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Basic Science

Conditioned media from contracting skeletal muscle potentiates insulin secretion and enhances mitochondrial energy metabolism of pancreatic beta-cells

Jonathan Barlow [a,*], Thomas P.J. Solomon [a,b]

[a] School of Sport, Exercise and Rehabilitation Sciences, University of Birmingham, UK
[b] Institute for Metabolism and Systems Research (IMSR), University of Birmingham, UK

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ABSTRACT

Aims/Hypothesis: In this study, we aimed to examine real-time effects of molecules released by contracting skeletal muscle on the insulin secretory function of β-cells using a novel perifusion platform. We hypothesised that media conditioned by contracting skeletal muscle will influence insulin secretion and mitochondrial energy metabolism in β-cells under normal and type-2 diabetic conditions.
Methods: INS-1 832/3 pseudoislets were perifused with media from C2C12 myotubes treated with or without electrical pulse stimulation (EPS; 40 V, 1.0 Hz, 2 ms). Insulin secretory function of pseudoislets was measured before, during, and after EPS to simulate pre-, during-, and post-exercise like effects. Additional experiments were completed in INS-1 832/3 cells under "healthy" and "diabetic-like" conditions as well as human pancreatic islets isolated from nondiabetic and type 2 diabetic donors.
Results: Insulin secretion increased significantly ($P < 0.05$) by pseudoislets when perifused with media from myotubes treated with but not without EPS. Conditioned media from EPS-treated myotubes also potentiated insulin secretion from INS-1 832/3 cell monolayers in the presence ($P < 0.05$) and absence of palmitate ($P < 0.001$) and in nondiabetic ($P < 0.01$) and type-2 diabetic ($P = 0.06$) isolated human islets. Pre-treatment of INS-1 832/3 cells to 24-hour high glucose ± palmitate dampened this effect. Moreover, conditioned media from myotubes treated with EPS significantly increased mitochondrial respiratory activity of INS-1 832/3 cells.
Conclusion/Interpretation: Conditioned media from myotubes treated with EPS potentiates acute insulin release from normal cultured β-cells, nondiabetic islets and Type-2 diabetic islets and is associated with enhanced mitochondrial substrate oxidation.

1. Introduction

Accelerated rates of prediabetes and T2D are far outpacing preventative measures. Importantly, increased dysregulated blood glucose is paralleled by an increase in global obesity, with 1 in 3 adults classified as overweight and 1 in 10 defined as obese [1]. Many cases of prediabetes and T2D are linked to poor lifestyle and physical inactivity, and regular exercise is essential for the prevention and treatment of T2D [2]. In patients with T2D and prediabetes, exercise improves peripheral insulin sensitivity and pancreatic beta-cell (β-cell) function [3–7]. Given that the demise of β-cell function is a key determinant in the development of T2D [8], understanding the molecular mechanisms linking exercise to improved β-cell function is of significant interest.

Since the discovery that skeletal muscle is an endocrine organ that secretes a variety of molecules capable of regulating the metabolic function of other tissues and organs [9,10], over the last decade, the concept of inter-organ communication involving skeletal muscle has developed. This discovery set a new paradigm, and more recently, the idea that skeletal muscle influences the function of β-cells through the secretion of muscle-derived molecules has emerged [11]. Given that muscle contraction is a key stimulus for the release of skeletal muscle-derived molecules [12], a means for skeletal muscle to β-cell crosstalk is likely and may underlie the exercise benefits associated with β-cell function in patients with prediabetes and T2D [13].

Evidence exists to support a role of muscle-secreted factors in influencing skeletal muscle to β-cell crosstalk in T2D. For example, conditioned media collected from TNF-α-induced insulin resistant skeletal muscle cells impairs glucose-stimulated insulin secretion (GSIS) from isolated human pancreatic islets [14]. Furthermore, serum obtained

Abbreviations: EPS, electrical pulse stimulation; T2D, type 2 diabetes; BAM 15, N5, N6-bis(2-fluorophenyl)[1,2,5]oxadiazolo[3,4-*b*]pyrazine-5,6-diamine; DMEM, Dulbecco's modified Eagle's Medium; RPMI, Roswell Parks Memorial Institute; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); β-cell, pancreatic beta-cell; BSA, bovine serum albumin; GSIS, glucose-stimulated insulin secretion; DAPI, 4′,6-diamidino-2-phenylindole.

* Corresponding author at: School of Sport, Exercise and Rehabilitation Sciences, University of Birmingham, B15 2TT, UK.
E-mail address: j.p.barlow@bham.ac.uk (J. Barlow).

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from blood of exercise trained mice protects against pro-inflammatory cytokine-induced apoptosis [15]. Moreover, we show that exercised human serum lowers accumulated basal insulin release from normal INS-1 cells [16]. Until now all previous muscle to β-cell crosstalk work has been limited to static incubation conditions, which are confounded by accumulated levels of released humoral factors that often yield supraphysiologic concentrations. Moreover, it remains unclear if acute skeletal muscle contraction mediates "real-time" crosstalk effects on β-cell function under type 2 diabetic-like conditions. Therefore in this study, we examined to what extent conditioned media from electrical pulse stimulated (EPS) C2C12 skeletal muscle myotubes influences real-time insulin release by INS-1 832/3 pseudoislets using a novel perifusion platform (Fig. 1). We also explored if conditioned media collected from myotubes treated with EPS influences the function of INS-1 832/3 cells exposed to type-2 diabetic-like conditions as well as isolated nondiabetic and type-2 diabetic human pancreatic islets. To understand how conditioned media collected from myotubes treated with EPS may influence insulin secretory function of β-cells we finally examined mitochondrial energy metabolism in INS-1 832/3 cells exposed to C2C12 conditioned media ± EPS.

2. Material and Methods

2.1. Cell Culture

2.1.1. INS-1 832/3 Pseudoislets

Rat INS-1 832/3 insulinoma cells (RRID: CVCL_0351), were maintained in RPMI-1640 medium as described previously [17], cultured and seeded into cell repellent 96-well U-bottomed plates (Greiner bio-one, Kremsmünster, Austria #650970) at a density of 2×10^3 cells/well. To allow for pseudoislet formation, cells were incubated at 37 °C with 5% CO_2 for 24 h and then washed into fresh RPMI-1640 culture media supplemented with 5 mM glucose. After another 24 h incubation at 37 °C with 5% CO_2, single pseudoislets which had formed in each well were pooled together and used for experiments as described in Section 2.2. Cell passages between 30 and 38 were used for experimentation. Confirmation of insulin responsiveness to glucose was confirmed in monolayer control cells over these passages by acute insulin release as described in Section 2.5 (Fig. S4).

