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Epigallocatechin-3-gallate (EGCG) activates AMPK through the inhibition of glutamate dehydrogenase in muscle and pancreatic ß-cells: A potential beneficial effect in the pre-diabetic state?

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ABSTRACT

Glucose homeostasis is determined by insulin secretion from the ß-cells in pancreatic islets and by glucose uptake in skeletal muscle and other insulin target tissues. While glutamate dehydrogenase (GDH) senses mitochondrial energy supply and regulates insulin secretion, its role in the muscle has not been elucidated. Here we investigated the possible interplay between GDH and the cytosolic energy sensing enzyme 5′-AMP kinase (AMPK), in both isolated islets and myotubes from mice and humans. The green tea polyphenol epigallocatechin-3-gallate (EGCG) was used to inhibit GDH. Insulin secretion was reduced by EGCG upon glucose stimulation and blocked in response to glutamine combined with the allosteric GDH activator BCH (2-aminobicyclo-[2,2,1] heptane-2-carboxylic acid). Insulin secretion was similarly decreased in islets of mice with ß-cell-targeted deletion of GDH (ß*Glud1*$^{-/-}$). EGCG did not further reduce insulin secretion in the mutant islets, validating its specificity. In human islets, EGCG attenuated both basal and nutrient-stimulated insulin secretion. Glutamine/BCH-induced lowering of AMPK phosphorylation did not operate in ß*Glud1*$^{-/-}$ islets and was similarly prevented by EGCG in control islets, while high glucose systematically inactivated AMPK. In mouse C2C12 myotubes, like in islets, the inhibition of AMPK following GDH activation with glutamine/BCH was reversed by EGCG. Stimulation of GDH in primary human myotubes caused lowering of insulin-induced 2-deoxy-glucose uptake, partially counteracted by EGCG. Thus, mitochondrial energy provision through anaplerotic input via GDH influences the activity of the cytosolic energy sensor AMPK. EGCG may be useful in obesity by resensitizing insulin-resistant muscle while blunting hypersecretion of insulin in hypermetabolic states.

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1. Introduction

In obese subjects, the pre-diabetic state is characterized by hyperinsulinemia secondary to insulin resistance (Kasuga, 2006), which may lead to diabetes in case of subsequent ß-cell failure (Prentki and Nolan, 2006). However, ß-cell-targeted genetic intervention in mice protects against obesity, either by the reduction of insulin gene dosage (Mehran et al., 2012) or by the ablation of the amplifying pathway of glucose-stimulated insulin secretion (Vetterli et al., 2016a). Clinical data have shown that pharmacological inhibition of insulin secretion in obese subjects can promote weight loss (Lustig et al., 2006; van Boekel et al., 2008), although drugs used in these studies are associated with undesired side effects, such as hyperglycemia. Weight control by lifestyle modification has a low success rate, therefore calling for alternative therapy. In this context, green tea has been widely investigated. It is rich in polyphenols including epigallocatechin-3-gallate (EGCG) and epicatechin-3-gallate (ECG), which cause weight reduction in various animal models (Sae-tan et al., 2011; Wang et al., 2014) and obese subjects (Basu et al., 2010). However, human trials have

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shown some inconsistencies in this regard probably due to the heterogeneity of both protocols and populations used (for review see (Wang et al., 2014; Hursel et al., 2009; Phung et al., 2010)).

The mechanism of action of EGCG remains obscure, although its antioxidant effect is regularly emphasized. In addition, inhibition of the mitochondrial enzyme glutamate dehydrogenase (GDH) by EGCG in the μmolar range has been reported (Li et al., 2012). GDH catalyses the following reaction: α-ketoglutarate + NH_3 + NADH ↔ glutamate + NAD^+. Its direct link to the TCA cycle makes GDH the pivotal regulator of, on the one hand, carbohydrate-induced carbon efflux (cataplerosis) and, on the other hand, amino acid-mediated influx into the TCA cycle (anaplerosis). Moreover, GDH is allosterically regulated by leucine as well as pyridine, adenine and guanine nucleotides; being inhibited by GTP and activated by ADP (Fisher, 1985; Smith et al., 2001). In pancreatic ß-cells, cataplerosis participates in the amplifying pathway of insulin secretion (Vetterli et al., 2016b). Conversely, GDH-mediated anaplerosis, evoked for instance by glutamine, is not favoured in ß-cells but can be prompted by the allosteric activator BCH (2-aminobicyclo-[2,2,1] heptane-2-carboxylic acid) resulting in insulin release (Carobbio et al., 2004; Sener et al., 1981) or by activating mutations causing a syndrome of hyperinsulinism (Stanley et al., 1998).

