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RESEARCH ARTICLE

A novel ATP-synthase-independent mechanism coupling mitochondrial activation to exocytosis in insulin-secreting cells

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ABSTRACT

Pancreatic β-cells sense glucose, promoting insulin secretion. Glucose sensing requires the sequential stimulation of glycolysis, mitochondrial metabolism and Ca^{2+} entry. To elucidate how mitochondrial activation in β-cells contributes to insulin secretion, we compared the effects of glucose and the mitochondrial substrate methylsuccinate in the INS-1E insulin-secreting cell line at the respective concentrations at which they maximally activate mitochondrial respiration. Both substrates induced insulin secretion with distinct respiratory profiles, mitochondrial hyperpolarization, NADH production and ATP-to-ADP ratios. In contrast to glucose, methylsuccinate failed to induce large [Ca^{2+}] rises and exocytosis proceeded largely independently of mitochondrial ATP synthesis. Both glucose- and methylsuccinate-induced secretion was blocked by diazoxide, indicating that Ca^{2+} is required for exocytosis. Dynamic assessment of the redox state of mitochondrial thiols revealed a less marked reduction in response to methylsuccinate than with glucose. Our results demonstrate that insulin exocytosis can be promoted by two distinct mechanisms one of which is dependent on mitochondrial ATP synthesis and large Ca^{2+} transients, and one of which is independent of mitochondrial ATP synthesis and relies on small Ca^{2+} signals. We propose that the combined effects of Ca^{2+} and redox reactions can trigger insulin secretion by these two mechanisms.

KEY WORDS: Mitochondria, Ca^{2+} signaling, Redox signaling, β-cells, Signal transduction

INTRODUCTION

The total pancreatic β-cells mass constitute a small endocrine tissue that is organized, together with other endocrine cells, in islets of Langerhans, which are dispersed throughout the pancreatic exocrine tissue (Rahier et al., 1983). β-cells sense glucose and other nutrients in the blood and secrete the appropriate amount of insulin to maintain circulating nutrient levels according to the metabolic requirements of the organism (Rutter et al., 2015). Insulin is the only blood glucose-lowering hormone. β-cell dysfunction or reduced mass leads to impaired glucose tolerance, type 2 diabetes and associated complications (Steffes et al., 2003; Weir and Bonner-Weir, 2004). Metabolism–secretion coupling describes the molecular mechanism linking nutrient sensing and signaling to insulin secretion (Groschner et al., 2014; Henquin, 2000). For glucose-stimulated insulin secretion this process has been relatively well characterized and requires the sequential activation of several biological processes (Fig. 1A). Glucose enters the β-cell by GLUT-mediated transport. In the cytosol it is metabolized by glycolysis to generate pyruvate, which is taken up by mitochondria. Mitochondrial pyruvate oxidation leads to the formation of reducing equivalents (NADH and $FADH_2$), which are substrates of the respiratory chain. Activation of respiration and mitochondrial ATP synthesis increase the cytosolic ATP-to-ADP ratio, which induces closure of the plasma membrane K_{ATP} channel, promoting plasma membrane depolarization and, therefore, Ca^{2+} entry through voltage-dependent Ca^{2+}-channels. The associated Ca^{2+} rises trigger insulin exocytosis. Mitochondrial metabolism linked to glucose stimulation also gives rise to coupling factors other than ATP, amplifying insulin secretion (Henquin, 2000). These amplifying factors explain increased insulin secretion during a graded glucose challenge, when Ca^{2+} signals are saturated (Gembal et al., 1993). Consistent with these findings, mitochondria in permeabilized cells can directly generate factors, distinct from Ca^{2+} and ATP, that are capable of inducing insulin exocytosis (Maechler et al., 1997). Following such early findings, a large number of mitochondria-derived coupling factors were proposed but the molecular mechanisms linking mitochondria to the amplification of insulin secretion remain elusive.

The observed cytosolic Ca^{2+} rise during glucose stimulation affects mitochondrial activity. Mitochondria take up and release the Ca^{2+} ions leading to a transient increase in matrix Ca^{2+}. These mitochondrial Ca^{2+} signals accelerate oxidative metabolism while at the same time stimulating ATP-synthase-dependent respiration. Coordinated activation of these two processes allows the respiratory rate to change several-fold with only small alterations of the NAD(P)H:$NAD(P)^+$ ratio (De Marchi et al., 2014b). Buffering or preventing mitochondrial Ca^{2+} rises impairs the second phase of insulin secretion, demonstrating that matrix Ca^{2+} transients serve as a signal in metabolism–secretion coupling (Tarasov et al., 2012; Wiederkehr et al., 2011).

Different nutrients and fuel secretagogues have been used to elucidate how β-cell mitochondria contribute to trigger and amplify insulin secretion (Antinozzi et al., 2002). Notably, this approach has been taken by several studies comparing the effect of the nutrient glucose with the mitochondrial substrate succinate (Kennedy et al., 1998; Zawalich and Zawalich, 1992; Zawalich et al., 1993). Succinate (Tretter et al., 2016) is oxidized at complex II, and therefore bypasses glycolysis and complex I of the respiratory chain. Succinate is among the most frequently used substrates to stimulate respiration in isolated mitochondria (De Marchi et al., 2004; Scorrano et al., 1999) but in intact cells, the methylester of succinate must be used instead to allow cellular uptake. Intracellularly, methylsuccinate is hydrolyzed to give rise to succinate to serve as a substrate for mitochondrial respiration. In this form, succinate promotes insulin secretion in pancreatic β-cells in several β-cell

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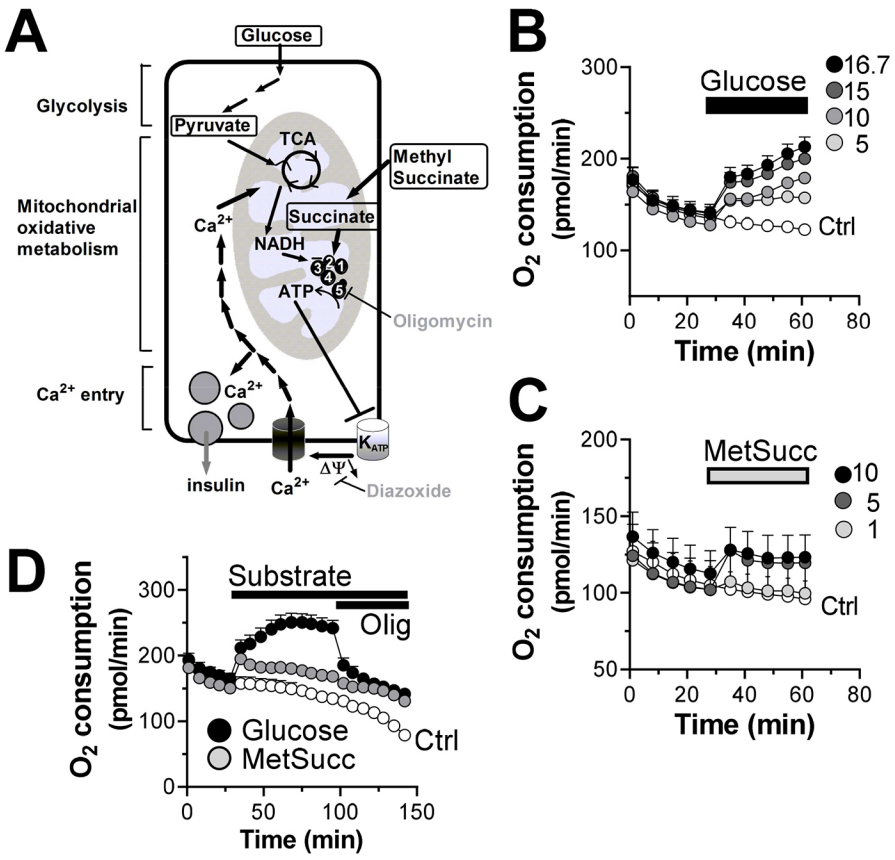

