitochondrial function show much promise in this respect [16] and the islet OCR, in particular, correlates positively with transplant outcomes in mice and pigs when normalised to islet size [41], and, without such normalisation, in humans too [42]. Bioenergetic parameters correlate generally much better with clinical transplant outcome than GSIS measures [16]. Our data may explain this perhaps surprising 'prediction discrepancy' as they demonstrate that mitochondrial oxygen consumption is not proportional to insulin secretion under all circumstances. For example, 'early' palmitate-induced GSIS impairment in isolated mouse islets (Fig 1A) is not foreshadowed by any respiratory changes (Figs 1D-H). As mitochondrial defects may equally arise without negative consequences for GSIS [33], we feel some caution is due when using 'bioenergetic health' as sole predictor of islet transplantation outcome.

Concluding remarks

Although mitochondria are central to the main role of pancreatic beta cells, i.e., coupling glucose sensing to insulin release, our results show that altered mitochondrial function does not necessarily predict GSIS changes. More work is needed to identify the roots of glucolipotoxic defects in oxidative phosphorylation reported here and elsewhere, to identify the molecular nature of the lipid species responsible for such defects, and to clarify their consequences for beta cell physiology.

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AUTHOR CONTRIBUTIONS

JB and CA conceived and developed the project idea; JB, VH-J, CA and MJ designed all experiments; JB and VH-J executed and analysed all experiments; JB, CA and MJ wrote the paper. As guarantor, CA takes responsibility for the contents of this paper.

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FIGURES

Fig 1.