Glucose-stimulated insulin secretion from the pancreatic ß-cell comprises two modalities, the obligatory calcium-mediated signalling and the more long-lasting amplifying pathway. The latter depends on the cataplerotic activity of GDH (Vetterli et al., 2012) and its genetic abrogation protects against obesity (Vetterli et al., 2016a). In the present study, we tested the pharmacological inhibition of GDH on insulin secretion in mouse and human islets. Moreover, the putative implication of GDH in insulin action was monitored through glucose uptake in human myotubes as well as the activity of the energy sensor AMPK, shown to be stimulated by EGCG (Wu et al., 2014).

2. Materials and methods

2.1. Pancreatic islet and cell preparations

Mouse pancreatic islets were obtained from either C57BL/6J wild type mice or ß-cell-specific GDH knockout mice (ß*Glud1*$^{-/-}$) described previously (Carobbio et al., 2009). Islets were isolated by collagenase digestion (collagenase P, Roche) as detailed elsewhere (Carobbio et al., 2004) and cultured overnight free floating in RPMI-1640 medium (Gibco) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 10 mM Hepes, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate and 50 μM 2-mercaptoethanol. Human islets were isolated from pancreases of deceased multiorgan donors, who had provided written informed consent (ECIT consortium), and maintained in CMRL-1066 before experiments (Vetterli et al., 2011). Rat insulinoma INS-1E ß-cells were cultured in RPMI-1640 as detailed previously (Merglen et al., 2004).

2.2. Epigallocatechin-3-gallate (EGCG) preparation

EGCG stock solution (4 mM, Sigma-Aldrich) was prepared in 1 mM ascorbic acid and stored at −20 °C. Since EGCG is unstable and its concentration decreases rapidly in cell culture medium, with a half-life of about 30 min (Sang et al., 2005), 0.5 mM ascorbic acid was added freshly in all media and buffers used for treatment of islets, INS-1E cells, and myotubes (Lambert et al., 2006).

2.3. Insulin secretion, calcium measurements, ATP and amino acid measurements

Mouse islets were maintained for 2 h in glucose- and glutamine-free RPMI-1640 medium in the absence or presence of 20 μM EGCG and then washed in Krebs Ringer bicarbonate HEPES buffer (KRBH, containing in mM: 135 NaCl, 3.6 KCl, 10 HEPES (pH 7.4), 5 $NaHCO_3$, 0.5 NaH_2PO_4, 0.5 $MgCl_2$, 1.5 $CaCl_2$, and 0.1% bovine serum albumin, BSA) containing basal (2.8 mM) glucose concentration. Then, islets were handpicked and incubated (10 islets per tube) for 1 h at basal glucose, 22.8 mM glucose, and the combination of 5 mM glutamine plus 10 mM BCH (glutamine + BCH) in the absence or presence of 20 μM EGCG. At the end of the incubation, islets were put on ice before collection of supernatant for insulin measurements by radioimmunoassay (Linco), and finally re-suspended in acid-EtOH to determine their insulin content. Values were expressed as percent of total islet insulin content (Vetterli et al., 2012).

Cytosolic calcium concentration was monitored in INS-1E cells by ratiometric measurements of Fura-2 fluorescence with filters set at 340/380 nm for excitation and 510 nm for emission in multi-well mode as detailed previously (Rubi et al., 2005). ATP levels were assessed in INS-1E cells using a Seahorse XF^e24 Flux Analyzer (Seahorse Biosciences) as described (Vanderperre et al., 2016). Amino acid measurements were done by reverse-phase high-performance liquid chromatography (HPLC) after derivatization with O-phthalaldehyde (Vetterli et al., 2012).

