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Free fatty acid receptor 1 (FFAR1/GPR40) signaling affects insulin secretion by enhancing mitochondrial respiration during palmitate exposure

Hjalti Kristinsson, Peter Bergsten, Ernest Sargsyan *

Department of Medical Cell Biology, Uppsala University, Uppsala SE-75123, Sweden

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ABSTRACT

Fatty acids affect insulin secretion via metabolism and FFAR1-mediated signaling. Recent reports indicate that these two pathways act synergistically. Still it remains unclear how they interrelate. Taking into account the key role of mitochondria in insulin secretion, we attempted to dissect the metabolic and FFAR1-mediated effects of fatty acids on mitochondrial function. One-hour culture of MIN6 cells with palmitate significantly enhanced mitochondrial respiration. Antagonism or silencing of FFAR1 prevented the palmitate-induced rise in respiration. On the other hand, in the absence of extracellular palmitate FFAR1 agonists caused a modest increase in respiration. Using an agonist of the M3 muscarinic acetylcholine receptor and PKC inhibitor we found that in the presence of the fatty acid mitochondrial respiration is regulated via $G\alpha_q$ protein-coupled receptor signaling. The increase in respiration in palmitate-treated cells was largely due to increased glucose utilization and oxidation. However, glucose utilization was not dependent on FFAR1 signaling. Collectively, these results indicate that mitochondrial respiration in palmitate-treated cells is enhanced via combined action of intracellular metabolism of the fatty acid and the $G\alpha_q$-coupled FFAR1 signaling. Long-term palmitate exposure reduced ATP-coupling efficiency of mitochondria and deteriorated insulin secretion. The presence of the FFAR1 antagonist during culture did not improve ATP-coupling efficiency, however, it resulted in enhanced mitochondrial respiration and improved insulin secretion after culture. Taken together, our study demonstrates that during palmitate exposure, integrated actions of fatty acid metabolism and fatty acid-induced FFAR1 signaling on mitochondrial respiration underlie the synergistic action of the two pathways on insulin secretion.

1. Introduction

Free fatty acids (FFAs) play an essential role in the regulation of insulin secretion. At low glucose levels, FFAs are used as a substrate for generation of ATP and maintain insulin secretion [1]. At high glucose conditions, β-oxidation is inhibited by a product of the glycolytic pathway, malonyl-CoA, and fatty acids are directed towards formation of triacylglycerol (TAG) [2,3]. The anabolic and catabolic reactions between long-chain acyl Co-As (LC-CoA) and TAG, known as glycerolipid/free fatty acid (GL/FFA) cycle, produce lipid signaling molecules including LC-CoAs, phosphatic acids, monoacylglycerol and diacylglycerol (DAG), all of which stimulate insulin secretion [4]. In addition to its role as a nutrient, FFAs serve as ligands and influence insulin secretion by interacting with G-protein coupled receptors (GPCRs) on the plasma membrane [5,6]. One of the GPCRs that is highly expressed in beta cells is the free fatty acid receptor 1 (FFAR1 or GPR40) [5,6]. Activation of the receptor leads to activation of phospholipase C (PLC) and hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into DAG and inositol triphosphate (IP_3). DAG and IP_3 potentiate insulin secretion by activating protein kinase C (PKC) and triggering ER Ca^{2+} release, respectively [7,8]. Recently, FFAR1 agonists have been developed as potential therapeutic agents for the treatment of type 2 diabetes [9–12].

In contrast to short-term effects, long-term exposure of beta cells to FFAs impairs insulin secretion and triggers apoptosis [13]. The deleterious effects of FFAs have been linked to altered glucose/fatty acid oxidation cycle [13], decreased NADPH content [14], endoplasmic reticulum (ER) stress [15] and partitioning towards formation of toxic ceramide species [16]. Also, FFAR1 signaling has been implicated in the long-term deleterious effects of FFAs [17–20].

We have recently demonstrated that fatty acid metabolism and FFAR1 signaling act synergistically on insulin secretion [17]. Reduced β-oxidation of fatty acids in the presence of a FFAR1 antagonist pointed out mitochondria as a site where the two pathways may converge [17].