Fig. 1. Glucose and methylsuccinate promote distinct oxygen consumption pathways in pancreatic INS-1E β-cells. (A) Canonical signal transduction pathway of pancreatic β-cells. Metabolism–secretion coupling of β-cells requires the sequential activation of glycolysis, mitochondrial oxidative metabolism and plasma membrane Ca^{2+} entry. Glucose stimulates glycolysis and pyruvate production. Pyruvate triggers mitochondrial metabolism and formation of NADH (by the TCA cycle), which is the fuel for the respiratory complexes (1,2,3,4), allowing ATP production by ATP-synthase (5). ATP inhibits K_{ATP} channels, inducing membrane depolarization (ΔΨ↓), and Ca^{2+} entry through voltage-gated Ca^{2+} channels, promoting insulin secretion. Inhibitors of mitochondrial ATP synthase (oligomycin) and membrane depolarization (diazoxide) are shown in gray. (B) Oxygen consumption was assayed in standard KRBH, containing basal, non-stimulatory 2.5 mM glucose. Cells were then stimulated by adding glucose (B) or methylsuccinate (MetSucc, C) at the indicated concentrations (in mM). Note that when glucose is used, the indicated amount reflects the final sugar concentration. When methylsuccinate is shown, the substrate is added in the presence of basal 2.5 mM glucose. (B,C) Control (ctrl, open circles). Representative results (mean±s.e.m.) from one of three different experiments performed in triplicate are shown. (D) The maximal respiration profiles were compared by stimulating the cells with 16.5 mM glucose (black circles) and 5 mM methylsuccinate (gray circles). Oligomycin (Olig, 2.5 µg/ml) was added to block ATP-synthase activity.

models as well as primary pancreatic β-cells (Alarcon et al., 2002; Fahien and MacDonald, 2002; Heart et al., 2007; Maechler et al., 1997; Mukala-Nsengu et al., 2004; Zawalich and Zawalich, 1992; Zawalich et al., 1993). As with glucose, succinate-induced insulin secretion is biphasic (Zawalich et al., 1993). There are however conflicting results regarding the mechanism linking the stimulation of mitochondria by methylsuccinate to insulin secretion. While Mukala-Nsengu and collaborators have reported a strong methylsuccinate-stimulated Ca^{2+} rises in mouse islets (Mukala-Nsengu et al., 2004), a total absence of a Ca^{2+} response characterized the recordings of Heart and collaborators using the same biological system (Heart et al., 2007). Rat islets, in contrast, showed substantial methylsuccinate-stimulated Ca^{2+} rises that were indistinguishable from the ones induced by glucose. However, only representative traces were reported in those studies and a real quantification of Ca^{2+} was missing. Furthermore, it has been proposed that stimulus–secretion coupling may still occur in the absence of extracellular Ca^{2+} and the absence of intracellular Ca^{2+} rises (Komatsu et al., 1995), suggesting the possibility that alternative signals stimulate insulin granule exocytosis.

To clarify whether Ca^{2+} signaling and mitochondrial activation are a prerequisite for insulin secretion independently from the nutrient secretagogue, we studied metabolism–secretion coupling using the three fuel secretagogues glucose, pyruvate and methylsuccinate in the INS-1E insulin-secreting cell line. For direct comparison of mitochondrial activation, we studied the three substrates at the minimal concentration required to obtain maximal activation of respiration as opposed to using them at equimolar concentrations (Heart et al., 2007). Based on our results, we describe two distinct mechanisms linking nutrient activation of mitochondria to insulin secretion. The two pathways differ in their dependency on cytosolic Ca^{2+} rises and redox signaling, and they are respectively dependent on and independent of mitochondrial ATP production.

RESULTS

Glucose and methylsuccinate induce distinct respiratory responses in INS-1E cell mitochondria

Metabolism–secretion coupling depends on mitochondrial activation and the generation of ATP and other factors linking metabolism to the initiation of Ca^{2+} signals and insulin secretion (Fig. 1A). During glucose stimulation, mitochondrial respiration accelerates in a biphasic manner (De Marchi et al., 2014b). In INS-1E cells, a step increase from 2.5 to 16.7 mM glucose accelerates respiration rapidly followed by a slower gradual increase of the respiratory rate (Fig. 1B).

Journal of Cell Science

Journal of Cell Science (2017) 130, 1929-1939 doi:10.1242/jcs.200741

This respiratory response to 16.7 mM glucose is maximal because lower concentrations of glucose induced qualitatively similar but less-pronounced responses (Fig. 1B). Stimulation of INS-1E cells with methylsuccinate (5 mM) rapidly augmented respiration but subsequently failed to produce a further increase, in contrast to the glucose response (Fig. 1C,D). Doubling the concentration of methylsuccinate did not further augment respiration, demonstrating that a maximal response to this substrate was achieved at 5 mM. Our results show that the profiles of respiration are clearly distinct (Fig. 1D). Blocking ATP-synthase with oligomycin caused a rapid inhibition of glucose-induced respiration. In INS-1E cells activated by methylsuccinate, the ATP-synthase-dependent respiration was surprisingly small (Fig. 1D). Although methylsuccinate is able to increase respiration, only a small fraction of this response is due to the stimulation of ATP-synthase.

Insulin secretion induced by methylsuccinate is K_{ATP} channel-dependent but insensitive to oligomycin

Given the distinct profile of methylsuccinate-induced respiration and in particular the poor stimulation of ATP-synthase, we compared insulin secretion induced by the two different substrates. Glucose and methylsuccinate were used at the concentration we found above to have maximal stimulatory effect on mitochondria. Consistent with previous reports (Alarcon et al., 2002; Heart et al., 2007), we found that methylsuccinate was able to promote insulin secretion (Fig. 2A). Glucose-induced insulin secretion depends on modulation of the K_{ATP} channel, which is inhibited by mitochondrial ATP and triggers depolarization-induced Ca^{2+} influx. We tested whether methylsuccinate-dependent insulin secretion was able to bypass such regulation of the K_{ATP} channel and the necessity for voltage-dependent Ca^{2+} influx. For this we studied insulin secretion following addition of diazoxide, which maintains the K_{ATP} channel in its open state and induces hyperpolarization of the plasma membrane, thereby preventing Ca^{2+} influx. In control experiments (Fig. 2B), diazoxide inhibited glucose-stimulated insulin exocytosis at 100 µM, consistent with published data (De Marchi et al., 2014b). Diazoxide also blocked methylsuccinate-stimulated insulin secretion (Fig. 2C). These results suggest that insulin secretion in response to both substrates depends on Ca^{2+} signaling. Consistent with this interpretation, both glucose and methylsuccinate-induced insulin secretion were reduced in the absence of extracellular Ca^{2+} (Fig. 2D).

The poor stimulation of ATP-synthase-dependent respiration by methylsuccinate (Fig. 1D), suggests that methylsuccinate-induced insulin secretion may not depend on canonical mitochondrial activation. To clarify the role of mitochondrial ATP synthesis on methylsuccinate-dependent insulin secretion, we measured the effect of the ATP-synthase inhibitor oligomycin (Fig. 2B,C). Glucose-induced insulin secretion was prevented when mitochondrial ATP synthesis was blocked with oligomycin (Fig. 2B), consistent with our earlier findings (De Marchi et al., 2014b). In contrast, the methlysuccinate response was largely insensitive to oligomycin (Fig. 2C). We conclude that the role of mitochondrial activation in metabolism–secretion coupling is distinct for the two secretagogues. Although the diazoxide dependence indicated that a threshold Ca^{2+} concentration is required for methylsuccinate-induced secretion, the observed oligomycin insensitivity demonstrates that factors other than mitochondrial ATP must be generated to promote methylsuccinate-induced insulin secretion.

Substrate-specific activation of INS-1E cell mitochondria

To better understand the mechanism by which methylsuccinate induces insulin secretion, we compared glucose- and