2.4. Immunoblotting

About 200 mouse or human islets were treated as mentioned above for insulin secretion. At the end of incubation islet lysates were prepared using lysis buffer (20 mM Tris-HCl, 1 mM EGTA, 1 mM EDTA, 50 mM NaCl, 25 mM NaF, 2 mM orthovanadate, 1% glycerol, 1% Triton X100 and a protease inhibitor cocktail from Roche). Proteins were separated by SDS-PAGE, then blotted onto nitrocellulose membrane Hybond-ECL (Amersham Bioscience), and blocked with 5% nonfat milk in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.02% Tween 20), finally probed with antibodies against phospho-AMPKα Thr172, AMPKα (Cell Signaling Technology), and actin (Sigma-Aldrich); then incubated with secondary horseradish peroxidase-coupled antibody, donkey anti-rabbit IgG ECL (Amersham Bioscience), goat anti-mouse IgG (Sigma-Aldrich), respectively. The target proteins were visualized by chemiluminescence (ECL Super- Signal West Pico Chemiluminescent, Pierce), analyzed using the ChemiDoc XRS System (Bio-Rad), and quantified by ImageJ (Schindelin et al., 2015).

2.5. Preparation of myotubes

Mouse C2C12 skeletal muscle cells were grown in Dulbecco's modified Eagle's medium (DMEM 25 mM glucose, Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (referred to as growth medium) before differentiation by incubating cells in DMEM supplemented with 2% horse serum (referred to as differentiation medium). C2C12 skeletal myotubes were used 7 days after induction of differentiation as described previously (Hirabara et al., 2010). C2C12 myotubes were incubated in the presence of 100 nM insulin for 1 h at 1 mM glucose and glutamine + BCH in the absence or presence of 20 μM EGCG before collection for immunoblotting performed according to the procedure described for islets.

Human skeletal muscle myoblasts were received from Dr. K. Bouzakri (biopsies obtained during abdominal surgery from Rectus abdominus muscle of donors who signed the informed consent at the Geneva University Hospital). The cells were grown in human skeletal muscle medium (Promo cell) before allowed to fuse and

