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Conjugated Linoleic Acids Mediate Insulin Release through Islet G Protein-coupled Receptor FFA1/GPR40^{*S}

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Among dietary components, conjugated linoleic acids (CLAs) have attracted considerable attention as weight loss supplements in the Western world because they reduce fat stores and increase muscle mass. However, a number of adverse effects are also ascribed to the intake of CLAs such as aggravation of insulin resistance and the risk of developing diabetes. However, the mechanisms accounting for the effects of CLAs on glucose homeostasis are incompletely understood. Herein we provide evidence that CLAs specifically activate the cell surface receptor FFA1, an emerging therapeutic target to treat type 2 diabetes. Using different recombinant cellular systems engineered to stably express FFA1 and a set of diverse functional assays including the novel, label-free non-invasive dynamic mass redistribution technology (Corning[®] Epic[®] biosensor), both CLA isomers *cis*-9, *trans*-11-CLA and *trans*-10, *cis*-12-CLA were found to activate FFA1 *in vitro* at concentrations sufficient to also account for FFA1 activation *in vivo*. Each CLA isomer markedly increased glucose-stimulated insulin secretion in insulin-producing INS-1E cells that endogenously express FFA1 and in primary pancreatic β -cells of wild type but not *FFA1*^{-/-} knock-out mice. Our findings establish a clear mechanistic link between CLAs and insulin production and identify the cell surface receptor FFA1 as a molecular target for CLAs, explaining their acute stimulatory effects on insulin

secretion *in vivo*. CLAs are also revealed as insulinotropic components in widely used nutraceuticals, a finding with significant implication for development of FFA1 modulators to treat type 2 diabetes.

The prevalence of obesity has risen considerably during recent decades in the Western world and is now a serious global health burden due to the many secondary complications and comorbidity associated with this condition such as increased susceptibility to cardiovascular events and aggravation of diabetes mellitus (1–3). Although treatment of obesity is best achieved by lifestyle changes such as dietary modifications and increased physical activity, this is often insufficient, and pharmacological intervention is required. Existing pharmacological options to lower body weight include inhibitors of gastrointestinal lipases (Orlistat) or centrally acting β -phenethylamine derivatives such as fenfluramine, dexfenfluramine, or sibutramine, but most of these medications are associated with either serious side effects and/or lack of efficacy on a long term basis (1, 4). Because weight loss in obese people is difficult to achieve and maintain, dietary, pharmaceutical, and nutraceutical approaches have been an area of intense investigation because of the growing need and interest to find molecules capable of fighting obesity.

Conjugated linoleic acids are conjugated dienolic isomers of linoleic acid (see Fig. 1A). Humans acquire CLAs³ through diet from dairy products, ruminant meat, and partially hydrogenated vegetable oils. Most biological actions have been ascribed to *cis*-9, *trans*-11-CLA and *trans*-10, *cis*-12-CLA, which represent the two predominant isomers found in foods and commercial preparations (5–8). CLAs (commercial preparations often contain equal amounts of both isomers) have emerged as highly popular food supplements in the Western world to achieve beneficial effects on health such as reduction of body fat gain and increase of muscle mass among many other salutary properties (5–9).

However, supplementation of diets by CLAs to attempt weight loss has become a subject of intense debate due to the potential influence of CLAs on glucose homeostasis and insulin sensitivity (8). Although a series of studies indicates that CLAs attenuate the development of impaired glucose tolerance and hyperinsulinemia (6, 10, 11), an at least equal number of studies support the notion that CLA intake is associated with serious adverse effects such as impaired insulin sensitivity, and ultimately, insulin resistance (8, 12, 13). Importantly, the molecular mechanisms underlying the effects of CLAs on glucose homeostasis are not completely understood.

Herein we tested the hypothesis that CLAs may exert insulinotropic effects via activation of the cell surface receptor FFA1, which is highly expressed on pancreatic β -cells and which has been shown previously to specifically respond to medium and long chain fatty acids *in vitro* and *in vivo* (14–16). We identify

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^S The on-line version of this article (available at <http://www.jbc.org>) contains supplemental text, Table 1, and supplemental Figs. 1–6.