2.1.2. INS-1 832/3 Monolayers

Rat INS-1 832/3 insulinoma cells (RRID: CVCL_0351), maintained in RPMI-1640 medium as described previously [17] were cultured and seeded at a density of 1×10^5 cells/well in 96-well microplates. At 75–85% confluence, cells were treated with RPMI-1640 medium containing 5 or 20 mM glucose supplemented with ±bovine serum albumin (BSA) conjugated palmitate and incubated at 37 °C with 5% CO_2 for 24 h. Palmitate was conjugated to BSA at a molar ratio of 5:1 (8 mM PA: 1.6 mM BSA) and was diluted 40-fold in culture medium yielding a total free fatty acid concentration of 200 μM and an estimated free fatty acid level of 20 nM assuming binding parameters reported by Huber et al. (2006) [18]. FBS was omitted from growth medium for palmitate experiments and BSA (40 μM) was used as vehicle control. Cell passages between 30 and 38 were used for experimentation. Confirmation of insulin responsiveness to glucose was confirmed in monolayer control cells over these passages by acute insulin release as described in Section 2.5 (Fig. S4). Moreover we can confirm that no significant differences ($P = 0.156$) in cell density were observed in cells cultured for 24 h in 5 or 20 mM glucose (data not shown).

2.1.3. C2C12 Myotubes

Mouse skeletal muscle C2C12 myoblasts (CRL-1772) were maintained in Dulbecco's Modified Eagle's Medium as described previously [17]. For differentiation to myotubes, cells seeded at a density of 2×10^5 cells in a total volume of 800 μL DMEM on 25 mm ⌀ tissue culture coverslips (Sarstedt, Nümbrecht, Germany, #83.1840) were placed in individual wells of a 6-well microplate. Cells were incubated at 37 °C with 5% CO_2 for 30 min to allow for cell adherence. At this stage, 2.2 mL of culture media was added to each well and plates were incubated for a further 24 h at 37 °C with 5% CO_2. At 85–95% confluence (~24 h post seeding), culture medium was exchanged with 8 mL/well differentiation medium [17]. Differentiation medium was replaced every 48 h until myotubes had formed. Visual inspection using an inverted light microscope indicated that complete differentiation took 5 days. Cell passages between 8 and 15 were used for experimentation.

2.2. Perifusion of C2C12 Skeletal Muscle Cell Media ± EPS and INS-1 832/3 Pseudoislet Insulin Secretion Kinetics

Coverslips containing C2C12 myotubes as described in Section 2.1.3 were transferred to a multi-welled reinnervate perfusion plate (AMSBIO, #AVP011, Cambridge, UK) and incubated in 3 mL/well Krebs Ringer HEPES buffer (KRH) [18]. During this incubation step, 90 pseudoislets were collected from harvested stocks as described in Section 2.1.1 and placed into 200 μL KRH before pipetting into a Swinnex 13 mm filter holder (Merck Millipore, Darmstadt, Germany) fitted with a 0.1 μM mesh. KRH with 5 mM glucose was pumped continuously through 2 paralleled EPS-electrode (IonOptix, Westwood, USA) containing reinnervate perfusion plates with C2C12 myotubes connected to 2 paralleled pseudoislet-containing chambers at a flow rate of 0.5 mL min^{-1} using a peristaltic pump (ISMATEC REGLO ICC Peristaltic Pump, Channel: 4, Cole-Parmer, St. Neots, UK) and pharmed

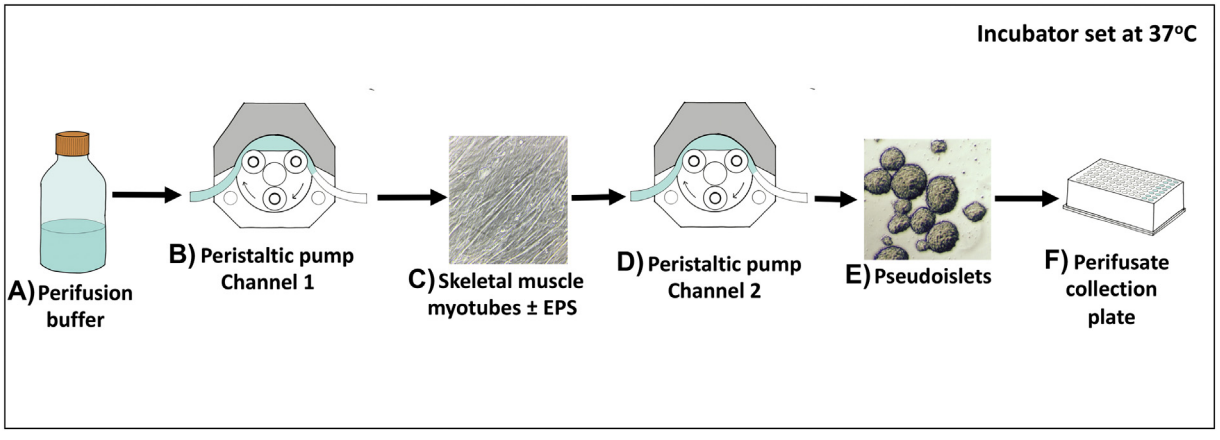

Fig. 1. Schematic of continuous perifusion platform for the assessment of skeletal muscle to pancreatic beta-cell crosstalk. Perifusion buffer (A) is transferred to C2C12 cultured skeletal muscle myotubes ± EPS (C) using peristaltic pumping from channel 1 (B). Perifusate solution from C2C12 myotubes (C) is pumped directly to INS-1 832/3 pseudoislets (E) using a second peristaltic pumping channel (D). Perifusate from pseudoislets (E) is collected into masterblock 96-well plates (F). Collected perifusate (F) is stored at −80 °C and later assessed for insulin. Electrical pulse stimulation (EPS).

ismaprene ISMATEC tubing (ID: 1.52 mm, OD: 3.02 mm, Cole-Parmer, St. Neots, UK). The perifusion setup was placed in a bench top incubator (Stuart SI60D) to maintain a constant temperature of 37 °C. For clarity, a schematic of this setup is presented in Fig. 1. Following a 32-min wash, perifusate was collected in 4-min intervals for 216 min. EPS of C2C12 myotubes was initiated as described in the legends of Fig. 2. Perifusate fractions were collected using an automated fraction collection system (OMNICOLL, Lambda Laboratory Instruments, Baar, Switzerland) into 96-well MASTERBLOCK plates (Greiner bio-one) and at the end of the experiment stored at −80 °C prior to being assayed for insulin by homogeneous time-resolved fluorescence (#62IN1PEG, Cisbio Bioassays, Codolet, France). Secreted insulin was normalized to DNA content as determined from PicoGreen assessment of pseudoislet lysates prepared in RIPA lysis buffer using Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies).