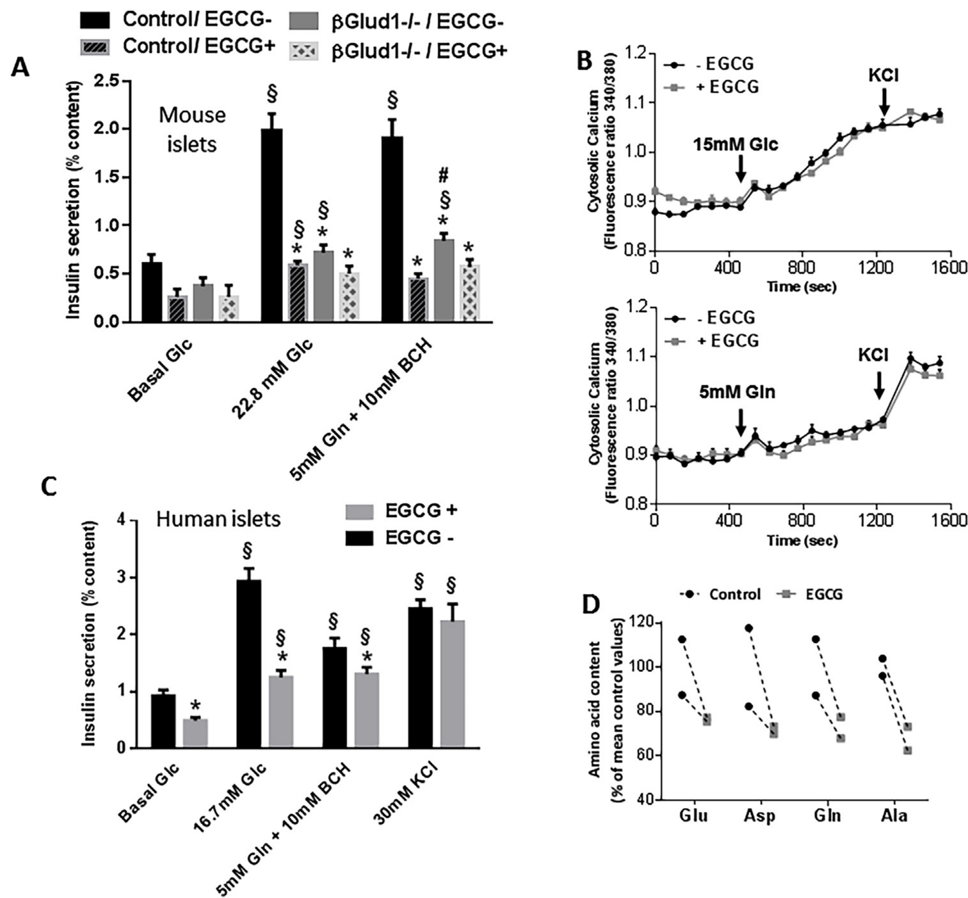

Fig. 1. EGCG targets the GDH enzyme to reduce insulin secretion in mouse and human pancreatic islets. (A) Islets isolated from control and ß-cell specific GDH knockout mice (ß*Glud1*$^{-/-}$) were pre-incubated 2 h in glucose- and glutamine- free RPMI-1640 in the absence or presence of 20 μM EGCG before measurement of insulin secretion over 1 h incubation period at 2.8 mM glucose (Basal), 22.8 mM glucose (Glc), and 5 mM glutamine (Gln) plus 10 mM BCH in the absence or presence of 20 μM EGCG as indicated. §P <0.03 vs. corresponding Basal of the same genotype; *P < 0.002 vs. wild type Control minus EGCG under corresponding condition; #P < 0.01 vs. wild type Control plus EGCG under corresponding condition. Data are means ± SE of 4 independent experiments. (B) Cytosolic calcium levels monitored in INS-1E cells without or with 20 μM EGCG started at basal 2.5 mM Glc followed by stimulation with either 15 mM Glc (upper panel) or 5 mM Gln (lower panel) and then addition of 30 mM KCl (means + SD, n = 5 per group). (C) In human islets, insulin secretion was measured after 1 h incubation at 2.8 mM glucose (Basal Glc), 16.7 mM glucose (Glc), 5 mM glutamine (Gln) plus 10 mM BCH, and 30 mM KCl in the absence or presence of 20 μM EGCG and pre-incubation as for mouse islets. *P < 0.05 vs. minus EGCG group under the same corresponding condition; §P <0.05 vs. Basal of corresponding group. Data are means ± SEM of three organ donors and in every independent experiment 500–1000 isolated islets from each donor were utilized. (D) Amino acid contents of human islets from (C) at the end of the incubation period in response to 16.7 mM Glc. Data are from 2 individual human islet batches expressed relative to means of control values and showing corresponding intra-experiment changes.

differentiate into myotubes in DMEM (5.5 mM glucose, Invitrogen), 2% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Human myotubes were used 7 days after induction of differentiation when most cells are polynucleated (Bouzakri et al., 2011).

2.6. Glucose transport assay

Human myotubes cultured in 6-well plates were starved for 5 h at day 7 post-differentiation in serum-free DMEM and EGCG (20 μM) was added during the last 2 h of starvation. Cells were then pre-incubated 40 min in glucose-free KRBH-BSA 0.1% with 120 nM insulin (Actrapid^{TM}) and glutamine + BCH in the absence or presence of 20 μM EGCG at 37 °C with 5% CO2, before glucose uptake assay over 10 min using [^{3}H]2-deoxyglucose (10 μM, 37′000 Bq/well) as described previously (Bouzakri et al., 2004). At the end of the incubation period medium was rapidly aspirated and cells washed with ice-cold PBS and lysed in NaOH 0.4 N before scintillation counting. Aliquots of cell lysates were used for determination of protein contents by Bradford assay and transport expressed as percent of basal control without insulin.

2.7. Statistical analysis

Statistics were done using the IBM statistics SPSS 21 software. Data are represented as the means ± SEM for at least 3 independent experiments. Differences between ß*Glud1*$^{-/-}$ and control were assessed by one-way ANOVA analysis followed by post hoc multiple comparisons Tukey. P < 0.05 was considered statistically significant.

3. Results

3.1. GDH-dependent effects of EGCG on insulin secretion and calcium levels in rodent and human ß-cells

The concentration of 20 μM EGCG was selected based on both measurements of GDH activity in insulinoma INS-1E ß-cell lysates (data not shown) and previous reports on glutamine oxidation in rat islets (Li et al., 2006). To study whether GDH is the main target of EGCG in ß-cells we first compared the insulin secretory responses of wild type islets with those of ß-cell-specific GDH knockout islets isolated from ß*Glud1*$^{-/-}$ mice (Carobbio et al., 2009). In wild type control mouse islets, 22.8 mM glucose and the combination of 5 mM glutamine plus 10 mM BCH stimulated insulin secretion 3.3-fold