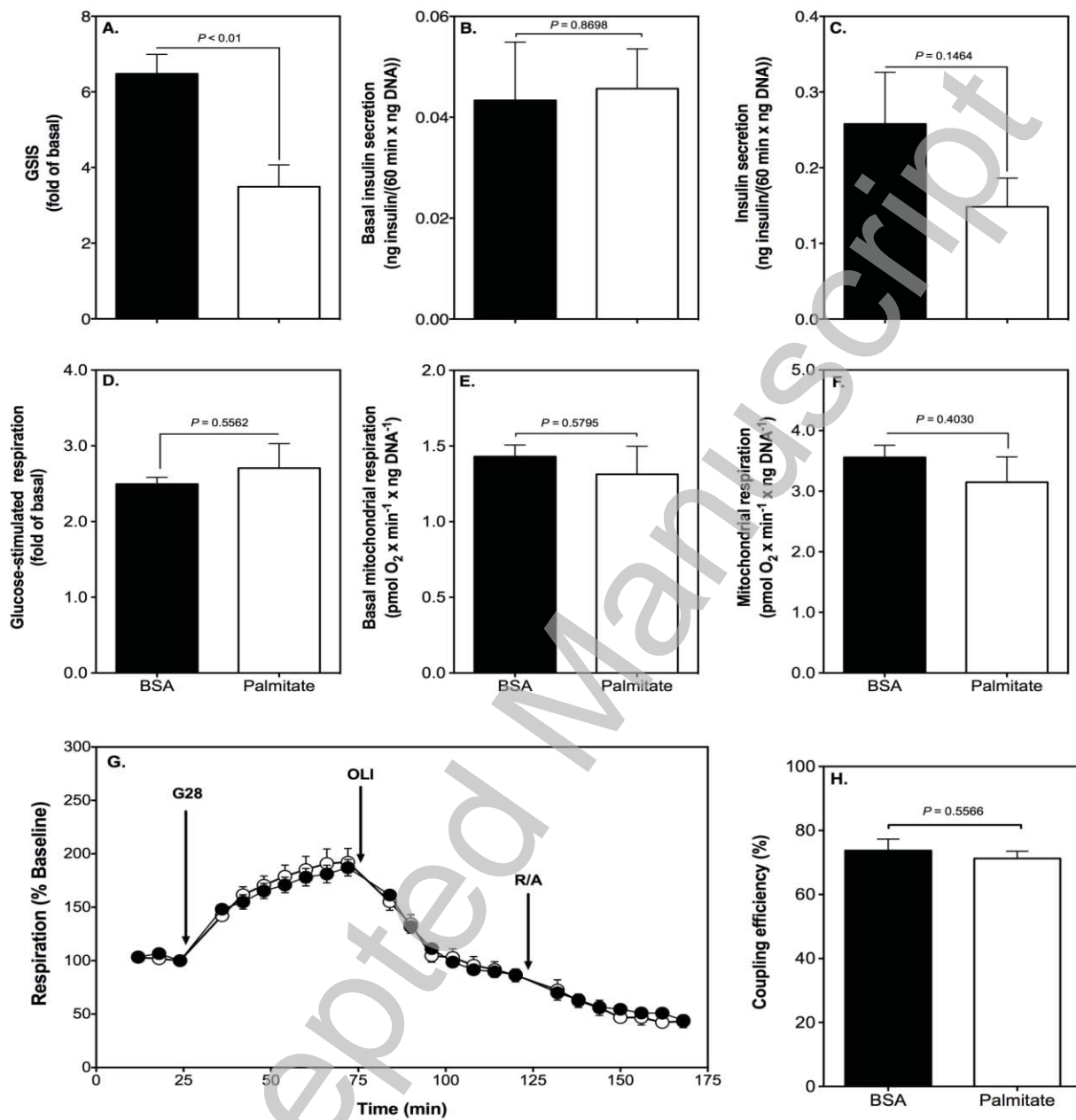


Fig 2.