Abbreviations: 2-DG, 2-deoxyglucose; BSA, bovine serum albumin; DAG, diacylglycerol; FBS, fetal bovine serum; FFA, free fatty acid; FFAR1, free fatty acid receptor 1; GL/FFA, glycerolipid/free fatty acid; GPCR, G-protein coupled receptor; GSIS, glucose-stimulated insulin secretion; IP_3, inositol triphosphate; LC-CoA, long-chain acyl CoAs; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; OCR, oxygen consumption rate; CPT1, carnitine palmitoyltransferase 1; TAG, triacylglycerol.

* Corresponding author.
E-mail address: ernest.sargsyan@mcb.uu.se (E. Sargsyan).

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It is known that this organelle is pivotal in beta-cell function. Uncoupling of respiration from ATP synthesis is essential for the regulation of ATP/ADP ratio and insulin secretion [21]; and beta cells depleted of mitochondria are unable to properly change insulin secretion in response to metabolic changes [22,23]. Taking into account the aforementioned, we decided to investigate the effects of fatty acid metabolism and FFAR1 signaling on mitochondrial function.

2. Materials and methods

2.1. Culture of cells and human islets

Mouse insulinoma MIN6 cells (a kind gift from Prof. Jun-Ichi Miyazaki, Osaka University, Japan) and human HEK293 cells were cultured in Dulbecco's Modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 25 mM glucose and supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 55 μM β-mercaptoethanol at 37 °C and 5% CO_2. Experiments on MIN6 cells were performed between passages 21–30.

Human islets were obtained from brain-dead otherwise healthy individuals from the Islet Transplantation Unit at Uppsala University. Islets were cultured in CMRL 1066 medium (Invitrogen) containing 5.5 mM glucose and supplemented with 10% FBS. Ethical permission to use human islets was obtained from the Regional Ethical Review Board in Uppsala (EPN number 2010/006; 2010-02-10).

2.2. Free fatty acid preparation

Palmitate (Sigma Aldrich, St. Louis, MO, USA) was prepared as 100 mM stock solution dissolved in 50% ethanol. Stock solution was diluted in culture medium to 0.5 mM concentration and allowed to complex for 30 min at 37 °C with fatty acid free bovine serum albumin (BSA) (Boehringer Mannheim GmbH, Mannheim, Germany) to a final molar ratio of 6.6 to 1 [17].

2.3. Short- and long-term treatment of cells and human islets

Cells/islets were treated with 0.5 mM palmitate (Sigma Aldrich) in the absence or presence of FFAR1 antagonists; 2 μM ANT203, 2 μM ANT825 (compound 39 in [24]) (both compounds from AstraZeneca, Macclesfield, UK) or 10 μM DC260126 (Tocris Bioscience, Bristol, UK).

Short-term treatment was performed for 1 h in XF assay medium (Seahorse Biosciences, North Billerica, MA, USA) set to pH 7.4 and supplemented with 25 mM glucose. Insulin secretion and oxygen consumption rate (OCR) were measured during culture.

Long-term treatment was performed for 48 h in complete DMEM culture medium. Glucose-stimulated insulin secretion (GSIS) and OCR were determined after treatment.

2.4. Down-regulation of FFAR1 by short hairpin RNA

FFAR1 was down-regulated by using the short hairpin RNA (shRNA) 5′-CCGGGCCCGTCTCAGTTTCTCCATTCTCGAGAATGGAGAAACTGAGACGG GCTTTTT-3′ (Sigma Aldrich). pLKO.1-puro non-Mammalian shRNA control plasmid DNA (Sigma Aldrich) was used as a negative control. Transfection was performed in 96-well plates by adding 50,000 cells to a mixture containing 1 μl Lipofectamine 2000 (Invitrogen) and 0.3 μg DNA in 50 μl OptiMEM (Invitrogen). After overnight incubation, the transfection medium was replaced with the culture medium for another 72 h.