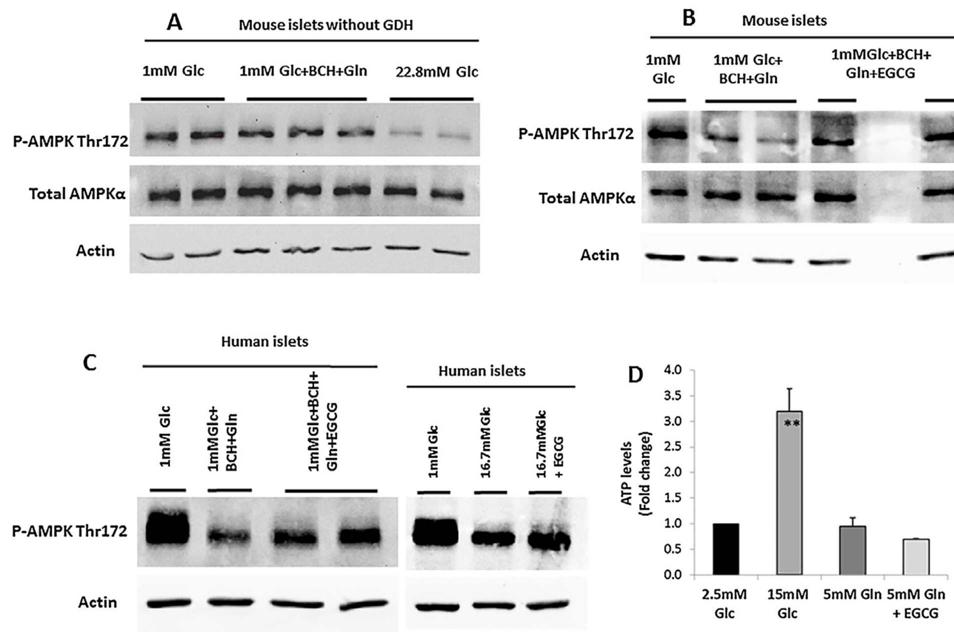

Fig. 2. Contribution of GDH activity to AMPK signalling in mouse and human islets. Isolated islets were pre-treated 2 h in glucose- and glutamine- free RPMI-1640 medium in the absence or presence of 20 μM EGCG, then treated for 1 h as indicated before assessment of phosphorylation state of AMPKα at Thr 172 (p-AMPK) by immunoblotting. (A) Islets lacking beta-cell GDH were isolated from ß*Glud1*$^{-/-}$ mice and incubated at 1 mM glucose (Glc), 5 mM glutamine (Gln) plus 10 mM BCH, and 22.8 mM glucose (Glc). (B) Wild type mouse islets were incubated at 1 mM Glc and 5 mM Gln plus 10 mM BCH in the absence or presence of 20 μM EGCG. (C) Human islets were incubated at 1 mM Glc, 5 mM Gln plus 10 mM BCH and 16.7 mM Glc in the absence or presence of 20 μM EGCG. Blots are representative of 3 (mouse) and 2 (human) independent experiments each performed in duplicate. (D) ATP levels in INS-1E cells incubated at basal 2.5 mM Glc, 15 mM Glc, 5 mM Gln, and 5 mM Gln plus 20 μM EGCG (means +SE of 3 independent experiments, **P < 0.01 vs. basal).

and 3.1-fold over basal release, respectively (Fig. 1A). EGCG inhibited glucose-stimulated insulin secretion by 70% and that induced by glutamine + BCH by 76%. As reported previously (Vetterli et al., 2012; Carobbio et al., 2009), insulin secretion stimulated by either glucose or glutamine + BCH was reduced respectively by 63% and 56% in islets lacking ß-cell GDH (ß*Glud1*$^{-/-}$). Of note, EGCG did not alter insulin secretion in ß-cell GDH knockout islets (Fig. 1A); suggesting that EGCG acts by inhibiting GDH. Measurements of calcium concentrations in INS-1E ß-cells showed that, unlike glutamine, glucose and KCl efficiently raised cytosolic calcium, an effect that was not modified by the presence of EGCG (Fig. 1B). In human islets EGCG reduced basal insulin release, as well as the secretory response to both glucose and glutamine + BCH (Fig. 1C). On the contrary, EGCG did not affect insulin secretion evoked by KCl, which does not require activation of metabolic pathways. Next, we measured the human islet contents in amino acids closely associated with GDH activity. As in ß-cell GDH knockout islets (Vetterli et al., 2012), EGCG lowered glutamate and aspartate levels, as well as glutamine and alanine contents (Fig. 1D).