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³ The abbreviations used are: CLA, conjugated linoleic acid; 9c, 11t, *cis*-9, *trans*-11; 10t, 12c, *trans*-10, *cis*-12; GSIS, glucose-stimulated insulin secretion; PTX, pertussis toxin; HBSS, Hanks' balanced salt solution; DMR, dynamic mass redistribution; ANOVA, analysis of variance.

CLAs as potent enhancers of glucose-stimulated insulin secretion (GSIS) and provide evidence that this mechanism requires activation of FFA1 because it is absent in FFA1-null mice. Our findings lead to a better understanding of the molecular signaling mechanisms of CLAs, in particular of their side effect profile, and question the value and widespread use of this nutraceutical.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Human astrocytoma 1321N1 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 1% sodium pyruvate, 100 units/ml penicillin, and 100 μ g/ml streptomycin. 1321N1 cells stably expressing the FFA1 receptor were kindly provided by Euroscreen (Gosselies, Belgium). For FFA1-1321N1 cells, medium was completed with 400 μ g/ml G418 (Invitrogen). Cells were kept at 37 °C in a 5% CO₂ atmosphere. CLAs (90% purity) were obtained via CPS Chemie Service GmbH, Aachen, Germany. The FFA1 antagonist PPTQ (trans-1-oxo-3-(4-phenoxyphenyl)-2-propyl-1,2,3,4-tetrahydroisoquinoline-4-carboxylic acid) was synthesized as described in Ref. 22.

Generation of Stable Flp-In T-REx 293 Cells—The Flp recombinase-mediated homologous recombination system (Flp-InTM T-RExTM, Invitrogen) was used to generate cell lines stably expressing human FLAG-tagged FFA1 (FFA1-HEK), FFA3 (FFA3-HEK), or FFA2 (FFA2-HEK) receptors in a doxycycline-dependent manner, as described previously (17).

Measurements of Intracellular [Ca²⁺]_i—FFA1-1321N1 and FFA1-HEK cells were seeded in poly-D-lysine-coated 96-well tissue culture plates, and intracellular Ca²⁺ levels were quantified with the Ca²⁺-sensitive fluorescence dye Oregon Green 488 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid-1/AM (1.5 μ M, Molecular Probes, Eugene, OR) using previously published protocols (17). For desensitization assays, FFA1-1321N1 cells were preincubated with the agonist for either 100 s or 50 min at 37 °C in the NOVOstar microplate reader (BMG Labtech, Offenburg, Germany) prior to injection of the test compound. To investigate the effect of the G $\alpha_{i/o}$ inhibitor pertussis toxin (PTX), cells were pretreated with 50 ng/ml PTX for 22 h.

Inositol Phosphate Accumulation Assays—FFA1-1321N1 cells were seeded in 96-well tissue culture plates and loaded with *myo*-[2-³H]inositol (0.5 μ Ci; TRK911; Amersham Biosciences). The next day, cells were washed twice in HBSS buffer supplemented with 1 mM CaCl₂ and 0.5 mM MgCl₂ and then incubated for 45 min at 37 °C with either of the two CLA isomers in HBSS buffer containing 10 mM LiCl. After termination of the reaction with 50 μ l/well of ice cold formic acid (10 mM), 20 μ l of the resulting cell extract were transferred to 80 μ l of yttrium silicate scintillation proximity assay beads (12.5 mg/ml; Amersham Biosciences) and shaken for 60 min at 4 °C. After overnight incubation at 4 °C, scintillation counting was performed on a TopCount (PerkinElmer Life Sciences).