2.3. Collection of C2C12 Myotube Conditioned Media ± EPS

Coverslips containing C2C12 myotubes were transferred to a multi-welled reinnervate perfusion plate as described in Section 2.2 and incubated in 3 mL/well KRH supplemented with 5 or 20 mM glucose for 1 h at 37 °C under air. Next, KRH with 5 mM or 20 mM glucose was pumped continuously through 2 paralleled EPS-electrode (IonOptix, Westwood, USA) containing reinnervate perfusion plates with C2C12 myotubes at a flow rate of 0.5 mL min^{-1} using a peristaltic pump and pharmed ismaprene ISMATEC tubing. The perifusion setup was placed in a bench top incubator (Stuart SI60D) to maintain a constant temperature of 37 °C. Following a 32-min wash, perifusate was continually pumped through both perfusion plates for a further 32 min at which stage EPS (40 V, 1 Hz, using 2 millisecond pulses; C-Pace EP, IonOptix, Westwood, USA) was initiated for 64 min in one of the two perfusion plates. After this period, perifusion was maintained without EPS for 120 min. During this time perifusate was collected into 50 mL Falcon tubes placed on ice. In myotubes treated with EPS, muscle contraction was confirmed by visual inspection using an inverted light microscope. The EPS protocol of 40 V, 1 Hz, with 2 millisecond pulses used for our experiments is widely accepted as a model for in vitro exercise without any significant loss of cell integrity [19,20] and mimics aspects of muscle contraction, for example, enhanced 2-deoxyglucose glucose uptake (Fig. S1). Collected myotube culture medium was filter-sterilised using 0.45 μM Millipore syringe filters, aliquoted into 1.5 mL volumes and stored at −80 °C until used for INS-1 832/3 cell and human islet functional experiments.

2.4. Glucose Uptake

2-Deoxyglucose (2DG) uptake was measured as described previously by [21]. Briefly, C2C12 myotubes treated with or without EPS for 1 h (cf. Section 2.3) were washed and incubated in serum free DMEM for 2 h at 37 °C. Cells were then incubated for 20 min in Krebs Ringer Bicarbonate buffer supplemented with 2-Deoxyglucose and then washed twice with phosphate buffered saline before lysis in 0.1 N NaOH. Lysates were solubilised with 0.1 N HCl and transferred to a 96-well plate containing assay reaction medium supplemented with resazurin. 2DG-dependent reduction of resazurin to the fluorescent resorufin