3.2. GDH impacts on AMPK activity in mouse and human islets

AMPK is inactive unless phosphorylated on the α-subunit activation loop at Thr172. Since EGCG activates AMPK (Wu et al., 2014) and inhibits GDH (Li et al., 2006), we probed for a possible relationship between these two metabolic enzymes in pancreatic islets. In islets lacking ß-cell GDH (ß*Glud1*$^{-/-}$), stimulation by glutamine + BCH failed to affect the P-AMPK state, while 22.8 mM glucose efficiently reduced (−76%, $P < 0.005$) the phosphorylated level seen at 1 mM glucose (Fig. 2A). Of note, there was no alteration in total AMPK protein. Conversely, in wild type control mouse islets (Fig. 2B) glutamine + BCH markedly lowered the P-AMPK compared to low glucose (−77%, $P < 0.02$), an effect largely prevented by the presence of EGCG (−22% vs. low glucose, $P < 0.01$; +235% vs. glutamine + BCH, $P < 0.05$). Like for mouse islets, in human islets (Fig. 2C) glutamine + BCH attenuated P-AMPK versus low glucose (−49%) and EGCG partially counteracted this effect (+34%). In contrast, the glucose-induced lowering of P-AMPK (−52%) was not altered by EGCG. Because P-AMPK state depends on the production of ATP, this parameter was measured in INS-1E ß-cells (Fig. 2D). This showed that glucose stimulation increased cellular ATP, while glutamine alone or in combination with EGCG did not.

These results indicate that the anaplerotic activity of GDH resulting from glutamine stimulation impacts on AMPK. On the other hand, the cataplerotic effect of glucose on GDH does not affect P-AMPK since, under these conditions, ATP production occurs independently of GDH.

3.3. Role of GDH in AMPK activation and glucose transport in myotubes

To extend our findings to one of the main insulin target tissues, we investigated two skeletal muscle models, *i.e.* mouse C2C12 and human primary cells differentiated from myoblasts. In C2C12 myotubes, stimulation of GDH by glutamine + BCH reduced the P-AMPK (Fig. 3A). This effect was completely reversed by EGCG. These results reveal a relationship between the activities of GDH and AMPK, similar to islets (Fig. 2). Next, glucose clearance by human myotubes was monitored using 2-deoxyglucose uptake and insulin (120 nM) as stimulus (Fig. 3B). Remarkably, insulin-induced glucose uptake was abolished in the presence of glutamine + BCH. This inhibition was partially reverted by the presence of EGCG, suggesting that GDH anaplerotic activation competes with metabolic pathways favoring glucose uptake.

4. Discussion

The present work shows that EGCG decreases ß-cell insulin secretion and increases muscle insulin sensitivity through the same molecular target, *i.e.* GDH (Fig. 3C). In EGCG treated pancreatic