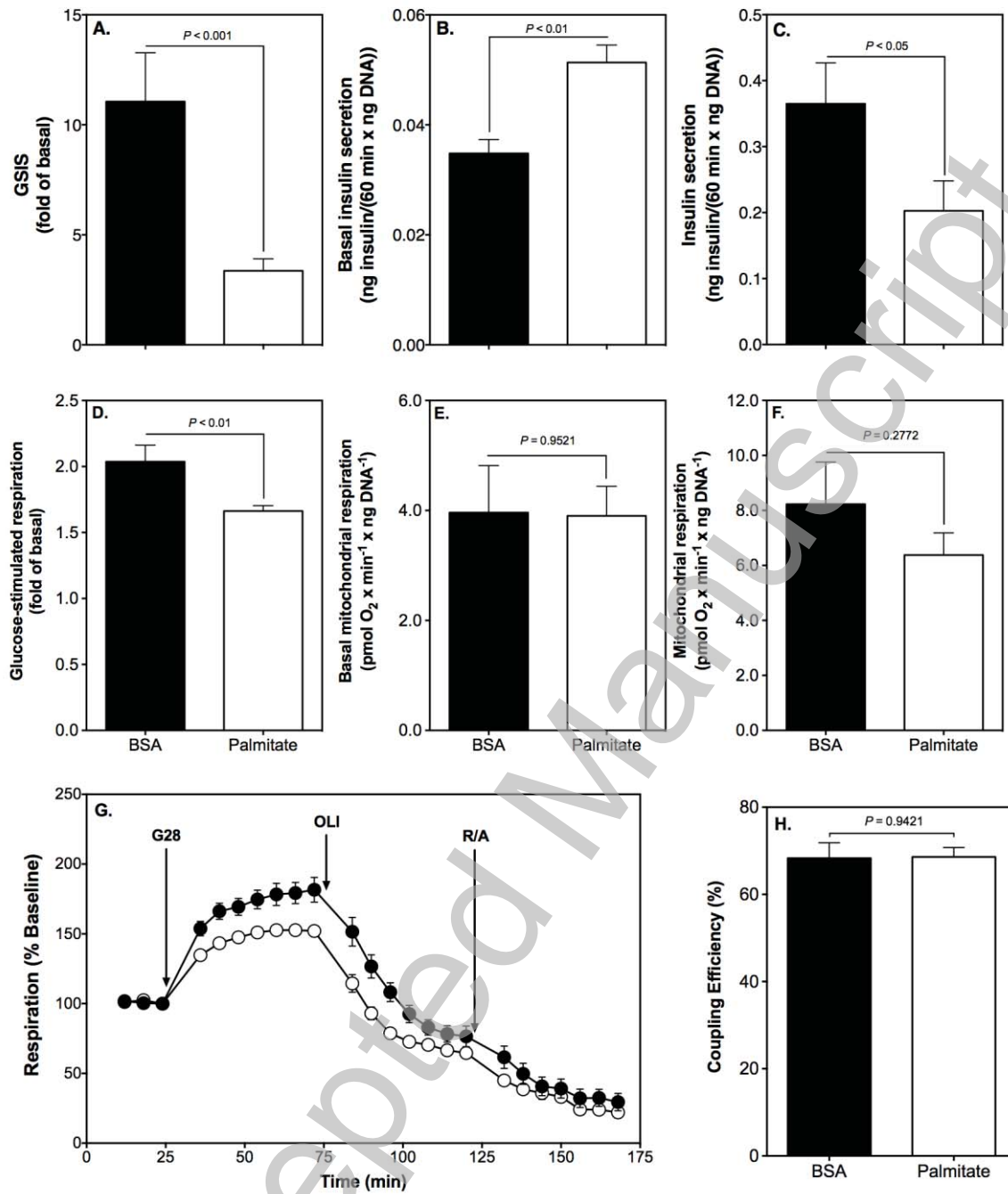


Fig 3.

