Brief Communication

MOLECULAR METABOLISM

Point mutation of *Ffar1* abrogates fatty acid-dependent insulin secretion, but protects against HFD-induced glucose intolerance

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ABSTRACT

Objective: The fatty acid receptor 1 (FFAR1/GPR40) mediates fatty acid-dependent augmentation of glucose-induced insulin secretion (GIIS) in pancreatic β-cells. Genetically engineered *Ffar1*-knockout/congenic mice univocally displayed impaired fatty acid-mediated insulin secretion, but *in vivo* experiments delivered controversial results regarding the function of FFAR1 in glucose homeostasis and liver steatosis. This study presents a new coisogenic mouse model carrying a point mutation in *Ffar1* with functional consequence. These mice reflect the situations in humans in which point mutations can lead to protein malfunction and disease development.

Methods: The Munich *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis-derived F1 archive containing over 16,800 sperms and corresponding DNA samples was screened for mutations in the coding region of *Ffar1*. Two missense mutations (R258W and T146S) in the extracellular domain of the protein were chosen and homozygote mice were generated. The functional consequence of these mutations was examined *in vitro* in isolated islets and *in vivo* in chow diet and high fat diet fed mice.

Results: Palmitate, 50 μM, and the FFAR1 agonist TUG-469, 3 μM, stimulated insulin secretion in islets of *Ffar1*$^{T146S/T146S}$ mutant mice and of wild-type littermates, while in islets of *Ffar1*$^{R258W/R258W}$ mutant mice, these stimulatory effects were abolished. Insulin content and mRNA levels of *Ffar1*, *Glp1r*, *Ins2*, *Slc2a2*, *Ppara*, and *Ppard* were not significantly different between wild-type and *Ffar1*$^{R258W/R258W}$ mouse islets. Palmitate exposure, 600 μM, significantly increased *Ppara* mRNA levels in wild-type but not in *Ffar1*$^{R258W/R258W}$ mouse islets. On the contrary, *Slc2a2* mRNA levels were significantly reduced in both wild-type and *Ffar1*$^{R258W/R258W}$ mouse islets after palmitate treatment. HFD feeding induced glucose intolerance in wild-type mice. *Ffar1*$^{R258W/R258W}$ mutant mice remained glucose tolerant although their body weight gain, liver steatosis, insulin resistance, and plasma insulin levels were not different from those of wild-type littermates. Worth mentioning, fasting plasma insulin levels were lower in *Ffar1*$^{R258W/R258W}$ mice.

Conclusion: A point mutation in *Ffar1* abrogates the stimulatory effect of palmitate on GIIS, an effect that does not necessarily translate to HFD-induced glucose intolerance.

Keywords FFAR1/GPR40; Free fatty acids; Insulin secretion; ENU-mutated *Ffar1*; FFAR1 deficient mice; High fat diet

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Abbreviations: CD, regular chow diet; ENU, *N*-ethyl-*N*-nitrosourea; FFAR1, free fatty acid receptor 1; GIIS, glucose-induced insulin secretion; GLP-1, glucagon like peptide-1; Glut-2, glucose transporter 2; GTT, glucose tolerance test; HEK-EM 293 cells, human embryonic kidney macrophage scavenger receptor-expressing (TRH-R) cells; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; HFD, high fat diet; ipITT, intraperitoneal insulin tolerance test; PAX6, paired box protein, also known as aniridia type II protein (AN2) or oculorhombin; *Ppara*/*Ppard*, peroxisome proliferator activated receptor α/δ genes; *Slc2a2*, solute carrier family 2 member 2 gene; TAK875 and TUG-469, FFAR1 agonists

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1. INTRODUCTION

Free fatty acid receptor-1 (FFAR1, formerly GPR40) promotes long chain fatty acid-mediated augmentation of glucose-induced insulin secretion (GIIS) [1–3]. In humans and rodents, high expression of FFAR1 is restricted to pancreatic and gastric endocrine cells, while expression in other tissues, including brain, is much lower [1,2,4,5]. These features make FFAR1 an attractive drug target for the treatment of insufficient insulin secretion, which is the ultimate cause for the onset of hyperglycemia and type-2 diabetes mellitus [6,7]. Until today, multiple agonists have been generated and tested for their efficacy to treat hyperglycemia in humans [6]. Although FFAR1 agonists counteract glucose intolerance in mice and humans, the beneficial effect of these new therapeutic drugs is still a matter of debate [8,9]. Thus, the promising drug TAK875 was discontinued after clinical phase III due to its liver toxicity. Confirming this side effect, FFAR1-deficient mice are protected against diet-induced liver steatosis [10]. This observation prompted the investigation of FFAR1-antagonists as therapeutic tools against fatty liver disease.

In addition, different FFAR1 agonists exert their effects through different cellular pathways. Thus, fatty acids stimulate insulin secretion mainly via Gq proteins, while TAK875 stimulation is mediated by β-arrestin-2 [11]. An additional, but indirect, stimulatory effect of FFAR1-agonists on insulin secretion is caused by the activation of FFAR1 expressed in intestinal endocrine cells which leads to GLP-1 secretion [12].