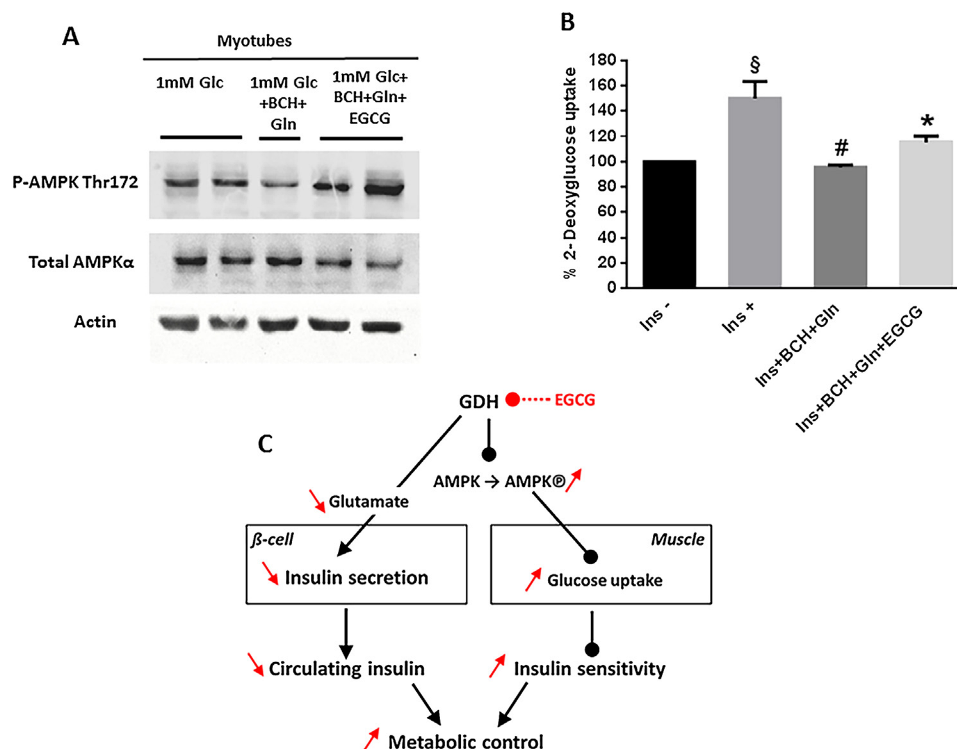

Fig. 3. Role of GDH in AMPK activation in C2C12 myotubes and glucose transport in primary human myotubes. (A) Phosphorylation of AMPKα at Thr 172 (p-AMPK) was determined by immunoblotting following 1 h incubation at 1 mM glucose (Glc) and 5 mM glutamine (Gln) plus 10 mM BCH in the absence or presence of 20 μM EGCG. Blot is representative of 2 independent experiments performed in duplicate. (B) [^{3}H]2-deoxyglucose uptake was measured in differentiated primary human myotubes following incubation in glucose-free KRBH with 120 nM insulin, 5 mM Gln plus 10 mM BCH in the absence or presence of 20 μM EGCG (for further details see Materials and Methods). Data are means ± SE of 3 independent experiments performed in duplicate. (C) Proposed model for interplay between GDH and AMPK in muscle and the pancreatic ß-cell and effects on glucose homeostasis, see text. Effects of EGCG are shown in red.

islets, insulin release evoked by pure calcium-mediated signalling was preserved. In contrast, glucose-induced amplification of insulin secretion was inhibited to the same extent as in islets lacking ß-cell GDH. The EGCG inhibition was associated with reduced glutamate levels, substantiating the cataplerotic activity upon glucose stimulation in human islets. Glutamate is the GDH cataplerotic product required for amplifying glucose-stimulated insulin exocytosis (Gheni et al., 2014; Maechler and Wollheim, 1999). When reverse anaplerotic activity of GDH was imposed in both mouse and human islets by the combination of glutamine + BCH, EGCG decreased insulin release, confirming previous observations in rat islets. Why glucose-stimulated insulin secretion was not affected by EGCG in rat islets could be due to differences in experimental protocols in our work and the previous report (Li et al., 2006).

A remarkable observation in our study is the relationship between the two energy sensing enzymes GDH and AMPK, located respectively in mitochondria and the cytosol. Induction of anaplerotic GDH activity was revealed by decreased P-AMPK upon glutamine + BCH stimulation, an effect absent in islets lacking ß-cell GDH. EGCG mimicked GDH deletion by preserving the phosphorylation state of AMPK. As expected, glucose stimulation reduced P-AMPK in mouse and human islets. GDH deletion and EGCG did not impair the glucose effect on P-AMPK, indicating the preserved ATP generation when GDH activity is suppressed (Carobbio et al., 2009).

Glucose homeostasis depends on the regulated balance of insulin secretion and action on its target organs. Among these, skeletal muscle ensures glucose clearance through insulin mediated glucose uptake, while insulin-independent glucose transport, occurring for instance during exercise, is promoted by AMPK activity (for review see (Ruderman et al., 2013)). In mouse myotubes, GDH activation by glutamine + BCH decreased P-AMPK, an effect inhibited by EGCG. This suggests that GDH participates in energy provision from amino acids in muscle. The physiological consequence of GDH activation was the reduced glucose uptake, partially reverted by EGCG.

The pre-diabetic state is typically characterized by obesity and insulin resistance, compensated by hyper-secretion from the pancreatic ß-cells leading to further lipid storage (Defronzo, 2009). Overt type 2 diabetes develops when the ß-cells decompensate by exhaustion and plasma insulin declines (Kasuga, 2006). Therefore, preservation of ß-cell function and maintenance of efficient glucose clearance are essential goals in the prevention of diabetes. Here we provide *in vitro* evidence that EGCG could fulfill these criteria by dampening the hyper-secretion of insulin, thereby improving insulin sensitivity of target tissues, while simultaneously increasing glucose uptake by skeletal muscle (Fig. 3C). Future clinical studies with EGCG or other GDH inhibitors should document whether such an approach can prevent the deterioration of glucose homeostasis in pre-diabetic subjects.

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