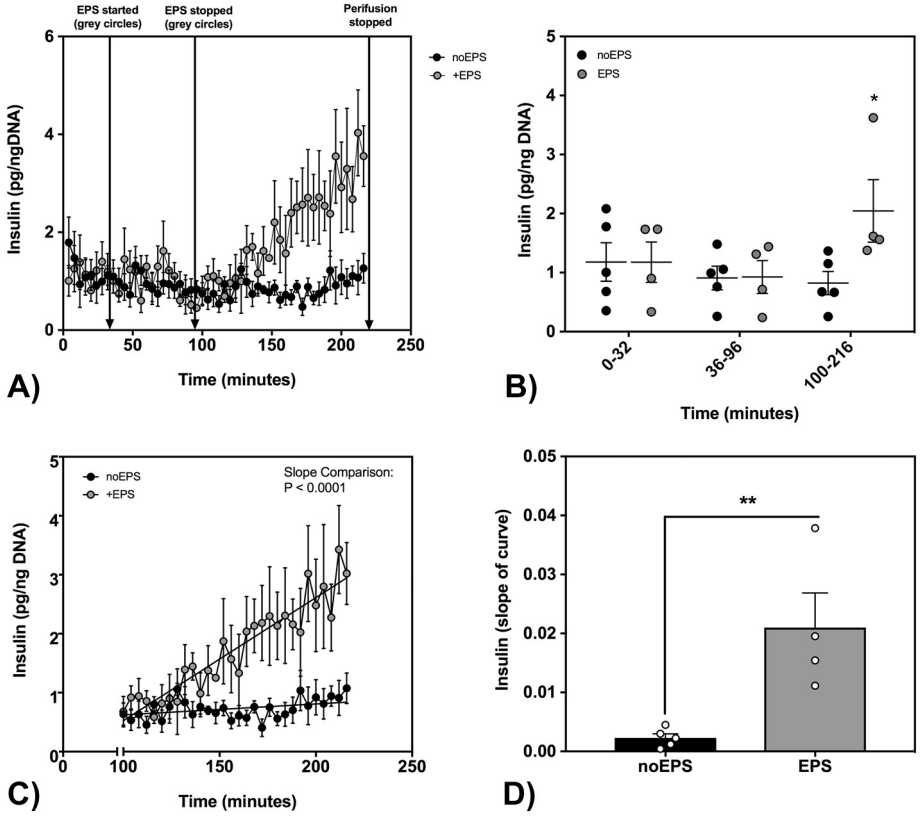

Fig. 2. Real-time effect of conditioned media from C2C12 myotubes treated with or without EPS on insulin secretion by INS-1 832/3 pseudoislets. Pseudoislets cultured in RPMI media containing 5 mM glucose were assessed for real-time insulin release during perifusion with myotube conditioned media ± EPS. [A] Real-time insulin release from pseudoislets perifused with conditioned media directly from intact C2C12 myotubes treated with (grey circles) and without (black circles) EPS. [B] Average insulin secreted from pseudoislets perifused with conditioned media from C2C12 myotubes over each perifusion phase; 0–32 min (no EPS – grey and black circles), 36–96 min (no EPS – black circles, EPS – grey circles), 100–216 (no EPS – black circles, post EPS – grey circles). [C] Rates of insulin secreted by pseudoislets perifused with conditioned media post EPS (grey circles) or post noEPS (black circles) – data extracted from [A]. [D] Slopes of insulin secreted by pseudoislets perifused with conditioned media post EPS (grey bar) or post noEPS (black bar) – calculated from data shown in [C]. Data are means ± SEM from 4 to 5 independent experiments. Statistical significance of mean differences was tested by 2-way ANOVA [B], linear regression analysis [C] and unpaired *t*-test [D]: asterisks indicate statistical significance in insulin secreted between pseudoislets perifused with conditioned media from myotubes with or without EPS treatment ($^{*}P < 0.05$ and $^{**}P < 0.01$).

(λex/em = 540/590 nm), was detected using a BMG LABTECH FLUOstar Omega plate reader.

2.5. Insulin Secretion by INS-1 832/3 Cells

INS-1 832/3 cells seeded and exposed to the presence or absence of BSA conjugated palmitate as described in Section 2.1.2 were washed twice into KRH for 1 h intervals between washes at 37 °C under air. Assay buffer was replaced with 100 μL C2C12 myotube conditioned medium ± EPS supplemented with 5 or 20 mM glucose as collected in Section 2.3. Cells were incubated in this medium for 60 min with shaking (250 rpm, Stuart plate shaker) at 37 °C. Next, supernatants were collected on ice and assayed for insulin by homogeneous time-resolved fluorescence (#62IN1PEG, Cisbio Bioassays, France). Absolute insulin secretion levels were normalized to cell density using 4′,6-diamidino-2-phenyl-indole (DAPI) fluorescence as previously described [22]. To control for EPS media conditioning effects indirect from the presence of C2C12 muscle cells, insulin secretion of INS-1 832/3 cells was also examined when exposed to media conditioned with or without EPS in the absence of C2C12 myotubes (Fig. S2).

2.6. Mitochondrial Respirometry

Mitochondrial respiration was measured in intact attached INS-1 832/3 cells as previously described [23]. Briefly, INS-1 832/3 cells seeded on XFe24 cell culture plates (Seahorse Bioscience, Agilent Technologies) were washed into C2C12 myotube conditioned medium ± EPS supplemented with 5 mM glucose as collected in Section 2.3. Cells were incubated for 50 min at 37 °C under air before transferring to a Seahorse XFe24 extracellular flux analyzer (controlled at 37 °C) for a 10-min calibration and 4 measurement cycles to record basal cellular respiration. Subsequently, 2 μg/mL oligomycin, 3 μM BAM 15 and a mixture of 2 μM rotenone plus 2 μM antimycin A were added sequentially to inhibit ATP synthase, uncouple oxidative phosphorylation and determine non-mitochondrial respiration. Absolute mitochondrial oxygen uptake values were normalized to cell density using DAPI fluorescence as previously described [22].

2.7. Human Islets

Nondiabetic human islets were freshly isolated from 5 donors including 2 males and 3 females with mean age 50.6 ± 6.6 y and BMI 29.8 ± 5.6 kg/m^2. Type 2 diabetic islets were thawed from cryopreserved islet preparations, as described previously [24], collected from 4 donors including 2 males and 2 females with mean age 64.3 ± 4.8 and BMI 35.7 ± 2.0. All islets were isolated, cryopreserved and thawed at the Alberta Diabetes Institute IsletCore or the Clinical Islet Laboratory at the University of Alberta, Edmonton, Canada, by previously described methods [24–26]. All studies were approved by the Human Research Ethics Board at the University of Alberta (Pro00013094; Pro 00001754).

2.8. Human Islet Insulin Secretion

Human islets were cultured in DMEM medium containing 10% FCS, 100 units/mL penicillin, 100 μg/mL streptomycin, supplemented with 5.5 mM glucose for 24 h at 37 °C with 5% CO_2. The following day, islets were washed into Krebs-Ringer HEPES bicarbonate buffer (KRHB) comprising of 115 mM NaCl, 5 mM KCl, 24 mM $NaHCO_3$ (pH 7.4), 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 0.1% BSA and 5 mM glucose at 37 °C with 5% CO_2 for 2 h. Subsequently, islets were hand-picked into groups of 15 islets/well of a 96 'V' bottomed microplate and incubated in 200 μL C2C12 myotube conditioned medium ± EPS supplemented with either 5 or 20 mM glucose (cf. Section 2.3) for 1 h at 37 °C. Each condition (conditioned media from myotubes treated with EPS and without EPS supplemented with either 5 or 20 mM glucose) was run in triplicate totaling to 180 islets per donor which were picked from 1800 IEQ. Supernatants were collected and stored at −20 °C before being assayed for insulin using homogeneous time-resolved fluorescence (#62IN1PEG, Cisbio Bioassays, Codolet, France). Secreted insulin was normalized to islet DNA content as determined from PicoGreen assessment of islet cell lysates prepared in RIPA lysis buffer using Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies).

2.9. Statistics

Significance of mean differences was tested for by unpaired *t*-tests (Figs. 2D, 3A–D, 4A–D, 6A, B and D), paired *t*-tests (Fig. 5), linear regression analysis (Fig. 2C) or two-way ANOVA applying Fishers LSD (Fig. 2B) or Sidak's post hoc test (Fig. 6C) using GraphPad Prism Version 7.0 for Mac OS X (GraphPad software, San Diego, CA, USA). Data are presented as means ± SEM.

3. Results

In this study we investigated if molecules secreted by acute skeletal muscle contraction impact insulin secretion by β-cells using a novel dynamic perifusion platform (Fig. 1). We hypothesised that skeletal muscle myotube conditioned media following EPS will increase insulin secretion from INS-1 832/3 pseudoislets. To test our hypothesis we developed a dynamic perifusion system (Fig. 1) to examine direct effects of skeletal muscle conditioned media at rest, during 1 h EPS and up to 2 h post contraction on insulin secretion kinetics by INS-1 832/3 pseudoislets (Fig. 2). To confirm our EPS parameters of 40 V, 2 ms and 1 Hz as an acute model for 'exercise' in vitro we measured glucose uptake by myotubes treated with and without EPS. Mimicking acute effects of exercise in vivo we show that following EPS, myotubes significantly increase their uptake of glucose (Fig. S1).