Label-free Dynamic Mass Redistribution (DMR) Assays (Corning® EPIC® Biosensor)—DMR assays were performed on a beta version of the Corning® Epic® biosensor as described previously in detail (18). Briefly, HEK cells transfected to inducibly express FFA1, FFA2, and FFA3 were grown to confluence for 20–24 h on fibronectin-coated Epic® biosensor 384-well

microplates. Cells were then washed twice with HBSS containing 20 mM HEPES and kept for at least 1 h in the Epic® reader at 28 °C. DMR was monitored before (baseline read) and after the addition of compound solutions for 3,600 s. A detailed description of the optical biosensor assay is available in the [supplemental material](#).

Insulin Secretion from Rat INS-1E Cells and Isolated Mouse Islets—INS-1E cells (kindly provided by C. B. Wollheim, University of Geneva, Switzerland) and islets isolated from wild type and FFA1 knock-out mice (a kind gift of Klaus Seuwen, Novartis Pharma AG, Basel, Switzerland) were cultured and incubated as described previously (19, 20). Insulin release into the supernatant and insulin content after acid ethanol extraction were determined by radioimmunoassay (Millipore).

Cell Preparation, mRNA Extraction, and Reverse Transcription PCR Analysis of FFA1—To verify that FFA1 transcripts are present in FFA1-1321N1, FFA1-HEK, and INS-1E cells, total RNA was extracted, and RNA integrity was confirmed prior to reverse transcription to cDNA. A detailed protocol for FFA1 mRNA detection including primer sequences and PCR conditions is given in the [supplemental material](#). Representative PCR profiles are depicted in [supplemental Fig. 1](#).

Calculations and Data Analysis—Quantification of DMR signals for concentration-response curves was performed by calculation of the maximum value between 300 and 1,200 s. All optical DMR recordings were buffer-corrected. EC₅₀ value determination by non-linear regression was performed using GraphPad Prism 4.02 (GraphPad Software, San Diego, CA). Where appropriate, differences in means were examined by one- or two-way analysis of variance (ANOVA) with Bonferroni's multiple comparison post hoc test using GraphPad Prism 4.02. A *p* value < 0.05 was considered statistically significant.

RESULTS

CLAs Are Full FFA1 Agonists in Recombinant Expression Systems—FFA1 is known to signal through G $\alpha_{q/11}$ proteins, leading to elevation of intracellular calcium (14–16, 21). We therefore tested CLAs for their ability to increase the cytosolic Ca²⁺ concentration, [Ca²⁺]_i, in 1321N1 cells transfected to stably express FFA1 (FFA1-1321N1 cells). Both CLA isomers evoked robust and concentration-dependent [Ca²⁺]_i increase, which was unaffected by pretreatment of cells with the G $\alpha_{i/o}$ inhibitor PTX (Fig. 1, *B* and *C*), ruling out a contribution of this signaling pathway to the Ca²⁺ response. Native 1321N1 cells did not respond to CLAs but were sensitive to stimulation with carbachol, a ligand for endogenously expressed G α_q -coupled muscarinic receptors ([supplemental Fig. 2](#)). Having once responded to each individual CLA isomer or to the selective, small molecule FFA1 agonist TUG424 (17), FFA1-1321N1 cells did not show any significant increase in [Ca²⁺]_i in response to a second CLA stimulus irrespective of whether pre-exposure times for the first stimulus were short (100 s, Fig. 1*D*) or long (50 min, [supplemental Fig. 3](#)). In the presence of a small molecule FFA1 antagonist (PPTQ, Ref. 22), CLA-dependent Ca²⁺ mobilization was undetectable, further confirming the specific interaction between CLAs and the cell surface FFA1 receptor (Fig. 1*E*). The concomitant accumulation of inositol phosphates induced by CLAs in FFA1-1321N1 but not native 1321N1 cells