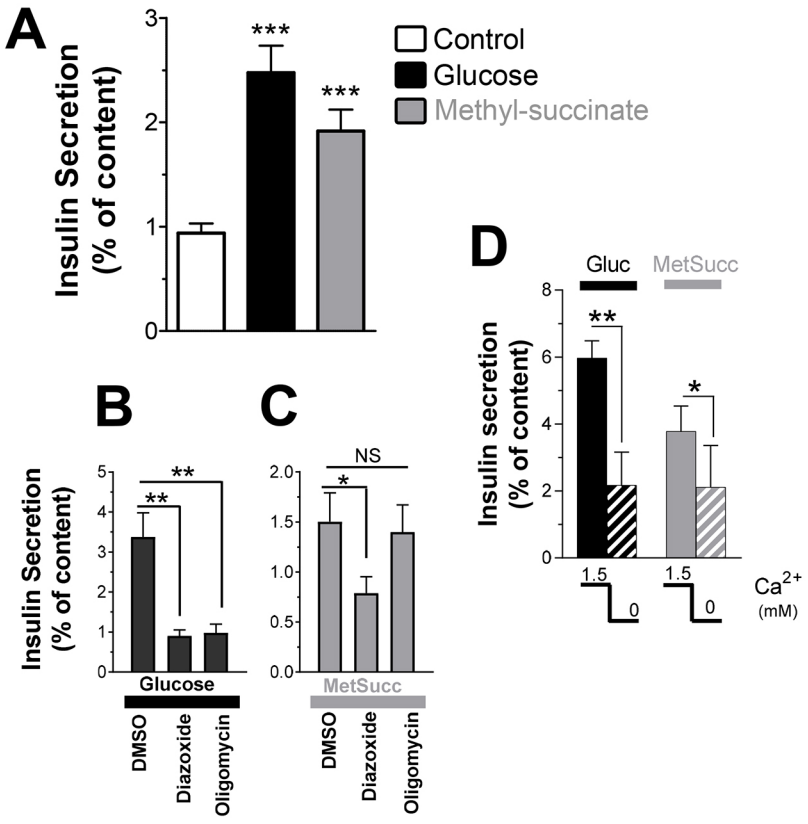

Fig. 2. Glucose- and methylsuccinate-induced insulin secretions are olygomycin-sensitive and -insensitive, respectively. (A–D) Static insulin secretion from INS-1E cells incubated for 30 min in the presence of resting (2.5 mM, white bar, control) or stimulatory (16.7 mM, black bars) glucose concentrations or methylsuccinate (5 mM in presence of 2.5 mM glucose, gray bars). Secreted insulin is expressed as a percentage of insulin content. (A) Mean±s.e.m. from 12 measurements from four independent experiments. (B,C) Inhibitory effects of diazoxide (100 µM) and oligomycin (2.5 µg/ml) on glucose (B, black) and methylsuccinate-induced (C, gray) insulin secretion. Mean±s.e.m. from nine measurements from three independent experiments. (D) Glucose- and methylsuccinate-induced insulin secretion in absence of extracellular Ca^{2+}. Insulin secretion was determined in KRBH or the same buffer lacking Ca^{2+}, in the presence of 0.4 mM EGTA. Mean±s.e.m. from six measurements. *P<0.05; **P<0.001; ***P<0.0001; NS, not significant (Student's t-test for paired samples).

Journal of Cell Science (2017) 130, 1929-1939 doi:10.1242/jcs.200741

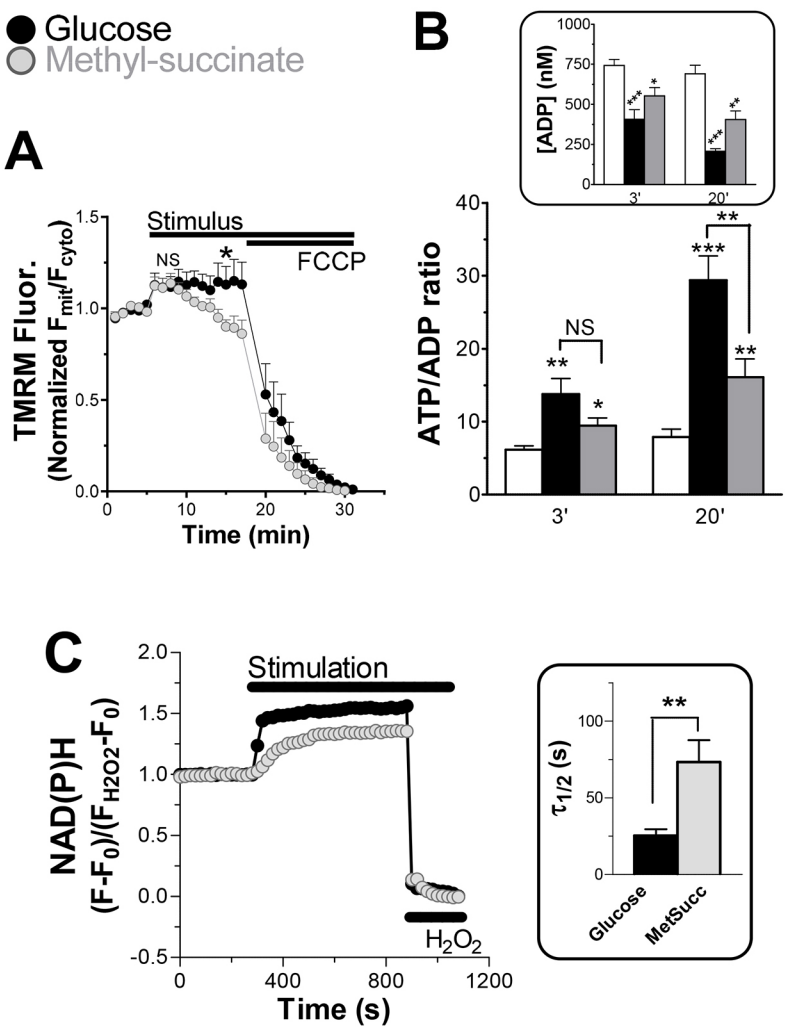

Fig. 3. Distinct mitochondrial membrane potential, ATP-to-ADP ratio and NAD(P)H responses to glucose and methylsuccinate. (A) The mitochondrial membrane potential in response to 16.7 mM glucose (black circles) or 5 mM methylsuccinate (gray circles) was measured in INS-1E cells by using the lipophilic cation TMRM. Changes in $\Delta\psi_m$ are expressed as the ratio of the fluorescence in mitochondria divided by the cytosolic fluorescence (F_{mito}/F_{cyto}) measured in the same cells. At the end of the recording the protonophore FCCP (2 µM) was used to dissipate $\Delta\psi_m$. The effect of the substrates on $\Delta\psi_m$ was measured on normalized responses, as a fraction of basal (set to 1) and after FCCP depolarization (set to 0). Data shown are the mean±s.e.m. from five (49 cells) and four (39 cells) independent experiments for glucose- and methylsuccinate-stimulated cells, respectively. (B) Glucose- and methylsuccinate-dependent ATP-to-ADP ratio responses in INS-1E cells. INS-1E cells were incubated for 20 min in standard KRBH at either resting (2.5 mM, white bars) or stimulatory (16.7 mM, black bars) glucose concentration or after stimulation with methylsuccinate (5 mM, gray bars). Inset: quantification of [ADP] after the indicated time for each condition. Shown is the mean±s.e.m. of nine measurements. The experiment was repeated three times. (C) Kinetics of NAD(P)H fluorescence changes were followed over time in standard KRBH medium containing 2.5 mM glucose. Glucose (raised from 2.5 to 16.7 mM, black circles) and methylsuccinate (5 mM, MetSucc, gray circles) was added as indicated (stimulation). Inset: statistical evaluation of the data shown in C. The half-time to reach a new steady-state of the NAD(P)H signal after stimulation was calculated. Shown is the mean ±s.e.m. from five and three independent experiments for glucose- (black) and methylsuccinate-stimulated (gray) cells, respectively. *P<0.05; **P<0.001; ***P<0.0001; NS, not significant (Student's t-test).

methylsuccinate-induced mitochondrial activation. Glucose causes a rapid hyperpolarization of the mitochondrial membrane potential in β-cells (Duchen et al., 1993) and mitochondria remain hyperpolarized in the continued presence of glucose (Fig. 3A). Methylsuccinate also caused rapid hyperpolarization of the mitochondrial membrane potential with kinetics similar to that seen with glucose. However, methylsuccinate-induced hyperpolarization was only transient. After 5 min of stimulation, the potential gradually returned to basal levels (Fig. 3A). The observed failure to maintain an elevated mitochondrial membrane potential may explain some of the observed differences of the respiratory response.

Mitochondria contribute to glucose-induced metabolism–secretion coupling primarily by raising the ATP-to-ADP ratio. This ratio controls the open state of the plasma membrane K_{ATP} channel. An increase in ATP-to-ADP ratio closes K_{ATP} channels leading to depolarization of the β-cell plasma membrane. Substrate-dependent activation of energy metabolism was therefore followed by measuring the concentration of ATP and ADP (Fig. 3B). Given the biphasic activation of mitochondria by glucose, the concentration of ATP and ADP was measured 3 min (initial activation) and 20 min (sustained activation) after addition of the secretagogues. Consistent with published work, glucose lowered intracellular ADP concentrations rapidly (inset, Fig. 3B). During continued stimulation with glucose, ADP levels further decreased to 36% of the value measured in INS-1E cells maintained under basal glucose conditions. Intracellular ATP concentrations are much larger than ADP concentrations and therefore the increase of ATP at the expense of ADP is marginal (data not shown). The ratio of ATP-to-ADP was increased from 6.8 (2.5 mM glucose) to 14 (16.7 mM glucose) and 30 (16.7 mM glucose) after 3 and 20 min, respectively, of glucose stimulation (Fig. 3B). Methylsuccinate had an equally rapid but less marked effect on ADP levels than glucose. After 3 min ADP concentrations were significantly reduced and after 20 min of substrate addition ADP levels were decreased to 63% of values measured in control cells (inset; Fig. 3B). The less-pronounced effects on ADP explain the weaker ATP-to-ADP response of INS-1E cells to methylsuccinate. The ATP-to-ADP ratio was 10 and 16 after 3 and 20 min, respectively, of stimulation with 5 mM methylsuccinate (Fig. 3B). The reduced ATP-to-ADP response to methylsuccinate is consistent with the poor activation of ATP-synthase-dependent respiration. These findings suggest succinate metabolism is less efficient at regulating the K_{ATP} channel-dependent pathway of insulin secretion.