In line with the inhibitory action of exercise on insulin secretion [27], we show that conditioned media from contracting skeletal muscle has no effect on insulin secretion by pseudoislets (Fig. 2A and B). Of interest, however, we found that insulin secretion was significantly increased from 0.8 ± 0.3 pg insulin/ng DNA to 3.6 ± 0.6 pg insulin/ng DNA by pseudoislets perifused with skeletal muscle conditioned media from the start (100 min) to the end (216 min) of the perifusion period after EPS had been immobilised (Fig. 2A and C). In contrast, insulin secretion from pseudoislets perifused with conditioned medium from skeletal muscle myotubes not treated with EPS was modestly increased over this period (Fig. 2A and C - 0.75 ± 0.2 pg insulin/ng DNA to 1.3 ± 0.3 pg insulin/ng DNA). Consistent with our raw data (Fig. 2A), when the rate of insulin release was averaged from each perifusion period, it was confirmed that insulin secretion from pseudoislets was significantly influenced by muscle conditioned media throughout the 2-hour perifusion period post EPS (Fig. 2B). This was also evident by examining the slope of the insulin curves from pseudoislets perifused with conditioned media over this period, whereby the slope of insulin secretion was significantly different from pseudoislets perifused with conditioned media from myotube treated with EPS compared with perifusion of conditioned media from myotubes without EPS treatment (Fig. 2C and D).

Confirming results from our perifusion experiments (Fig. 2), conditioned myotube media following 1 h EPS supplemented with 5 mM glucose significantly increased acute insulin release by intact INS-1 832/3 cells (Fig. 3A). Notably, this increase occurred without any significant effects on cell density (noEPS = 1.08×10^{-3} cells ± 0.15; EPS = 1.04×10^{-3} cells ± 0.10). Furthermore, insulin secretion by INS-1 832/3 cells exposed to 24 hour high palmitate was also significantly increased by conditioned media from myotubes treated with EPS (Fig. 3B). Importantly, conditioned media from myotubes treated with EPS had no significant effect on insulin secretion by INS-1 832/3 cells exposed to experimental 'glucotoxic' and 'glucolipotoxic' T2D-like conditions (Fig. 3C and D). Unlike the case with conditioned media supplemented with 5 mM glucose (Fig. 3A and B), there was only a modest insignificant effect of conditioned media from myotubes treated with EPS supplemented with 20 mM glucose on insulin release by INS-1 832/3 cells

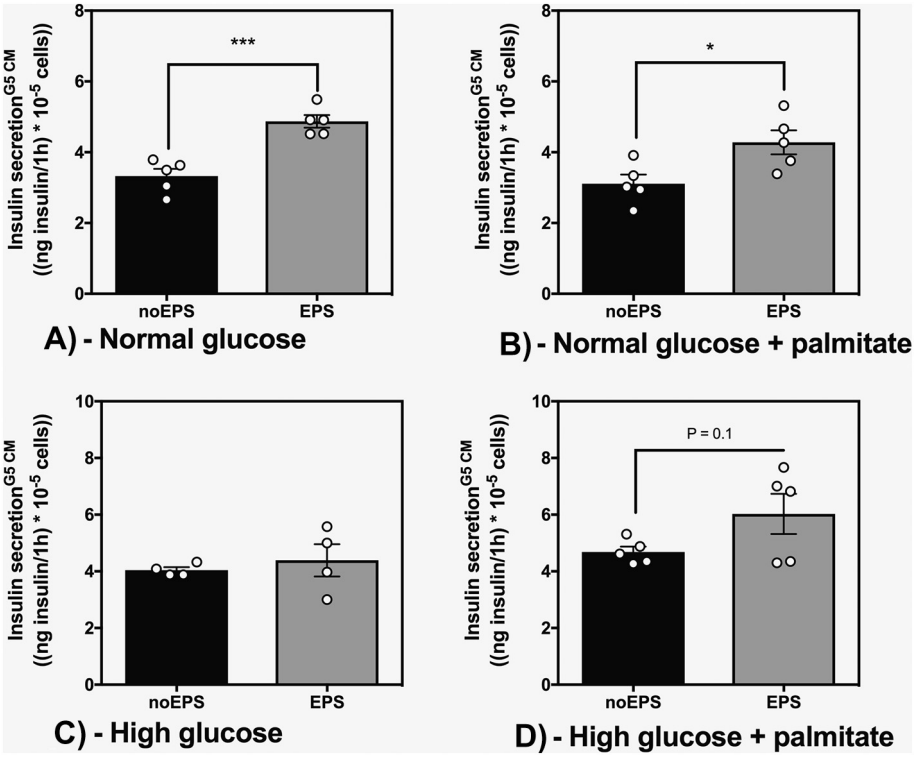

Fig. 3. Static insulin secretion by INS-1 832/3 cells exposed to C2C12 myotube conditioned media containing 5 mM glucose under normal and Type-2 diabetic-like conditions. Cells cultured for 24 h to RPMI media containing 5 mM glucose [A], 5 mM glucose plus palmitate [B], 20 mM glucose [C] or 20 mM glucose plus palmitate [D] were assessed for insulin release when exposed to media for 1 h containing 5 mM glucose conditioned by myotubes treated with (grey bars) or without EPS (black bars). Data are means ± SEM of 4–5 independent experiments with each condition repeated 3–4 times. Mean differences were tested for statistical significance by Unpaired *t*-tests: asterisks indicate statistical significance in insulin secreted by cells exposed to conditioned media from myotubes with or without EPS treatment ($^{*}P < 0.05$ and $^{***}P < 0.001$).