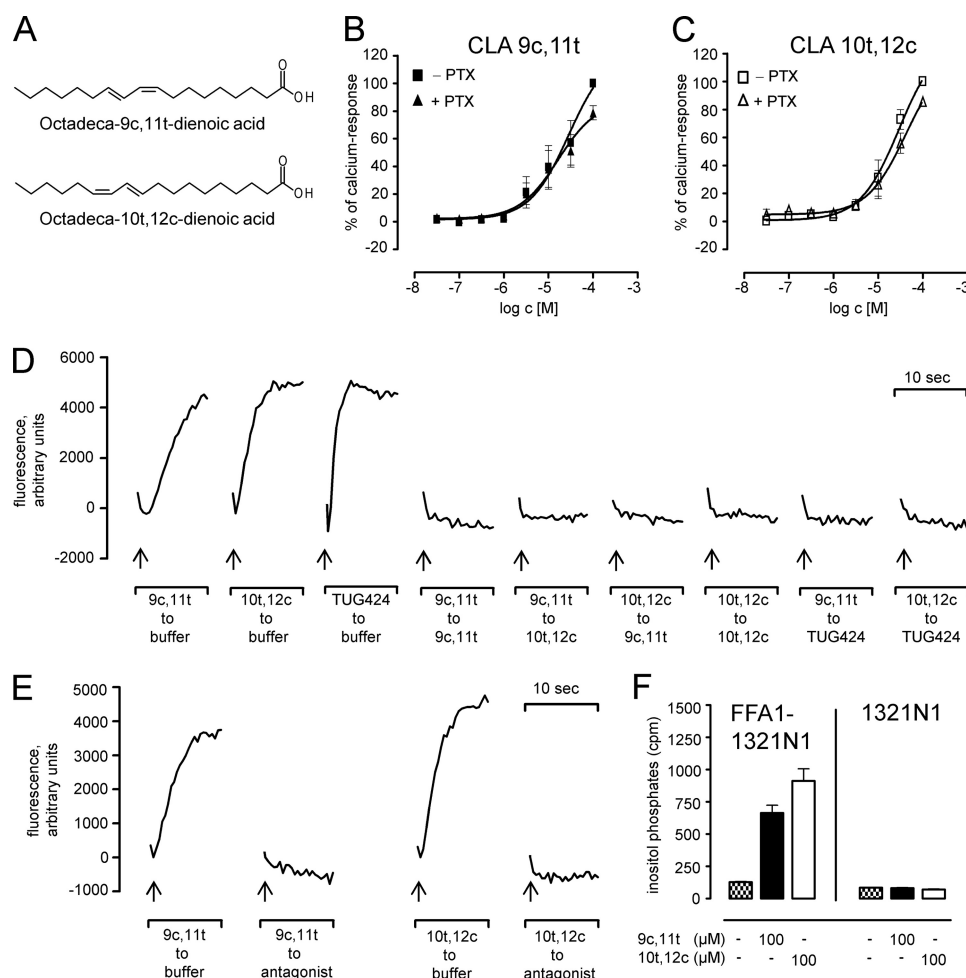


FIGURE 1. CLA isomers are functional agonists of the free fatty acid receptor FFA1. *A*, structures of CLA isomers octadeca-9c,11t-dienoic acid and octadeca-10t,12c-dienoic acid. *B–E*, calcium mobilization assays in 1321N1 cells stably expressing FFA1. *B* and *C*, concentration-response curves of CLA isomers in the presence or absence of PTX (50 ng/ml). *D*, cross-desensitization of FFA1 was determined by recording Ca^{2+} -dependent fluorescence changes. Cells were prestimulated with buffer or 100 μM of the indicated compound for 100 s, and the second compound addition (30 μM) was performed at the labeled point in time (arrow). All Ca^{2+} signals of desensitized FFA1 are significantly different from those obtained without prior desensitization of the receptor ($p < 0.001$) according to one-way ANOVA with Bonferroni's multiple comparisons test. *E*, Ca^{2+} flux in response to CLAs (30 μM) after pretreatment of cells with buffer or 10 μM FFA1 antagonist (PPTQ, Ref. 22) for 30 min is shown. Pretreatment of the cells with the FFA1 antagonist completely blunted CLA-induced Ca^{2+} traces ($p < 0.001$, unpaired Student's t test). *F*, CLA-induced inositol phosphate accumulation in FFA1-1321N1 and 1321N1 cells. Data shown are mean values $\pm$ S.E. of at least three (*B* and *C*) or two (*F*) independent experiments or representative of four such experiments (*D* and *E*).

further underlines the ability of CLAs to stimulate $\text{G}\alpha_{q/11}$ -coupled FFA1 signaling (Fig. 1*F*).