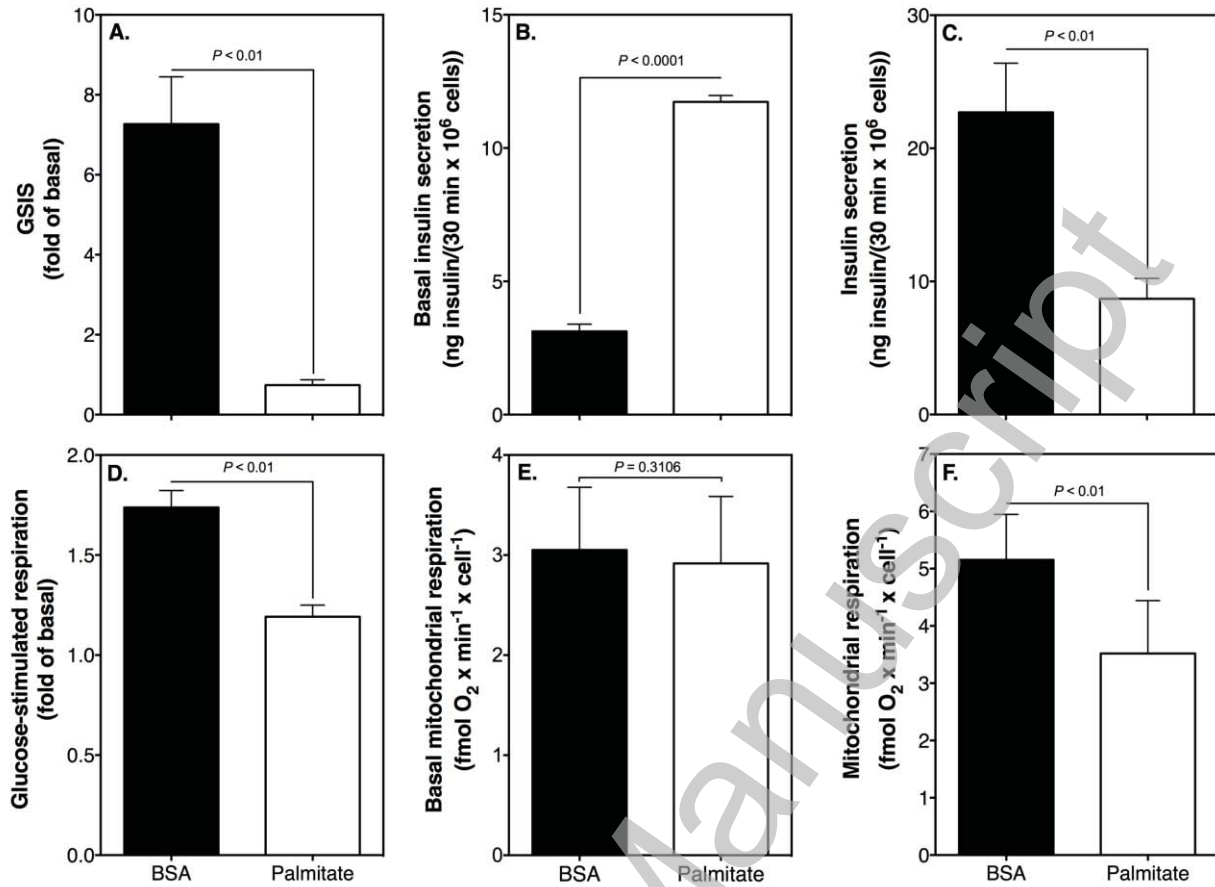


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