Raising the ATP-to-ADP ratio depends on accelerated mitochondrial respiration, which is linked to the sustained generation of reducing equivalents such as $FADH_2$ and NAD(P)H, formed as part of substrate oxidation in the tricarboxylic acid (TCA) cycle. The NAD(P)H autofluorescent signal can be used as an indirect read-out of oxidative metabolism. In its reduced form this co-factor is autofluorescent, while the oxidized form $NAD(P)^+$ is not. Glucose induces an almost immediate rise of the NAD(P)H signal with a rapid establishment of a new steady state (De Marchi et al., 2014b; Fig. 3C). Compared to these very rapid responses,

Journal of Cell Science

Journal of Cell Science (2017) 130, 1929-1939 doi:10.1242/jcs.200741

methylsuccinate-dependent NAD(P)H production was much slower (Fig. 3C). The half-time to reach a new steady-state was 2.6-fold larger in methylsuccinate- compared to glucose-stimulated cells (inset, Fig. 3C). In contrast to the mitochondrial membrane potential, the methylsuccinate-induced NAD(P)H increase was sustained.

Taken together, these results demonstrate that there is a small and transient mitochondrial activation in methylsuccinate-stimulated cells. They indicate that a previously unsuspected and partly non-bioenergetic mechanism (oligomycin-independent component of insulin secretion) is responsible for methylsuccinate dependent-insulin secretion.

Methylsuccinate fails to induce substantial cytosolic Ca^{2+} signals

In the light of the distinct effects of glucose and methylsuccinate on mitochondrial activation and the contrasting results of oligomycin on insulin secretion, we assessed the ability of glucose and methylsuccinate to initiate cytosolic Ca^{2+} transients. These Ca^{2+} signals establish the final link between nutrient metabolism and insulin granule exocytosis. For single-cell recordings of cytosolic Ca^{2+}, the cells were transfected with the cytosolic Ca^{2+} sensor $YC3.6_{cyto}$. Glucose stimulation of INS-1E cells induced an increase in the amplitude and/or frequency of cytosolic Ca^{2+} signals (Fig. 4A). Surprisingly, methylsuccinate did not promote Ca^{2+} signals in the majority of the cells analyzed (Fig. 4B).

Single-cell analysis of Ca^{2+} signaling is complicated by the fact that the amplitude and kinetics of the Ca^{2+} elevations are highly variable. Proper characterization of the differences between glucose and methylsuccinate-dependent Ca^{2+} signaling therefore requires an analysis taking into account this heterogeneity. For each cell, we therefore expressed the area under the curve for the Ca^{2+} transients during 5 min of stimulation as a function of the corresponding signal 5 min before stimulation (Fig. 4C). For glucose, a direct correlation between the area under the curve for the Ca^{2+} signals before and during stimulation was observed (Fig. 4C). Cells with no or small Ca^{2+} signals experienced a several-fold increase in the Ca^{2+} signals, but in absolute terms the amplitude of these signals remained rather small. However, for cells with high basal Ca^{2+} signals, glucose further increased these signals but the fold change was less pronounced. This relationship was strikingly different for methylsuccinate. Methylsuccinate-stimulated INS-1E cells showed only very small Ca^{2+} rises or even depression of the signals in cells experiencing large basal Ca^{2+} activity. Only in cells with little or no basal Ca^{2+} signals, could we observe a slight increase of Ca^{2+} signaling following methylsuccinate stimulation. Quantification of the area under the curve shows that, on average, glucose increase Ca^{2+} signals by 9.2-fold while in methylsuccinate-stimulated cells the average Ca^{2+} response was poor (1.5-fold). Methylsuccinate-dependent β-cell activation causes only a very small Ca^{2+} response. Nevertheless, Ca^{2+} signals are indispensable given that methylsuccinate-induced insulin secretion is blocked by diazoxide (Fig. 2). These results also suggest that the requirement for Ca^{2+} as a signal during methylsuccinate stimulation are different from those required upon stimulation with glucose.

Glycolysis does not explain the differences between the two pathways of β-cell activation

Compared to glucose, methylsuccinate bypasses glycolysis and complex I of the respiratory chain to activate energy metabolism. The distinct glucose- and methylsuccinate-dependent mechanisms of β-cell activation, could therefore be linked to glycolysis. Pyruvate can be used as a substrate to further dissect these differences as pyruvate bypasses glycolysis. INS-1E cells express the

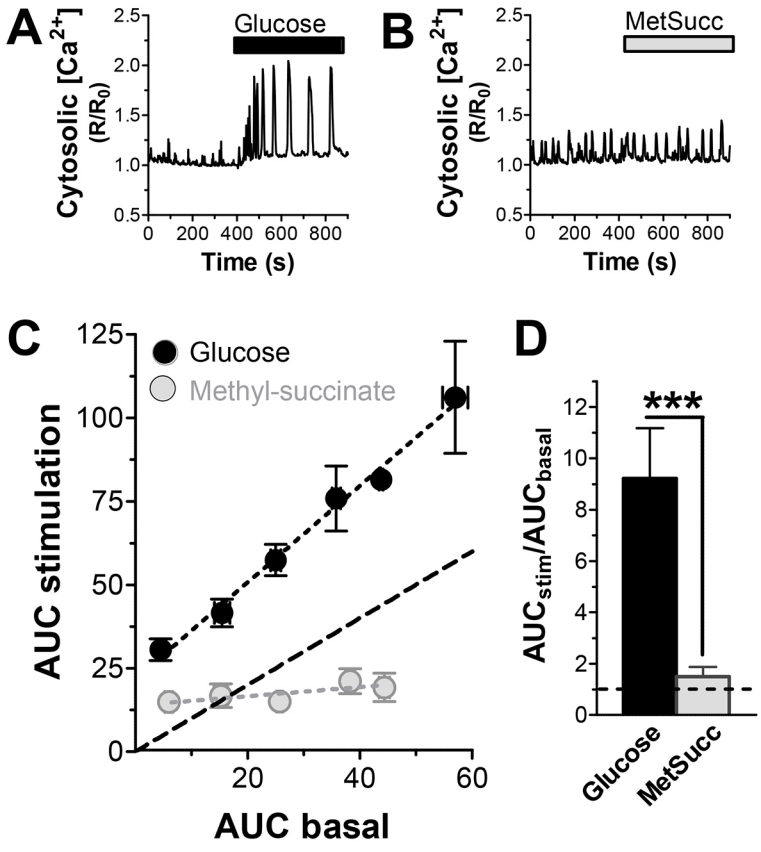

Fig. 4. Weak-to-absent intracellular Ca^{2+} responses to methylsuccinate. (A–D) Bi-parametric analysis of Ca^{2+} signals measured in INS-1E cells expressing the cytosolic Ca^{2+}-probe YC3.6, and stimulated with glucose or methylsuccinate. (A,B) Examples of cytosolic Ca^{2+} responses in individual INS-1E cells stimulated with 16.7 mM glucose (A) or 5 mM methylsuccinate (MetSucc, B) as indicated. The ratiometric signals were normalized to basal conditions (set to 1). (C) Single-cell amplitude of Ca^{2+} responses as a function of the basal Ca^{2+} signal amplitude, measured in glucose-stimulated (16.7 mM, black circles) or methylsuccinate-stimulated (5 mM, gray circles) cells. The total Ca^{2+} rises before and during stimulation were measured for each trace (cell) by integrating the area under the curve during 5 min. Data were aggregated for different ranges of $[Ca^{2+}]$ values and expressed as a function of basal $[Ca^{2+}]$. Dashed lines show the linear regressions through the data. (D) Statistical evaluation of the data shown in C, showing the total Ca^{2+} increase during stimulation, normalized to the related basal Ca^{2+} elevations. Data are mean±s.e.m. of 62 (n=5) and 53 cells (n=6) for glucose- (black) and methylsuccinate-stimulated (gray) cells, respectively. ***P<0.001 (Student's t-test).