Several transgenic and knockout/congenic mouse models have been generated in order to assess the role of FFAR1 for proper insulin secretion and maintenance of glucose homeostasis. The results obtained with three different receptor knockout mouse models were not consistent. The protection against high fat feeding-induced fatty liver and glucose intolerance, as observed by Steneberg and colleagues, could not be reproduced using other *Ffar1* KO mouse models [10,13,14]. Such differences may be explained by undesirable side effects generated by insertion of exogenous DNA, deletion of non-coding regions with specific functions, e.g. microRNA, and the additional role of the *Ffar1* promoter for the expression of FFAR2 (GPR43) and FFAR3 (GPR41) [15,16]. Congenic mice differ not only in the ablated gene but also in a flanking segment on either side of the ablated locus [17]. Furthermore, a complete deletion of a protein may generate a compensatory up-regulation of other proteins. To circumvent such problems, we searched for a coisogenic mouse model with a minimal genetic alteration producing a maximal effect. Using site-directed mutagenesis, several point mutations in *Ffar1* with functional consequences have been identified, including R258 [18,19]. We screened the Munich ENU-mutagenesis-derived F1 sperm and corresponding DNA archive for point mutations in *Ffar1*. The archive comprises more than 16,800 samples from individual F1-mutagenized mice on the C3HeB/FeJ genetic background [20,21]. Two mouse models carrying point mutations in the coding region of *Ffar1* are presented in this study of which the R258W mutation prevents the stimulation of insulin secretion by palmitate and the FFAR1 agonist TUG-469.

2. MATERIALS, ANIMALS AND METHODS

2.1. Materials

TUG-469, a specific FFAR1 agonist, was a kind gift of Trond Ulven, Southern University of Denmark, Odense M, Denmark. All other materials, unless otherwise stated, were from Sigma–Aldrich (Deisenhofen, Germany) and of analytical grade.

2.2. Generation of mice

ENU mutagenesis was performed as described previously [22]. Briefly, male C3HeB/FeJ mice were treated weekly by one 90 mg/kg ENU-injection for three consecutive weeks. First generation F1 mice were phenotyped, and male mice were cryo-archived by their sperm and spleen-derived DNA samples. The DNA archive was screened for alleles of interest using a LightScanner® device originally from Idaho Technology Inc. (distributed by Bioke, Leiden, Netherlands). *In vitro* fertilization, fusing sperm of mutated F1 mice and mating with wild-type C3HeB/FeJ mice were performed as described elsewhere [23]. During maintenance the mutant mice were repeatedly backcrossed to wild-type C3HeB/FeJ mice in order to eliminate unwanted ENU mutations. Mice were kept under a 12 h light/dark cycle and had *ad libitum* access to chow diet (CD) and water. High fat diet (HFD) containing 45% fat/kcal from lard and soybean (Research Diets D12451; New Brunswick; NJ; USA) was fed for 8 weeks starting at age of 4 weeks. Mouse holding and handling were done according to the federal animal welfare guidelines and the state ethics committee and approved by the governments of Upper Bavaria and Baden-Württemberg.

2.3. Glucose and insulin tolerance tests

Blood glucose concentrations were measured after intra-peritoneal injection of 2 g glucose/kg body weight (ipGTT) or of intra-peritoneal injection of 1 unit/kg body weight insulin (ipITT) in mice fed CD or HFD for 8 weeks. Before GTT, mice were fasted overnight. For determination of plasma insulin levels blood samples were collected in heparinized capillary from tail vein. Blood glucose was measured with a glucometer. During oral glucose tolerance test (oGTT), 2 g glucose/kg body weight was administered via gavage in overnight fasted animals. The tests were performed with the same animals keeping an interval of 1 week between the tests. Plasma insulin and glucagon were measured using ELISA kits (Mercodia, Sweden). Plasma leptin and resistin were quantified using a ProcartaPlex™ immunoassay (Luminex™ xMAP technology, Invitrogen).

2.4. Isolation of islets and insulin secretion

Mouse islets were isolated via collagenase digestion (1 mg/ml collagenase, Serva, Heidelberg, Germany) and thereafter purified by handpicking. Islets were cultured overnight in RPMI 1640 medium containing 11 mM glucose supplemented with 10% FCS, 10 mM HEPES, 2 mM L-glutamine, and 1 mM Na-pyruvate without antibiotics. Thereafter, islets were washed twice and pre-incubated for 1 h at 37 °C with Krebs Ringer buffer (KRB) containing (in mM): 135 NaCl, 4.8 KCl, 1.2 $MgSO_4$, 1.3 $CaCl_2$, 1.2 KH_2PO_4, 5 $NaHCO_3$, 2.8 glucose, 10 HEPES, and 5 g/l bovine serum albumin (fatty acid free, low endotoxin, Sigma, Deisenhofen, Germany), pH 7.4. Subsequently, islets were incubated in fresh KRB containing 0.5 g/l bovine serum albumin supplemented with test substances as indicated for 1 h at 37 °C. Palmitate was added from a stock solution of 50 mM in DMSO. Secreted insulin and islet insulin content after insulin extraction in acid ethanol (1.5% [vol/vol] HCl/75% [vol/vol] ethanol) were measured via radioimmunoassay (Millipore, Biotrend Chemikalien GmbH, Germany).

2.5. Semiquantitative analysis of cellular mRNA levels

Islets were isolated and directly lysed in RNA lysis buffer (Macherey–Nagel, Düren, Germany, Figure 1C), or islets were cultured before lysis in medium supplemented with 10% FCS ± 600 μM palmitate. Palmitate, from a stock solution of 100 mM in DMSO, was coupled to FCS at a final concentration of 6 mM before addition to the culture medium. 50–200 islets were collected for total RNA-isolation using the commercial RNeasy kit (Qiagen, Hilden, Germany). Residual DNA