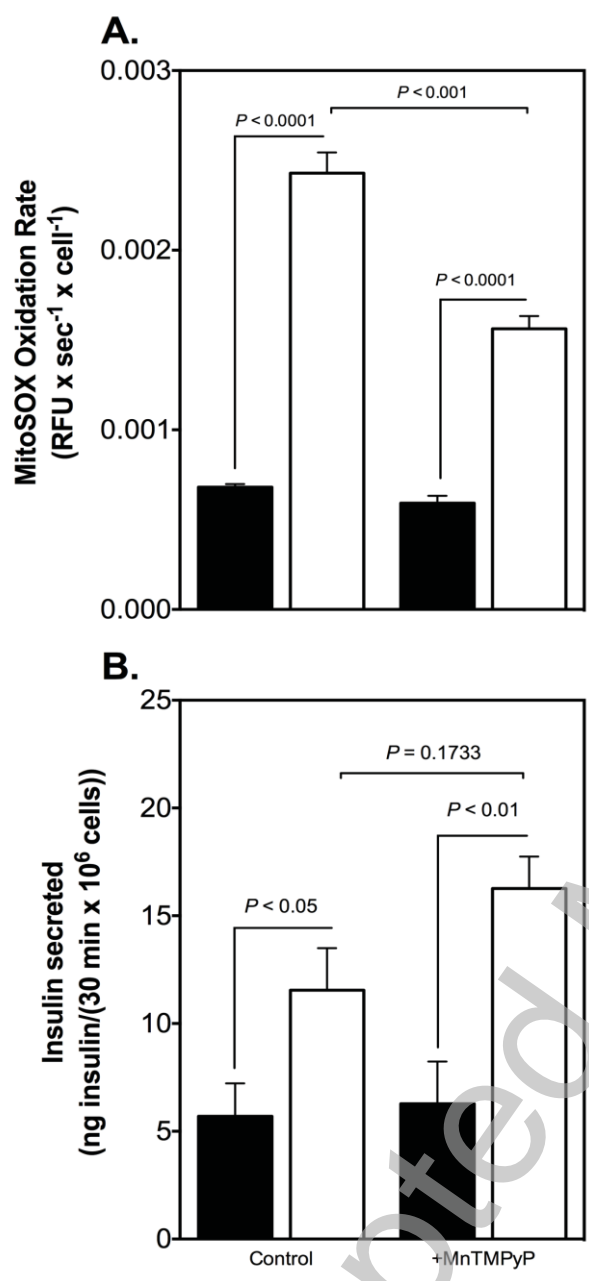