2.5. Measurement of FFAR1 mRNA level by real-time PCR

Total mRNA was isolated from MIN6 cells using NucleoSpin® RNA (Macherey-Nagel, Duren, Germany) and reversely transcribed into cDNA with SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen). The real-time PCR was performed in 10 μl volume using Dynamo Capillary SYBR Green qPCR kit (Finnzymes, Espoo, Finland). The following primers were used for amplification: FFAR1 (forward primer, 5′-CCATTCTGCTCTTCTTTCTG-3′ and reverse primer, 5′-GGGT TTATGAAACTAGCCAC-3′), β-actin (forward primer, 5′-TCTGTGTGGA TTGGTGGCTC-3′ and reverse primer, 5′-GACTCATCGTACTCCTGCTT GCT-3′). FFAR1 mRNA level was normalized to the housekeeping gene β-actin using the following formula: target amount $= 2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = [C_t\ (\text{GPR40 KO}) - C_t\ (\beta\text{-actin KO})] - [C_t\ (\text{GPR40 control}) - C_t\ (\beta\text{-actin control})]$ [25].

2.6. Oxygen consumption measurements

Mitochondrial respiration was determined by measuring OCR in the Extracellular Flux Analyzer XF96e (Seahorse Biosciences). Assays were performed in XF assay medium (Seahorse Biosciences) set to pH 7.4 and supplemented with 25 mM glucose.

Mitochondrial function was determined by measuring basal respiration, ATP-coupled respiration, proton leak and maximal respiratory capacity. Basal OCR was measured during the last 30 min of 1-h culture. All measurements were corrected for non-mitochondrial OCR, which was measured by adding inhibitors of electron transport chain; rotenone (5 μM) and antimycin (5 μM). Mitochondrial OCR was estimated by subtracting OCR measurement after rotenone/antimycin addition from OCR measurement before oligomycin addition. ATP-coupled respiration was assessed by the addition of ATP synthase inhibitor oligomycin (4 μM). OCR measurement after oligomycin addition was subtracted from OCR measurement before oligomycin addition. The drop in OCR induced by oligomycin addition reflects ATP-coupled respiration. Proton leak OCR was estimated by subtracting OCR measurement after addition of rotenone/antimycin from OCR measurement after addition of oligomycin. Maximal respiratory capacity was determined by adding 4 μM ionophore FCCP.

OCR was, in addition, measured in the presence of FFAR1 agonists TUG-499 (2 μM) (Merck Millipore, Darmstadt, Germany) and AS2034178 (2 μM) (Tocris Bioscience). Also, OCR was measured in the presence of 2-deoxyglucose (2-DG) (100 mM), carnitine palmitoyltransferase 1 (CPT1) inhibitor etomoxir (40 μM), agonist of M3 muscarinic acetylcholine receptor carbachol (100 μM) and PKC inhibitor chelerythrine (10 μM). Concentrations of the compounds were determined in optimization experiments. Compounds were obtained from Sigma Aldrich if not indicated.

2.7. Measurements of palmitate oxidation and glucose utilization

Palmitate oxidation and glucose utilization were determined by including during culture 2 μCi [^{3}H]palmitate and 2 μCi d-[5-^{3}H]glucose, respectively. Blanks for each condition were created by adding radioactive compounds to medium. After 1-h treatments, media were transferred to 1.5-ml tubes. Then tubes were placed inside scintillation vials containing 500 μl of H_2O. The scintillation vials were sealed and incubated at 56 °C overnight to permit ^3H_2O formed by the cells to evaporate and equilibrate with water in the vials [26]. The vials were then cooled to room temperature. After removing the tubes, 15 ml Ultima Gold™ scintillation fluid (PerkinElmer) was added to the water and ^3H_2O content determined by a liquid-scintillation spectrometer (Wallac System 1400™ PerkinElmer, Boston, MA). The average number of disintegrations in blank tubes was subtracted from experimental measurements.

2.8. Measurements of mitochondrial DNA

Total DNA was isolated using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The real-time PCR was performed using Dynamo Capillary SYBR Green qPCR kit (Finnzymes, Espoo, Finland). mtDNA was amplified using primers against ND1 gene (5′-ATTACTTCTGCCAGCCTGAC-3′