Journal of Cell Science

Journal of Cell Science (2017) 130, 1929-1939 doi:10.1242/jcs.200741

monocarboxylate transporter, which allows them to take up pyruvate (Ishihara et al., 1999). As a consequence pyruvate can act as a secretagogue in INS-1E cells. Consistent with these findings, pyruvate caused a rapid and marked acceleration of respiration (Fig. 5A). Maximal stimulation of INS-1E mitochondria required a concentration of 5 mM pyruvate with no further acceleration at higher concentrations (Fig. 5A). Direct comparison of glucose and pyruvate responses showed that both induced a biphasic activation of INS-E cell mitochondria (Fig. 5B), and the initial increase was even more pronounced when pyruvate was added. Like glucose, pyruvate was able to activate ATP-synthase-dependent respiration as oligomycin strongly reduced pyruvate-induced respiration (Fig. 5B). Consistent with the respiration experiments, insulin secretion (Fig. 5C), NAD(P)H production (Fig. 5D) and cytosolic Ca^{2+} signals (Fig. 5E) were strongly promoted by pyruvate, to a similar degree as seen with glucose.

We conclude that glycolysis is not the reason why glucose and methylsuccinate use distinct mechanisms to activate insulin-secreting cells. Signals downstream of pyruvate transport (e.g. mitochondrial reactions) appear to be the main drivers of the methylsuccinate-dependent pathway.

Glucose and methylsuccinate promote distinct mitochondrial and cytosolic redox signals

Based on the differences in NAD(P)H responses and on the conclusion that relevant reactions should occur inside mitochondria, we considered the possibility that the two substrates affect redox signaling differently. Matrix redox signals are of central importance in the regulation of mitochondrial function (Daiber, 2010; Handy and Loscalzo, 2012; Mailloux et al., 2014; Santo-Domingo et al., 2015) and can be regulated by Ca^{2+} (Santo-Domingo et al., 2015). Glucose and methylsuccinate promote distinct Ca^{2+} signals (Fig. 4), and therefore potentially different redox reactions. Nutrients can simultaneously activate mitochondrial redox reactions, which can have either oxidizing or reducing effects on the matrix redox state (Santo-Domingo et al., 2015). To shed further light on substrate-dependent redox signaling, we used the mitochondrial-targeted redox-sensitive sensor roGFP1 (Fig. 6A; Hanson et al., 2004). Two cysteine groups in roGFP1 undergo reversible changes between an oxidized and a reduced form, altering the emission spectrum of the probe. The sensor is in equilibrium with the mitochondrial glutathione redox couple (GSSG and 2GSH) (Schwarzlander et al., 2015). The fluorescence signal increased during glucose stimulation (Fig. 6B), indicating a more reduced state of the mitochondrial glutathione pool. For normalization, hydrogen peroxide and dithiothreitol were used at the end of each measurement to obtain a minimal (fully oxidized) and maximal (fully reduced) value, respectively (inset, Fig. 6B). Although methylsuccinate also promoted a reduction of the mitochondrial matrix (Fig. 6B), the redox response was ∼50% smaller compared to that seen with glucose (Fig. 6C). In β-cells, the cytosolic and mitochondrial redox state are coupled through the glycerophosphate and malate or aspartate shuttle systems (Eto et al., 1999a,b; Marmol et al., 2009). We therefore considered the possibility that those redox effects could also influence the cytosolic redox state. To test this, INS-1E cells were transfected with the cytosolic roGFP sensor (Fig. 6D). Glucose promoted a small but significant increase in the roGFP signal, indicative of the reduction of the cytosolic glutathione pool (Fig. 6E,F). Following methylsuccinate stimulation, cytosolic redox changes were much smaller than in response to glucose (Fig. 6E). To assess the importance of a change in redox environment in methylsuccinate-stimulated insulin secretion, we tested the effect of some well-known redox modulators on granule exocytosis (Fig. 7A). The presence of the ROS scavengers N-acetylcysteine (NAC, Zafarullah et al., 2003) promoted an ∼35% increase in methylsuccinate-stimulated insulin secretion. Our results show that a shift to a more reducing environment can favor metabolism–secretion coupling in response to methylsuccinate. Interestingly, no significant change was recorded in the presence of the pro-oxidant ter-Butylhydroquinone (Holowiecki et al., 2016), nor with the

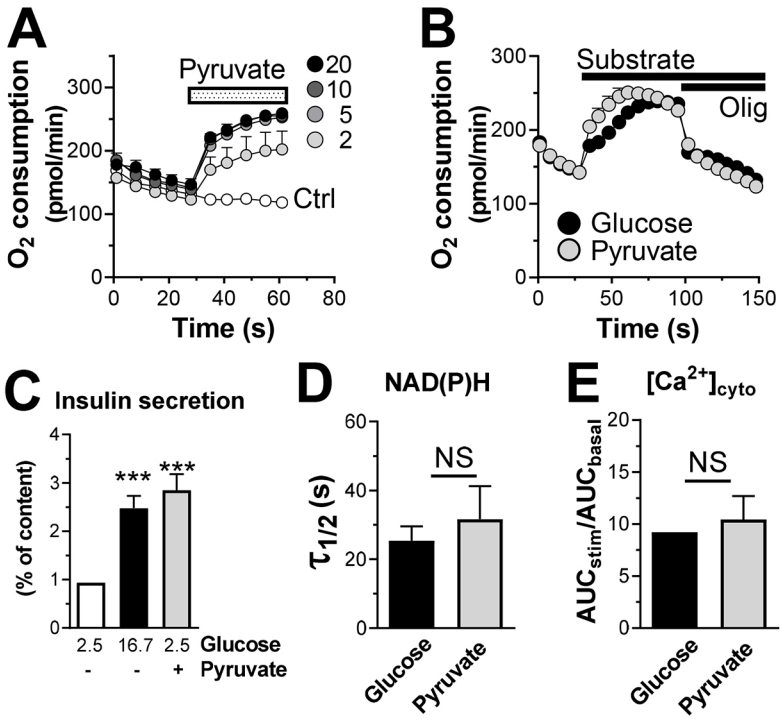

Fig. 5. Pyruvate, despite bypassing glycolysis, promotes the same signal transduction pathway as glucose. (A) Oxygen consumption was assayed in standard KRBH containing basal 2.5 mM glucose. Cells were stimulated by adding pyruvate at the indicated concentrations (in mM). (B) The maximal respiration profiles were compared by stimulating the cells with 16.7 mM glucose (black circles) or 5 mM pyruvate (gray circles). Oligomycin (Olig; 2.5 µg/ml) was added. In A and B, representative results (mean±s.e.m.) from one of three independent experiments performed in triplicate are shown. (C) Static insulin secretion from INS-1E incubated for 30 min in the presence of resting (white bar) and stimulatory glucose (black bar, 16.7 mM) concentrations, or pyruvate (5 mM, gray bar). Shown is the mean±s.e.m. from three measurements from one experiment. The experiment was repeated four times. (D,E) Effect of glucose (16.7 mM) and pyruvate (5 mM) on NADH(P)H production (D) and cytosolic Ca^{2+} rise (E). (D) The calculated half-time to reach a new steady-state of the NAD(P)H as the mean±s.e.m. from five or four independent experiments for glucose- (black) and pyruvate-stimulated (gray) cells, respectively. (E) The statistical evaluation of cytosolic Ca^{2+} rises quantified as described in Fig. 4D. Data are mean±s.e.m. of 62 (n=5) and 41 cells (n=3) for glucose- (black) and pyruvate-stimulated (gray) cells, respectively. ***P<0.0001; NS, not significant (Student's t-test).