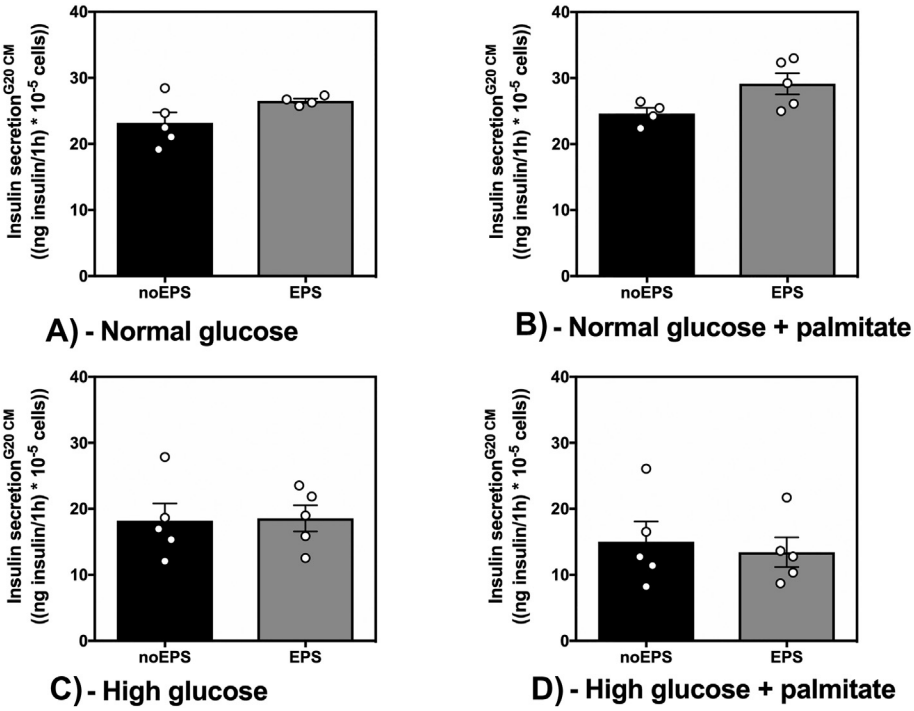

Fig. 4. Static insulin secretion by INS-1 832/3 cells exposed to C2C12 myotube conditioned media containing 20 mM glucose under normal and Type-2 diabetic-like conditions. Cells cultured for 24 h to RPMI media containing 5 mM glucose [A], 5 mM glucose plus palmitate [B], 20 mM glucose [C] or 20 mM glucose plus palmitate [D] were assessed for insulin release when exposed to media for 1 h containing 20 mM glucose conditioned by myotubes treated with (grey bars) or without EPS (black bars). Data are means ± SEM of 4–5 independent experiments with each condition repeated 3–4 times. Mean differences were tested for statistical significance by Unpaired *t*-tests.

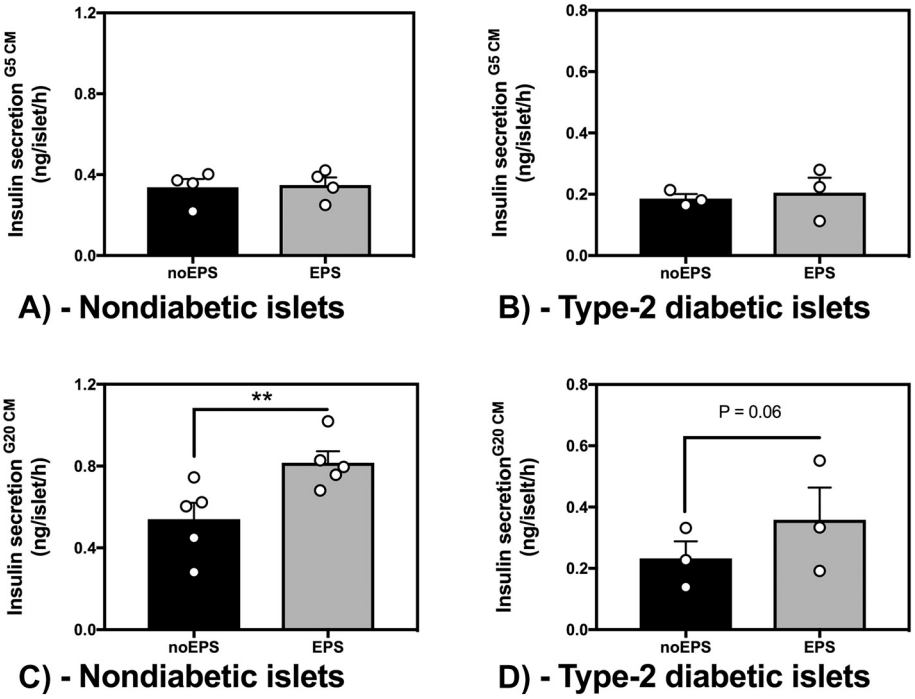

Fig. 5. Static insulin secretion by isolated nondiabetic and Type-2 diabetic human islets exposed to C2C12 myotube conditioned media containing 5 mM or 20 mM glucose. Nondiabetic islets [A and C] and Type-2 diabetic islets [B and D] cultured in DMEM media containing 5 mM glucose were assessed for insulin release when exposed to media for 1 h containing 5 mM glucose [A and B] or 20 mM glucose [C and D] conditioned by myotubes treated with (grey bars) or without EPS (black bars). Data are means ± SEM of 3–4 individual islet donors (Suppl. Fig. 2) each run in triplicate. Mean differences were tested for statistical significance by Unpaired *t*-tests: asterisks indicate statistical significance in insulin secreted by islets exposed to conditioned media from myotubes with or without EPS treatment ($^{**}P < 0.01$).