Activation of FFA1 by CLAs could also be recapitulated in assays detecting DMR of cellular proteins with the holistic label-free Epic® optical biosensor technology. DMR measurements have recently emerged as a valuable approach to analyze G protein-coupled receptor pharmacology and function non-invasively in real time and living cells (18, 23, 24). As opposed to the more traditional biochemical assays that are designed to detect specific alterations of intracellular second messengers such as Ca^{2+} or cAMP, DMR technology collates receptor activity into a single optical readout that reflects an integrated cellular response akin to tissue bioassays (18, 25, 26). FFA1-HEK293 cells engineered to inducibly express FFA1 (Fig. 2, *A* and *B*) but not untransfected control cells (supplemental Fig. 4) responded with robust and concentration-dependent DMR signals to CLA challenge. Interestingly, both CLAs were markedly more efficacious than the small molecule FFA1 agonist TUG424 in DMR assays (compare Fig. 2, *A* and *B* with Fig. 2, *C*

and *D*), a feature that was not observed in the single component functional assays based on the detection of changes in intracellular Ca^{2+} (supplemental Fig. 5). Both CLA isomers were inactive on the two other fatty acid-binding G protein-coupled receptors, FFA2 and FFA3, respectively, which are also expressed in pancreatic β -cells (27) and which are closely related to FFA1 but specifically activated by short chain fatty acids (Fig. 2, *E* and *F*) (28–31).

CLAs Are FFA1 Agonists in Immortalized and Primary Pancreatic β -Cells and Require FFA1 for Enhancement of GSIS—To define whether FFA1 ligation by CLAs is of physiological relevance, both isomers were examined for insulinotropic effects in the immortalized rat cell line INS-1E (Fig. 3*A*), which endogenously expresses the homologue of human FFA1 (32). Both CLA isomers markedly enhanced GSIS at high but not at low glucose levels in an FFA1- and concentration-dependent manner (Fig. 3*A* and supplemental Fig. 6). In primary islets, isolated from 8–10 week-old wild type mice, CLA-mediated potentiation of GSIS was only detectable at high glucose levels (Fig. 3*B*). Importantly, how-