Journal of Cell Science

Journal of Cell Science (2017) 130, 1929-1939 doi:10.1242/jcs.200741

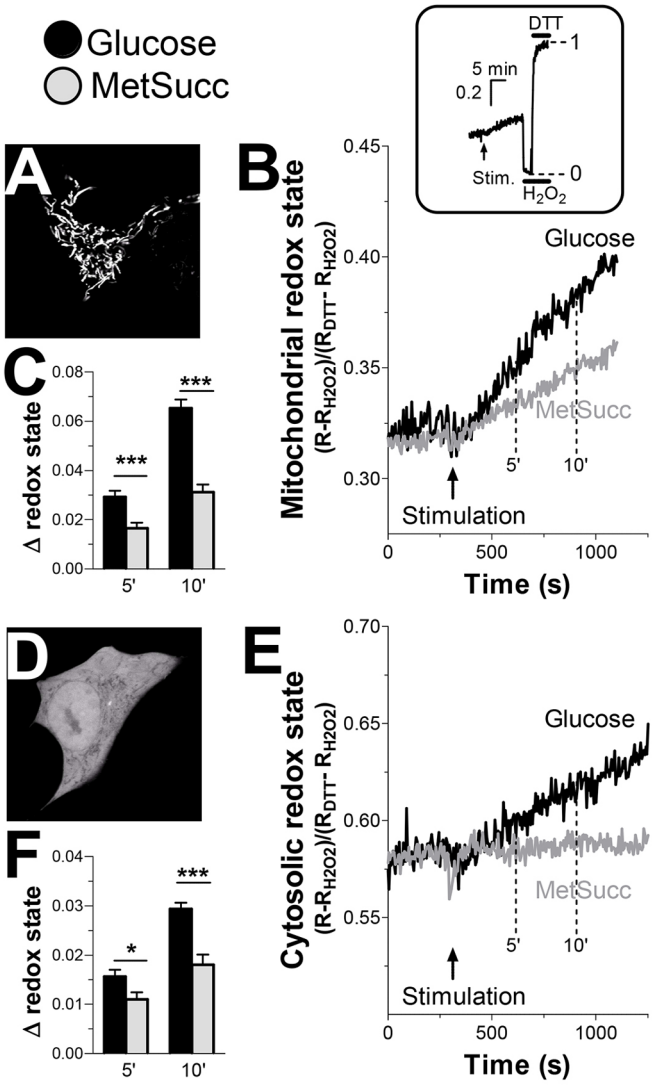

Fig. 6. Methylsuccinate promotes a less marked reduction of the redox state of thiols in both the mitochondrial matrix and the cytosol. (A) Mitochondrial ro-GFP fluorescence pattern in INS-1E cells. (B) Mitochondrial ro-GFP signals, recorded in a single cell at 490 nm. Changes in ratio of mitochondrial roGFP fluorescence (ratio of signal at 480 nm to that at 410 nm) evoked by glucose (raised from 2.5 to 16.7 mM, black trace) or methylsuccinate (MetSucc 5 mM, gray trace). The fluorescence ratio was normalized to the levels achieved subsequently with 10 mM H_2O_2 (value=0) and 100 mM DTT (value=1), as shown in the inset. (C) Statistical evaluation of the effect of glucose and methylsuccinate on the amplitude of the mitochondrial evoked response, measured 5 min and 10 min after addition. Data are mean±s.e.m. of 68 (n=9, black) and 83 cells (n=11) for glucose- (black) and methylsuccinate-stimulated cells (gray). (D–F) Cytosolic and nuclear ro-GFP fluorescence pattern in INS-1E cells. (D) Cytosolic and nuclear ro-GFP signals, recorded in a single cell at 490 nm. (E) Changes in cytosolic roGFP fluorescence (ratio of signal at 480 nm to that at 410 nm) evoked by glucose (raised from 2.5 to 16.7 mM, black trace) or methylsuccinate (5 mM, gray trace). The fluorescence ratio is normalized as shown in the inset to B. (F) Statistical evaluation of the effect of glucose and methylsuccinate on the amplitude of the evoked cytosolic response measured 5 min and 10 min after addition. Data are mean±s.e.m. of 77 (n=7), and 76 cells (n=7) for glucose- (black) and methylsuccinate-stimulated cells (gray). *P<0.05; ***P<0.001 (Student's t-test).

ROS generator menadione (De Marchi et al., 2003). These results indicate a complex relationship between redox signaling and methylsuccinate-dependent secretion.

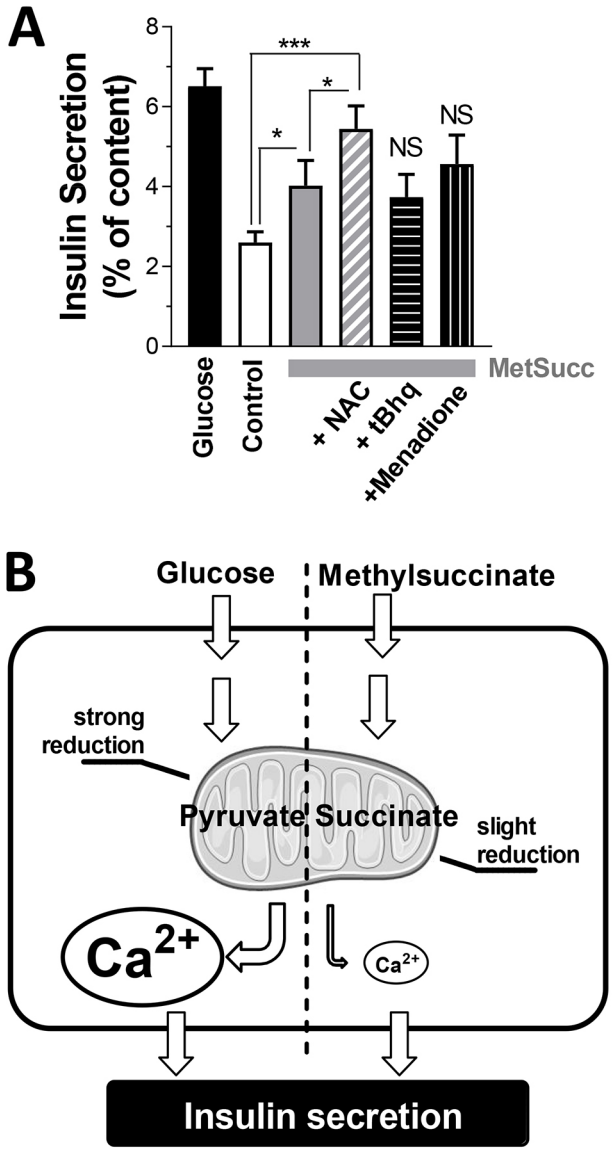

Fig. 7. Effect of redox-modulators on methylsuccinate-stimulated insulin secretion, and proposed model for two distinct mitochondrial pathways activating insulin-secreting cells. (A) Static insulin secretion from INS-1E cells incubated for 30 min with 16.7 mM glucose or 5 mM methylsuccinate in the presence of redox modulators. Insulin secretion was stimulated with methylsuccinate as indicated and co-incubated in the presence of 500 µM N-acetyl cysteine (NAC); 200 µM ter-Butylhydroquinone (tBhq) or 50 µM 2-methyl-1,4-naphtoquinone (Menadione). Mean±s.e.m. from nine measurements from three experiments. *P<0.05; ***P<0.0001; NS, not significant (Student's t-test). (B) Following nutrient stimulation, β-cell mitochondria can be activated to promote insulin secretion using at least two different mechanisms. The canonical oligomycin-dependent pathway activated by glucose (left) is characterized by massive Ca^{2+} rises and a strong reduction of the mitochondrial and cytosolic glutathione pools. An alternative oligomycin-independent pathway (right), depending on permissive cytosolic Ca^{2+} and accompanied by smaller mitochondrial glutathione reduction is achieved by activating mitochondrial complex II with succinate.

Taken together, these results show that glucose favors the formation of reduced glutathione in both compartments. Methylsuccinate induces a weaker matrix redox response and has almost no impact on the cytosolic redox state. Redox and Ca^{2+} signatures of nutrient-induced insulin secretion, demonstrate the existence of two distinct pathways of β-cell activation.

Journal of Cell Science (2017) 130, 1929-1939 doi:10.1242/jcs.200741

DISCUSSION

Mitochondria link nutrient metabolism to the generation of signals that induce insulin granule exocytosis (De Marchi et al., 2014b; Wiederkehr et al., 2011; Wiederkehr and Wollheim, 2008). The classical pathway induced by glucose depends on the stimulation of mitochondrial ATP synthesis and a pronounced rise in cytosolic Ca^{2+}, stimulating insulin exocytosis. This triggering pathway is amplified by other factors that further augment insulin secretion. The triggering pathway induces Ca^{2+} signals essential for insulin exocytosis. The elucidation of this mechanism and alternative pathways promoting insulin secretion may lead to the identification of novel targets to restore β-cell function in type 2 diabetes. Here, we report on the existence of an alternative fuel-dependent pathway leading to insulin granule exocytosis. This novel mechanism occurs largely independently of mitochondrial ATP synthesis, requires almost no rise in cytosolic Ca^{2+} and is characterized by a distinct redox signature (Fig. 7B). We have uncovered this unusual pathway while studying mitochondrial activation during methylsuccinate-induced insulin secretion.

For direct comparison of different fuel substrates, we determined the minimal concentration of the substrates that maximally activate oxygen consumption. To establish how fuels stimulate mitochondrial activation, this approach is more suitable than the comparison of substrates at equimolar concentration (Antinozzi et al., 2002). Methylsuccinate gives rise to succinate, which is a direct substrate of complex II of the respiratory chain. As such, methylsuccinate was able to cause a step increase in respiration. In the case of glucose, this early acceleration is followed by a slower gradual increase in respiratory rate. As a result, ATP-synthase-dependent respiration increases with time following glucose stimulation. In contrast, a second gradual increase was not observed in methylsuccinate-stimulated cells and no further acceleration of respiration could be achieved by using higher concentrations of methylsuccinate. More importantly, only a very small fraction of methylsuccinate-induced respiration was dependent on ATP-synthase. The results revealed that although methylsuccinate accelerates respiration, the augmentation of mitochondrial ATP synthesis may be small. Consistent with this interpretation, in response to methylsuccinate the ATP-to-ADP ratio increased slowly. At 20 min after methylsuccinate stimulation the ATP-to-ADP ratio was significantly higher than in control cells but the ratio remained about two-fold lower than in glucose-activated INS-1E cells.