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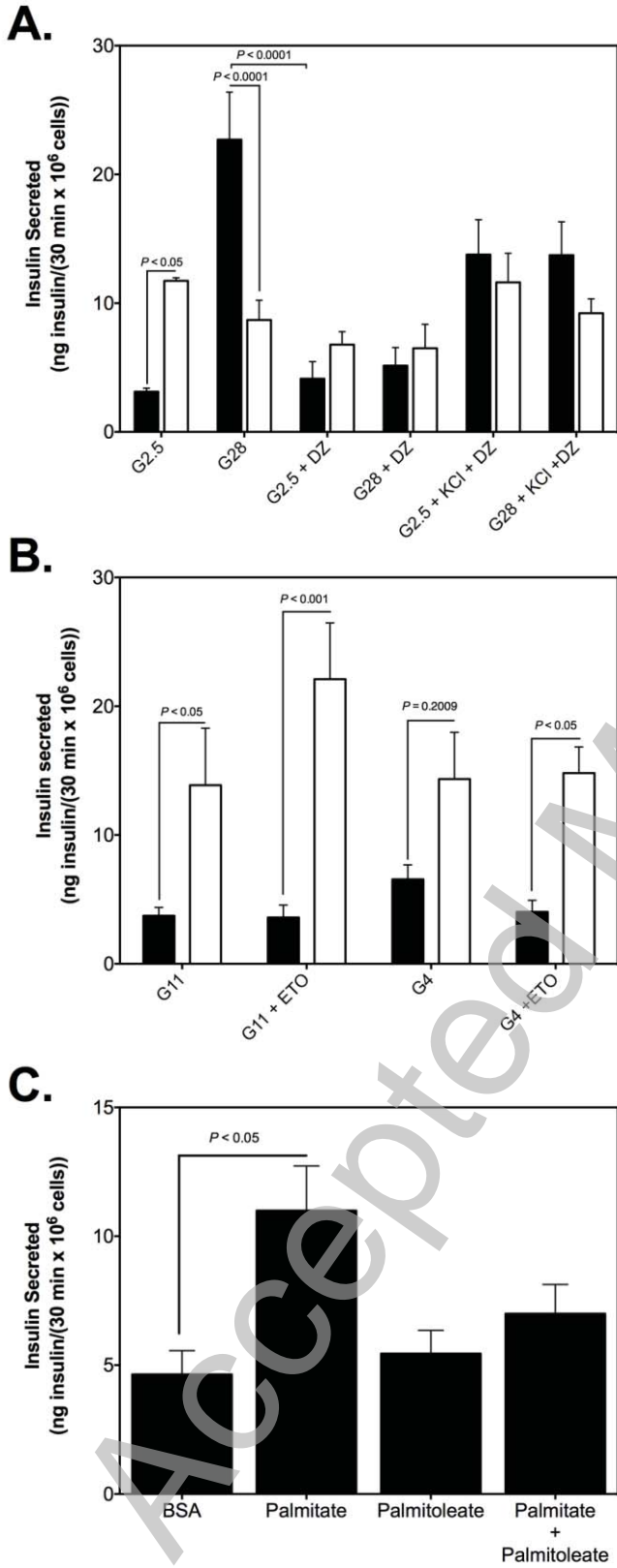


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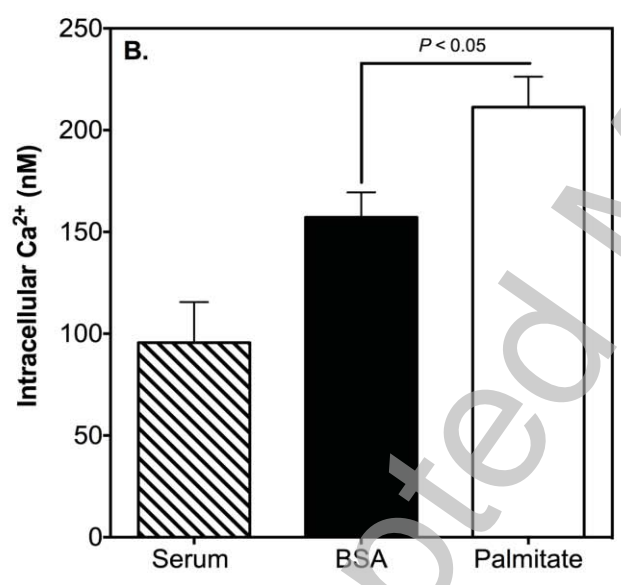
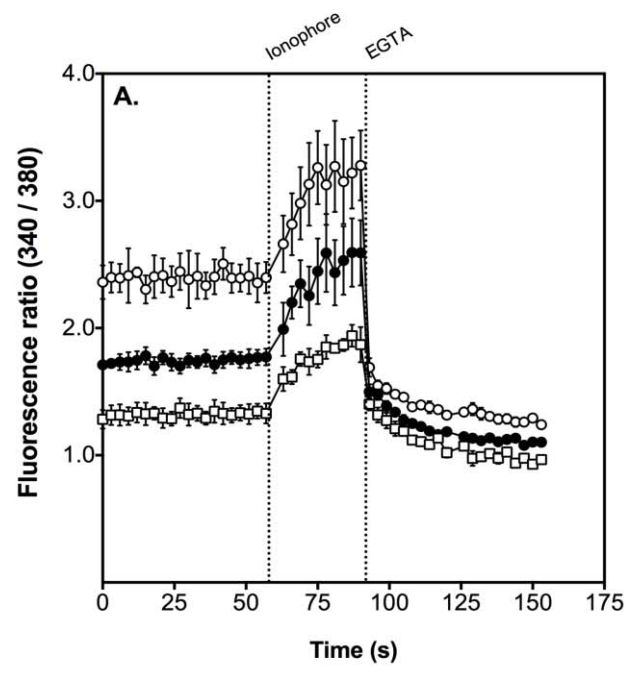