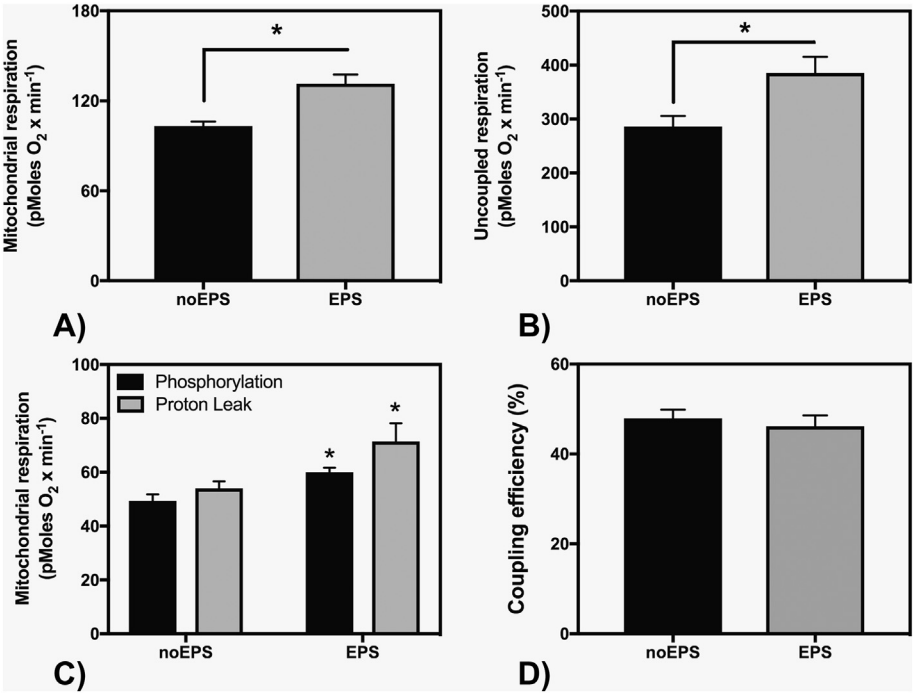

Fig. 6. Mitochondrial respiratory activity of INS-1 832/3 cells exposed to conditioned media from C2C12 myotubes treated with or without EPS. Absolute mitochondrial respiration normalized to cell number was assessed in cells exposed to myotube conditioned media with (grey bars) and without (black bars) EPS containing 5 mM glucose [A]. Subsequently, absolute uncoupled mitochondrial respiration was determined in cells as BAM 15 stimulated respiration [B] and mitochondrial respiration used to make ATP (black bars) or associated with proton leak (grey bars) was determined as oligomycin-sensitive or oligomycin-resistant mitochondrial oxygen uptake, respectively [C]. Coupling efficiency of oxidative phosphorylation was calculated as the percentage of mitochondrial oxygen uptake used to make ATP [D]. Data are means ± SEM from 3 individual experiments with each condition repeated 6 times. Statistical significance of mean differences was tested by Unpaired *t*-tests: asterisks indicate statistical significance in respiratory parameters by cells exposed to conditioned media from myotubes with or without EPS treatment ($^{**}P < 0.01$).

exposed with ($P = 0.06$) or without ($P = 0.11$) palmitate (Fig. 4A and B). Consistently, this effect was not evident in INS-1 832/3 cells following experimental T2D-like conditions (Fig. 4C and D).

To probe the effect of skeletal muscle conditioned media on a better model for human physiology, human pancreatic islets isolated from nondiabetic and type 2 diabetic human donors were exposed to myotube conditioned media treated with or without EPS. Confirming INS-1 832/3 pseudoislet (Fig. 2) and cell data (Fig. 3), conditioned media from myotubes treated with EPS significantly potentiated insulin secretion of nondiabetic (Fig. 5C) and type 2 diabetic (Fig. 5D) human islets. Different from INS-1 832/3 cells and pseudoislets, the potentiation of insulin secretion by conditioned media from myotubes treated with EPS on human isolated islets only occurred in conditioned media supplemented with 20 mM glucose (Fig. 5C and B). Insulin secretion by human islets was unaffected by conditioned media from EPS treated myotubes supplemented with 5 mM glucose (Fig. 5A and B). It should be noted that the effect of media conditioned with EPS in islets from type 2 diabetic donors was not statistically significant ($P = 0.06$). This is likely due to being underpowered with only an N of 3 donors. Despite not being significant, from individual donor response date (Fig. S3), it is clear that insulin release was increased with media conditioned by EPS in all 3 T2D donors. It should also be noted that from the individual donor responses (Fig. S3), one donor had a much greater response to the conditioned media from EPS treated myotubes. This difference may be related to the relative insulin secretory response of this specific donor at the stage of isolation. For example being in the hyperinsulinemic rather than hypoinsulinemic phase of the disease. Although we cannot confirm this conclusively, it is consistent with the lower HbA1c of 6.2 for this donor compared to 7.7 and 6.7 for the other two donors and also corroborates with the high GSIS index of 18.7-fold which was measured prior to islet cryopreservation for this donor.

To understand how conditioned media from myotubes treated with EPS impacts insulin secretion by INS-1 832/3 cells and human islets, we examined mitochondrial respiratory activity of INS-1 832/3 cells exposed to conditioned media from myotubes treated with or without EPS. We discovered that the basal rate of mitochondrial oxygen consumption was significantly increased by INS-1 832/3 cells exposed to conditioned media from myotubes treated with EPS (Fig. 6A). Consistent with effects on basal mitochondrial respiration, uncoupled mitochondrial respiration (Fig. 6B), phosphorylation respiration (Fig. 6C), and leak respiration (Fig. 6C) were all augmented in INS-1 832/3 cells exposed to conditioned media from myotubes treated with EPS. Accordingly, the coupling efficiency of oxidative phosphorylation (i.e. the percentage of respiration coupled to ATP synthesis) in INS-1 832/3 cells was not affected by conditioned media from myotubes treated with EPS (Fig. 6D).

4. Discussion

It is well established that exercise improves β-cell function in patients with prediabetes and T2D [3–7]. Moreover, it has recently been recognized that compounds released from skeletal muscle provide a means for inter-organ crosstalk during exercise [15,28–30]. Therefore, the beneficial effects of exercise on β-cell function in patients with prediabetes and T2D has been attributed to skeletal muscle-derived molecules. However, direct evidence supporting this idea remains to be established. Our data build on this story by revealing that media conditioned by acute skeletal muscle contraction increases the rate of insulin secretion by INS-1 832/3 pseudoislets (Fig. 2) and potentiates insulin secretion by cultured insulinoma cells with or without exposure to palmitate (Fig. 3) as well as from islets isolated from nondiabetic and type-2 diabetic donors (Fig. 5).

The disparity between the effects of conditioned media from EPS treated myotubes on insulin secretion by cultured insulinoma cells (Fig. 3) versus human islets (Fig. 5) is of interest and may arise due to differences in the sensitivity of cultured insulinoma cells and human islets for glucose. That said it is also possible that this finding is related to autocrine and/or paracrine events between other cells present within pancreatic islets which are absent in cultured INS-1 832/3 cell monolayers. For example if molecules present within conditioned media from EPS treated myotubes supplemented with 20 mM glucose influences the secretion of glucagon and/or GLP-1 from alpha cells then this in turn may potentiate insulin secretion by β-cells. Although we cannot exclude this possibility, based on the fact that mitochondrial respiratory activity is also increased by conditioned media from EPS treated myotubes (Fig. 6), paracrine effects alone are unlikely to explain the crosstalk effect.