Methylsuccinate hyperpolarizes the mitochondrial membrane potential by acting as a substrate of the respiratory chain. Surprisingly, this hyperpolarization was only transient despite the continued presence of the substrate. The reasons for this are unknown. A possible explanation is that following its accumulating in the mitochondrial matrix, succinate may slow upstream metabolism leading to impaired citric acid cycle flux. In addition, fumarate, the oxidation product of succinate, may accumulate with time, slowing succinate dehydrogenase-dependent formation of $FADH_2$. However, this interpretation does not seem likely given our NAD(P)H measurements. Methylsuccinate is able to raise NAD(P)H and the levels remain elevated in the continuous presence of the substrate. These data demonstrate that sufficient NADH is available as an electron donor for the respiratory chain. The NAD(P)H response to methylsuccinate is much slower, and at steady-state the NAD(P)H signal is significantly lower than in glucose- or pyruvate-treated cells. This lower steady-state NAD(P)H signal may in part explain reduced respiration and mitochondrial ATP synthesis but not the failure to cause continuous hyperpolarization of the mitochondrial inner membrane. Based on these marked differences in mitochondrial activation, in particular the poor stimulation of ATP synthase, we propose that methylsuccinate stimulates insulin secretion through a pathway that is less dependent on changes in the ATP-to-ADP ratio and the control of the K_{ATP} channel.

Consistent with the literature, the observed increase in ATP-to-ADP ratio following glucose stimulation resulted in enhanced Ca^{2+} signaling by increasing the amplitude and/or frequency of the Ca^{2+} spikes. Methylsuccinate failed to induce a Ca^{2+} response in most cells. The difference was most striking in cells displaying rather strong basal Ca^{2+} activity. In this subpopulation of cells, methylsuccinate seemed to even dampen Ca^{2+} signaling. In INS-1E cells Ca^{2+} signaling is very heterogeneous at the single-cell level. We therefore applied a detailed analysis describing the Ca^{2+} responses as a function of the Ca^{2+} signaling prior to nutrient stimulation. Glucose-dependent Ca^{2+} responses were proportional to basal Ca^{2+} signaling. Strong basal Ca^{2+} activity was associated with larger glucose-induced Ca^{2+} spikes. This was strikingly different for methylsuccinate, where only cells with little or no basal Ca^{2+} showed an increased the signal when methylsuccinate was added. In virtually all cells that displayed Ca^{2+} signaling under resting conditions, the Ca^{2+} spikes remained the same or were reduced. Previous reports comparing the effect of glucose and methylsuccinate on Ca^{2+} signaling have obtained conflicting results (Heart et al., 2007; Mukala-Nsengu et al., 2004), but quantification of single-cell Ca^{2+} responses was missing. The poor Ca^{2+} responses may explain the partial inability of methylsuccinate to efficiently activate mitochondrial ATP synthesis. We have previously demonstrated that matrix Ca^{2+} plays an important signaling function for sustained activation of mitochondria and, as a consequence, activation of second-phase insulin secretion (De Marchi et al., 2014b; Wiederkehr et al., 2011). When cellular Ca^{2+} uptake is blocked, glucose causes a rapid increase in respiration but the subsequent further acceleration of respiration is missing. In the absence of Ca^{2+} signaling, INS-1E cells and human islets also fail to show enhanced ATP-synthase-dependent respiration (De Marchi et al., 2014b). Similar reduced and transient activation of mitochondria is observed here after stimulation with methylsuccinate. The inability of methylsuccinate to strongly activate ATP-synthase-dependent respiration is likely due to missing mitochondrial Ca^{2+} signals. Although methlysuccinate is unable to induce the pathway triggering insulin secretion, Ca^{2+} influx, such as observed in resting β-cells, remains essential for methylsuccinate-induced insulin secretion. Maintaining the K_{ATP} channel in its open state by using diazoxide suppressed voltage-dependent Ca^{2+} influx and thereby methylsuccinate-dependent insulin secretion. In other words, for the pathway induced by methylsuccinate, a permissive Ca^{2+} concentrations remains essential in order to trigger insulin granule exocytosis, a property also observed by investigators studying the amplifying pathway of insulin secretion (Henquin, 2000). We propose that methylsuccinate prepares the insulin secretory machinery to induce granule exocytosis even in the case of small Ca^{2+} signals that would be inefficient with glucose stimulation, which requires the triggering pathway to promote insulin exocytosis. The mechanism leading to vesicle priming or other events promoting insulin release in response to methylsuccinate remains to be investigated. The unexpected strong component of oligomycin-insensitive insulin secretion triggered by methylsuccinate confirms that the signal transduction pathways are distinct from the one induced by glucose.

The large differences in the kinetics of NAD(P)H production prompted us to investigate the influence of the different substrates on redox signaling. Nutrient stimulation and associated mitochondrial Ca^{2+} rises are able to activate mitochondrial redox

Journal of Cell Science

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reactions, with opposite effect on the mitochondrial matrix redox state (Santo-Domingo et al., 2015). Increased availability of fuel substrates and the concomitant activation of Ca^{2+}-regulated matrix dehydrogenases favor oxidative metabolism, resulting in the enhanced production of NADH and an associated reduction of the redox state (Glancy and Balaban, 2012; Pralong et al., 1990). At the same time, stimulation of respiration generates reactive oxygen species (ROS), promoting a shift to a more oxidized redox environment (Brookes et al., 2004; Kowaltowski et al., 2009). We have previously demonstrated that histamine-induced mitochondrial Ca^{2+} rises have a net reducing effect on matrix glutathione in HeLa cells (De Marchi et al., 2014a). In pancreatic β-cells, glucose stimulation has also been shown to acutely reduce mitochondrial glutathione (Roma et al., 2012; Takahashi et al., 2014). By using the mitochondrial redox sensor mito-roGFP1, we confirm that glucose acutely changes the matrix redox balance, favoring glutathione reduction. Interestingly, the redox response to methylsuccinate was about two-fold slower. Differences in the mitochondrial redox state may help to explain differences in the pathways downstream of mitochondria that are linked to insulin secretion.

Earlier work has identified cytosolic NADPH as a candidate signaling molecule linking metabolism to insulin exocytosis (Ivarsson et al., 2005). These findings were linked to glutaredoxin, which is reduced by glutathione, a molecular process that directly affected the fusion of insulin granules with the plasma membrane. We have therefore also followed the cytosolic redox state. As assessed by using cytosolic roGFP, we find that glucose induced a slight but reproducible reduction also in the cytosolic redox state. Methylsuccinate failed to alter the cytosolic redox state. A possible explanation is that metabolic effects promoting reduction and the concomitant production of ROS induced by succinate are counteracting each other, canceling out a redox-mediated effect. The inability of methylsuccinate to raise cytosolic glutathione levels may be due to enhanced ROS production. ROS have been proposed by a number of investigators to also have signaling functions in the pancreatic β-cell. For example, Leloup and collaborators have provided evidence that mitochondrial ROS are obligatory signals for glucose-induced insulin secretion (Leloup et al., 2009). In addition, it has been recently reported that ROS stimulate basal insulin secretion in rat pancreatic islets, without increasing intracellular Ca^{2+} (Saadeh et al., 2012). Our data showing that methylsuccinate-dependent secretion does not induce cytosolic Ca^{2+} signaling would therefore be consistent with redox modulation of exocytosis. We propose that the large body of published evidence on redox signaling and insulin secretion could be rationalized by considering the combined effects of redox reactions and Ca^{2+}. Combining large cytosolic Ca^{2+} rises with a strong reduction of the redox environment leads to insulin secretion. Alternatively, a shift towards a less reduced state, including the possible formation of ROS at the level of mitochondria, may stimulate insulin secretion requiring only permissive Ca^{2+} levels (Fig. 7). Although several aspects of this new pathway proposed here (Fig. 7B) remain speculative, redox events are known to contribute to the regulation of insulin secretion (Ježek et al., 2012). Consistent with this, a significant effect of redox-active compounds on β-cell function has been demonstrated (Kaneto et al., 1999; Tanaka et al., 1999). Such compounds are even able to exert beneficial effect in animal models of diabetes. Succinate dehydrogenase has been shown to generate ROS at high rates (Quinlan et al., 2012). This led to the speculation that methylsuccinate may promote insulin secretion through enhanced ROS formation. Surprisingly, we observe that the well-known anti-oxidant NAC (Zafarullah et al., 2003) enhances insulin exocytosis in our model, supporting a role for reducing reactions on metabolism–secretion coupling. In contrast, the pro-oxidants menadione and ter-Butylhydroquinone were ineffective in promoting secretion in methylsuccinate-stimulated insulin-secreting cells, demonstrating the existence of additional levels of complexity in the redox modulation of exocytosis. The importance of the spatio-temporal control or the specificity of ROS generated may explain our unexpected results. We speculate that only ROS produced in association with the respiratory chain or more specifically succinate dehydrogenase have the potential to influence insulin secretion. Alternatively, to modulate the here-described novel pathway, specific ROS in the proper concentration may be required.