Fig 7.

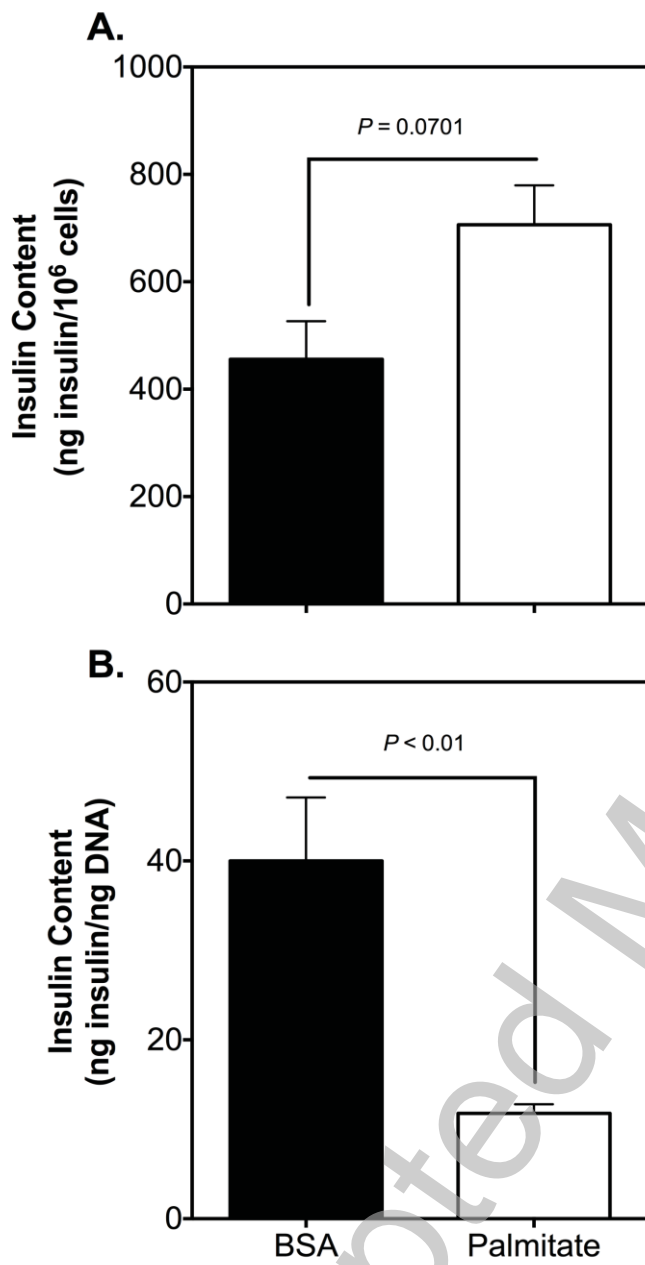
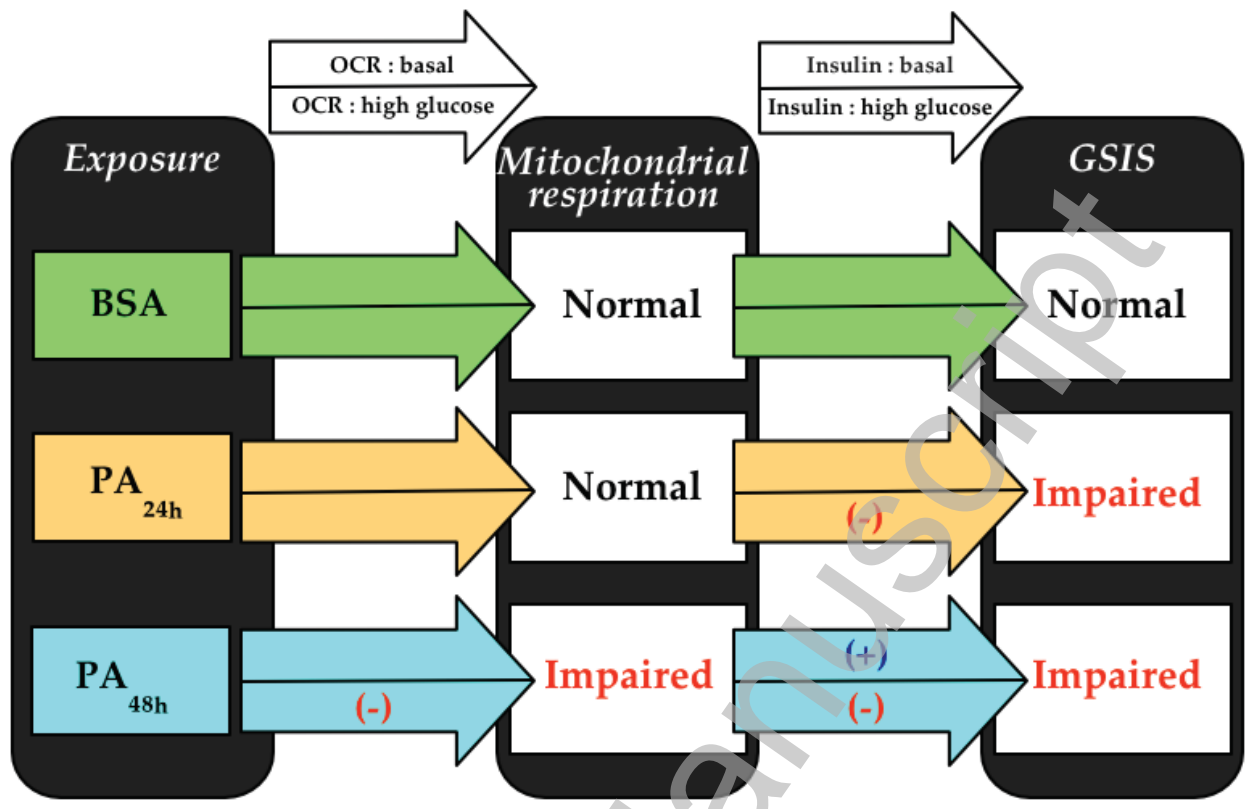