The finding that insulinoma cells pre-treated with high glucose with or without palmitate blunts the effect of EPS on insulin secretion (Fig. 3) indicates that excessive hyperglycaemia may restrict the insulin potentiating effect of acute exercise by β-cells. However, to conclusively determine this, investigation of acute hyperglycaemia and effects of exercise on insulin secretion in vivo are warranted. Nevertheless, our results signify that molecules released as a consequence of acute skeletal muscle contraction may improve insulin secretion by β-cells in obese prediabetic and T2D patients who have controlled hyperglycaemia.

Given that β-cell dysfunction and associated impaired insulin secretion is a key component in prediabetes and progression to T2D [8], the discovery of novel compounds with the potential to improve β-cell function is at the forefront of diabetes research. Yet, it is uncertain if improving a dysfunctional β-cell to secrete more insulin is a more effective strategy than simply reducing its workload. It is therefore suggested that a more favourable approach is to target both peripheral insulin resistance and β-cell dysfunction at the same time [1]. It is well recognized that exercise improves peripheral insulin sensitivity and the molecular mechanisms of this effect have been well studied [30,31]. However, less is understood about the molecular benefit of exercise for β-cell function, especially in regards to T2D. In healthy islets isolated from exercised trained rodents, glucose-stimulated insulin secretion appears to be lower than non-exercised animals [16]. However, in support of our data, in rodents, acute exercise in vivo appears to potentiate insulin secretion [31]. Myokines, such as IL-6, have also been linked with the protective effect of exercise against pro-inflammatory-induced β-cell loss [16]. In this regard our data provide additional experimental evidence that implicate a role of muscle-derived molecules in mediating the effects of exercise on β-cell function.

Although we are yet to determine the identity of the muscle-derived molecule(s) responsible for our observed insulin secretory effects, from our mitochondrial uncoupled respiration data (Fig. 6), we can exclude molecules that act on KATP channel activity or exocytosis and thus rule out amplifying signals of insulin secretion. Instead, molecules in conditioned media from myotubes treated with EPS appear to act on the triggering pathway of insulin secretion by stimulating mitochondrial substrate oxidation. Moreover, given that coupling efficiency of oxidative phosphorylation was unchanged in cells exposed to conditioned myotube media with or without EPS treatment (Fig. 6D), we can also rule out molecules that may lead to increased glucose uptake or that stimulate glucose or pyruvate oxidation; as these substrates increase coupling efficiency in insulinoma cells [32,33]. It is more likely that conditioned media from myotubes treated with EPS contains an unknown oxidizable compound that acts as an insulin secretagogue to potentiate insulin secretion through increased mitochondrial respiration. This concept is consistent with our observation that EPS-conditioned muscle cell media has no effect on insulin secretion in INS-1 832/3 cells exposed to experimental diabetic-like conditions (Fig. 3), that impair mitochondrial function in β-cells [34,35]. Moreover, given that conditioned media from myotubes treated with or without EPS has no effect on the density of INS-1 832/3 cells (data not shown) it is unlikely that factors present within myotube conditioned media influence the viability and/or proliferation of β-cells in our experiments.

When considering the validity of these data and previous work in this field, it is important to acknowledge the limitations that these studies present. A major limitation, especially from previous studies, is in the

experimental design, whereby conditioned muscle-cell media collected after a static culture period is used to culture β-cells or isolated pancreatic islets. While these experiments have been insightful, conditioned media collected from static cultures leads to accumulation of secreted products [12]. In some studies, this is overcome by using blood serum, however serum contains many factors not specific to skeletal muscle, making it impossible to pinpoint muscle as the source of the molecule involved in mediating a perceived crosstalk effect. To overcome this issue, in this study, we used a dynamic perifusion model that continuously and directly transports media between cultured skeletal muscle and cultured pseudoislets. It should be stressed, however, that the flow rate used in our system is almost ten times less that it would be in muscle in humans. Furthermore, due to the volume of media contained within the wells of the perifusion plates, there is a significant lag time of around 60 min for completely refreshing the culture media in the plate. Therefore it is difficult to delineate between molecules contained within conditioned media released during muscle contraction and post contraction on mediating changes in insulin secretion. That said, given that post contraction muscle cell media increases the rate of insulin secretion continuously for 2 h (Fig. 2), it is unlikely that molecules released by contracting muscle are associated with the stimulation in insulin secretion because they would have been washed out before the second hour of the post contraction phase. It is also worth note that our results are limited to in vitro models from a range of different species including rat, mouse and human; and thus we cannot exclude cross species effects. Therefore investigation into the acute effects of skeletal muscle contraction on β-cell function using cells and tissues from the same species of origin and further confirmation in vivo are warranted.

In summary, our perifusion crosstalk analysis reveals a clear functional crosstalk effect of skeletal muscle media conditioned by EPS on β-cell insulin secretion. Moreover, the bioenergetic behaviour of INS-1 832/3 cells exposed to conditioned media from skeletal muscle treated with EPS aligns with those observed for insulin secretion. Our findings offer novel insight in the real-time crosstalk effect of acute skeletal muscle contraction on β-cell function and suggest involvement of enhanced substrate oxidation in the mechanism by which skeletal muscle conditioned media influences β-cell insulin secretion. These data provide clues as to how exercise might improve β-cell function in patients with T2D and builds on prior knowledge in vivo that basal insulin secretion is suppressed during exercise due to α2-adrenoreceptor activation by norepinephrine [27,36]. As physical activity also targets peripheral insulin sensitivity and improves blood glucose tolerance, exercise interventions may prove to be more effective than pharmacological agents in the management of β-cell failure in T2D. Although the identity of the muscle-derived molecules and the mechanism by which they impact insulin secretion from β-cells during exercise in vivo remains to be established, our data support the requirement for future investigation in this area.

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Conflict of interest

The authors declare no competing interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2018.11.004.

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