In summary, we provide evidence for a novel pathway downstream of mitochondrial activation leading to the promotion of insulin secretion. The mechanism of this pathway is dependent on a very small intracellular Ca^{2+} increase, with small effects on mitochondrial ATP production, but that is essential for vesicles exocytosis. Although this mechanism is mainly non-bioenergetic, it relies on mitochondrial activation and is accompanied by distinct matrix redox changes. The study of this novel mechanism, may lead to the identification of novel targets to restore β-cell function in type 2 diabetes.

MATERIALS AND METHODS

Reagents and constructs

Chemicals were from Sigma, Invitrogen or VWR, unless otherwise indicated. The mitochondrial and the cytosolic redox indicators roGFP1 (Hanson et al., 2004) were provided by S. James Remington (University of Oregon, Eugene, OR). The $YC3.6_{cyto}$ pcDNA3 was kindly provided by Atsushi Miyawaki (Riken Brain Science Institute, Wako, Japan) (Nagai et al., 2004).

INS-1E cell culture

INS-1E cells, obtained from Claes Wollheim (University of Geneva, Geneva, Switzerland), were cultured at 37°C in a humidified atmosphere (5% CO_2) in RPMI-1640 medium (Invitrogen) containing 11 mM glucose, supplemented with 10 mM Hepes (pH 7.3), 10% (v/v) heat-inactivated fetal calf serum (FCS; Brunschwig AG, Switzerland), 1 mM sodium pyruvate, 50 µM β-mercaptoethanol, 50 µg/ml penicillin and 100 µg/ml streptomycin. Cells were authenticated and tested for contamination.

Oxygen consumption measurements

Oxygen consumption in INS-1E cells was measured by using a XF96 instrument (Seahorse Biosciences, MA). INS-1E cells were seeded into polyornithine-coated Seahorse tissue culture plates at a density of 20,000 cells per well. After 2 days, the cells were washed twice in Krebs–Ringer bicarbonate Hepes buffer (KRBH), containing (in mM): 140 NaCl, 3.6 KCl, 0.5 NaH_2PO_4, 0.5 $MgSO_4$, 1.5 $CaCl_2$, 10 Hepes, 5 $NaHCO_3$ pH 7.4, and 2.5 mM glucose. Respiration rates were determined every 6 min. All experiments were performed at 37°C. The ATP-synthase inhibitor oligomycin (2.5 µg/ml) was added as indicated in the figures.

Static insulin secretion

INS-1E cells were plated on a polyornithine-treated 24-well plate. After 48 h, cells were washed in KRBH containing 2.5 mM glucose. Then cells were stimulated for 30 min with 16.7 mM glucose, or 5 mM methylsuccinate or 5 mM pyruvate. Supernatant were collected. Cells were lysed with a mixture of ethanol (75%) and HCl (1.5%) to determine insulin release and cell content using Rat Insulin Enzyme Immunoassay kit (SpiBio, France).

Single-cell imaging of cytosolic Ca^{2+} signals

Cytosolic Ca^{2+} was measured with the genetically encoded cameleon sensors $YC3.6_{cyto}$ (Nagai et al., 2004). INS-1E cells were plated on polyornithin-treated 35-mm-diameter glass-bottom dishes (MatTek, MA) and transfected with pcDNA3 vector carrying the Ca^{2+} sensor, using

JetPRIME reagent (Polyplus transfection). At 2 days after transfection cells were washed four times and experiments were performed at 37°C in KRBH buffer. Glass coverslips were inserted in a thermostatic chamber (Life Imaging Services). Cells were imaged on a DMI6000 B inverted fluorescence microscope, using a HCX PL APO 40×1.30 NA oil immersion objective (Leica Microsystems) and an Evolve 512 back-illuminated CCD with 16×16 µm pixel camera (Photometrics, Tucson, Arizona). Cells were excited at 430 nm through a BP436/20 filter. The two emission images were acquired with BP480/40 and BP535/30 emission filters. Fluorescence ratios were calculated in MetaFluor 7.0 (Meta Imaging Series) and analyzed in Excel (Microsoft) and GraphPad Prism 5 (GraphPad). Images were taken every 2 s.

Mitochondrial membrane potential

Cells were loaded with 10 nM tetramethylrhodamine methyl ester (TMRM; Molecular Probes) for 45 min at 37°C, dissolved in KRBH buffer. Cells were excited at 545 nm through a BP 546/11 filter and fluorescence collected through a BP 605/75 filter. Changes in the mitochondrial membrane potential ($\Delta\psi_m$) were expressed as the ratio of the fluorescence in mitochondria divided by the cytosolic fluorescence (F_{mito}/F_{cyto}), measured in the same cells, as previously described (Da Cruz et al., 2010). At the end of the recording the protonophore carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) was used to dissipate $\Delta\psi_m$. Fluorescence ratios were determined as explained for Ca^{2+} measurements. Data were normalized to both basal fluorescence (=1) and the minimal value (FCCP=0).

Cellular ADP and ATP

ADP and ATP were measured enzymatically as described previously (Schultz et al., 1993). Cell extracts were prepared from INS-1E cells incubated in standard KRBH.

NAD(P)H measurements

Cells were allowed to adhere to glass-bottom dishes (MatTek, Ashland, MA) for 2 days and experiments were performed in the KRBH solution as described above. A laser-scanning confocal microscope (Leica TCS SP5 II MP, Mannheim, Germany) with a HCX IRAPO L 25x/0.95 water objective was utilized to monitor NAD(P)H autofluorescence at 37°C (Life Imaging Services). Laser-scans at 727 nm (IR Laser Chameleon ultra, Coherent) were used for two-photon NAD(P)H excitation. Each 512×512 pixel image represents an average of 16 scans taken with a resonant scanner at 8000 Hz. NAD(P)H emission was collected at 445–495 nm wavelength every 20 s. Data were processed with LAS AF software (Leica) and analyzed in Excel (Microsoft) and GraphPad Prism 5 (GraphPad). In order to normalize the NAD(P)H responses, each experiment was terminated by adding hydrogen peroxide, which results in an almost complete loss of autofluorescence (minimal value). Fluorescence intensity data were normalized to both basal autofluorescence (=1) and the minimal value (hydrogen peroxide=0). Change in NAD(P)H fluorescence responses were fitted with a one-phase exponential association function to extract the half-time (τ).

Mitochondrial and cytosolic redox signals measurements

Ratiometric measurements of the mitochondrial redox state were performed using the same instrument as described for $[Ca^{2+}]_{mt}$ measurements with the mitochondrial-targeted, genetically encoded sensor roGFP1 (Hanson et al., 2004). Cells were excited at 410 and 480 nm and emission was collected at 535 nm (535DF45, Omega Optical) through a 505DCXR (Omega Optical) dichroic mirror. Images were acquired every 2 s. The 480-to-410 fluorescence ratios were normalized on both the minimum of fluorescence (obtained after addition of 1 mM H_2O_2) and the maximum (obtained after addition of 10 mM dithiothreitol, DTT).

Statistics

The significance of differences between means was established using the Student's *t*-test for unpaired samples *P<0.05; **P<0.01; ***P<0.001; NS, not significant.

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Competing interests

The authors are employees of Nestlé Institute of Health Sciences, which is part of the Nestlé Group.

Author contributions

Conceptualization: U.D.M., A.W.; Methodology: J.S., D.B.; Validation: A.H.; Formal analysis: A.H., J.T., Y.R.; Investigation: U.D.M., A.H., J.T., Y.R.; Writing - original draft: U.D.M.; Writing - review & editing: U.D.M., D.B., A.W.; Visualization: U.D.M.; Supervision: U.D.M., A.W.; Project administration: U.D.M.

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Journal of Cell Science

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