Fig 8.



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LEGENDS

Fig.1: 24-h palmitate exposure impairs islet GSIS without sign of mitochondrial dysfunction.

Insulin secretory and mitochondrial respiratory responses to glucose were normalised, respectively, to basal insulin secretion (A) and basal mitochondrial oxygen consumption (D). Absolute insulin secretion and mitochondrial respiratory rates were determined at both 5.5 (B and E) and 28 mmol/l (C and F) glucose by normalising rates to DNA content. As described in the Methods, respiratory rates and coupling efficiencies (H) were calculated from extracellular flux traces (G) in which glucose (G28), oligomycin (OLI), and a mixture of rotenone and antimycin A (R/A) were added as indicated. Open and closed symbols: islets exposed to palmitate or BSA, respectively (n = 7-13 wells from 3-4 assays).

Fig.2: 48-h palmitate exposure stimulates basal islet insulin secretion without effect on basal mitochondrial respiration.

Insulin secretory and mitochondrial respiratory responses to glucose were normalised, respectively, to basal insulin secretion (A) and basal mitochondrial oxygen consumption (D). Absolute insulin secretion and mitochondrial respiratory rates were determined at both 5.5 (B and E) and 28 mmol/l (C and F) glucose by normalising rates to DNA content. As described in the Methods, respiratory rates and coupling efficiencies (H) were calculated from extracellular flux traces (G) in which glucose (G28), oligomycin (OLI), and a mixture of rotenone and antimycin A (R/A) were added as indicated. Open and closed symbols: islets exposed to palmitate or BSA, respectively (n = 7-13 wells from 3-4 assays).

Fig.3: Palmitate-induced GSIS impairment is disconnected from mitochondrial dysfunction in INS-1E cells.

Secretory and respiratory responses to glucose were normalised, respectively, to basal insulin secretion (A) and basal mitochondrial oxygen uptake (D). Absolute insulin secretion and mitochondrial respiratory rates were determined by normalising the respective activities to INS-1E cell number both in the absence (B and E) and the presence (C and F) of 28 mmol/l glucose. Open and closed bars: 24-h exposure to palmitate or BSA, respectively, at 11 mmol/l glucose (n = 3-4).

Fig.4: Effect of palmitate on basal insulin release by INS-1E cells is unaffected by scavenging ROS.

MitoSOX oxidation (A), measured as time-dependent increase in relative fluorescence units, RFU, and insulin secretion (B) were normalised to cell number and measured \pm 20 μ mol/l MnTMPyP. Open and closed bars: 24-h exposure to palmitate or BSA, respectively, at 11 mmol/l glucose (n = 3-4).

Fig.5: Palmitate stimulation of basal insulin release by INS-1E cells does not involve GSIS amplification, GSIS potentiation or lipid oxidation, but is attenuated by palmitoleate.

Cells were exposed for 24 h to palmitate (open bars in panels A and B), palmitoleate \pm palmitate, or BSA (black bars in panels A and B.) at 11 (panels A and C), or at 4 or 11 mmol/l glucose (panel B – G4 and G11, respectively) \pm 0.2 mmol/l etomoxir (ETO). After 1-h glucose starvation, insulin secretion was measured at 2.5 (panels B and C), or at 2.5 or 28 mmol/l glucose (panel A – G2.5 and G28, respectively). Diazoxide (DZ) and KCl were included in the assay at 0.25 and 50 mmol/l, respectively, as indicated. Insulin secretion rates were normalised to cell number (n = 3-8).

Fig.6: Palmitate increases the basal calcium level of INS-1E cells.

Intracellular calcium levels (B) were calculated from background-corrected Fura-2 AM fluorescence intensity ratios (A – 340/380 nm excitation) in INS-1E cells grown in fully-supplemented RPMI ('serum', open squares), or in serum-deprived cells exposed for 24 h to palmitate or BSA (open and black circles, respectively) at 11 mmol/l glucose. Cells were loaded with 1 μ mol/l Fura-2 AM and fluorescence was measured at 3-sec intervals for 3 min. Average fluorescence ratios measured over the first 60 sec of the assay were calibrated by sequential addition of 5 μ mol/l 4-bromo A-23187 ('ionophore') and 20 mmol/l EGTA in every assay to saturate and deplete calcium levels, respectively (n = 6).

Fig.7: Effect of palmitate on insulin content. INS-1E cells (A) and mouse islets (B) were exposed for 24 h and 48 h, respectively, to palmitate or BSA (open and black bars, respectively) at 11 mmol/l glucose (n = 2-3). Values were normalised to cell number (A) or DNA content (B).

Fig.8: Palmitate-induced GSIS impairment is disconnected from mitochondrial respiratory dysfunction. Control islets (BSA) exhibit 'normal' mitochondrial respiration and GSIS phenotypes as they increase both their mitochondrial OCR and insulin secretion rate in response to high glucose. 24-h palmitate exposure (PA_{24h}) impairs GSIS exclusively because of a lowered insulin secretion at high glucose - mitochondrial respiration is unaffected at this point. 48-h palmitate exposure (PA_{48h}) impairs both mitochondrial respiration and GSIS because of a dampened glucose response. GSIS impairment results in part from palmitate-induced stimulation of basal insulin release - the basal mitochondrial OCR remains unaffected by palmitate.

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