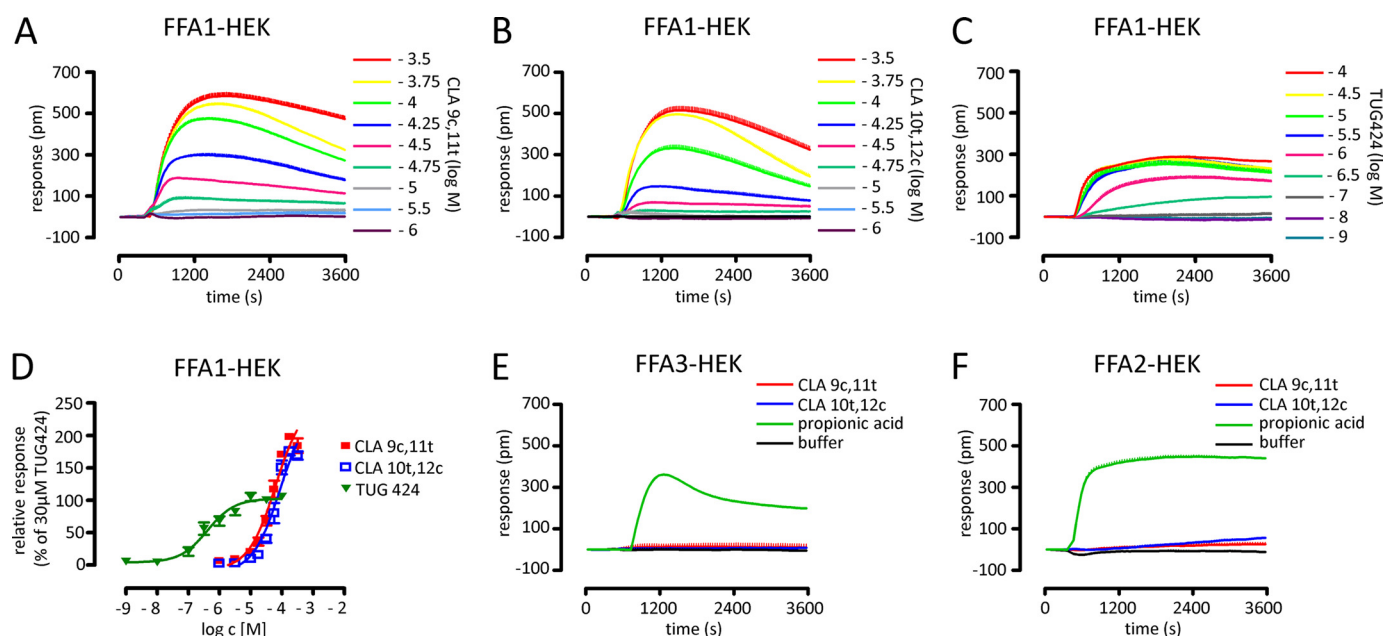


FIGURE 2. Label-free real time DMR analysis of CLA isomers in HEK cells. A–C, HEK cells stably expressing FFA1 were challenged with the indicated concentrations of CLAs (A and B) and the small molecule FFA1 agonist TUG424 (compound **20** in Ref. 17) (C), and wavelength shift was monitored over time as a measure of receptor activation. D, dose-response curves for the indicated ligands in FFA1-HEK cells derived from DMR traces. E and F, DMR recordings of CLAs in HEK cells stably expressing FFA3 (E) or FFA2 (F). Functional response to propionic acid, an agonist of both receptors, is shown as control. Depicted are representative data (mean values + S.E.) of at least three independent experiments (A–C, E, and F) or mean values ± S.E. of four independent experiments (D).

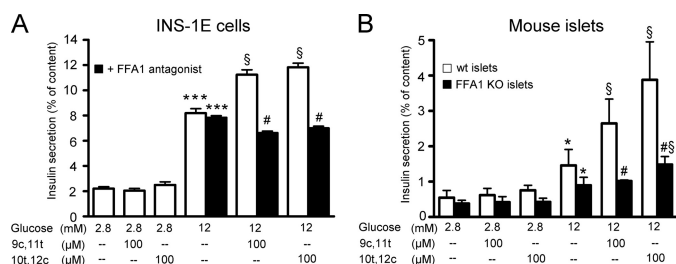


FIGURE 3. Effect of CLAs on glucose-stimulated insulin secretion in the immortalized rat INS-1E β -cell line (A) and in islets of wild type or FFA1^{-/-} mice (B). CLAs potentiate GSIS only at high glucose levels in an FFA1-dependent manner (A). The FFA1 antagonist (PPTQ, Ref. 22) was applied at a final concentration of 10 μ M. Shown are mean values ± S.E. of 3–6 independent experiments, each performed in quadruplicate. * and ***, significant effect to 2.8 mM glucose; \$, significant effect to 12 mM glucose; #, significant effect to 12 mM glucose and the respective CLA analog; *, \$, and #, $p < 0.05$; ***, $p < 0.001$ according to one-way ANOVA with Bonferroni's multiple comparisons test.

ever, this effect was blunted for the 9c, 11t-isomer or markedly reduced for the 10t, 12c-isomer in islets of the corresponding FFA1^{-/-} littermates. These data suggest that CLA-mediated insulinotropic effects *in vivo* are specifically mediated through FFA1 and that 10t, 12c-CLA but not 9c, 11t-CLA acutely amplifies insulin secretion via an additional mechanism not involving FFA1.

DISCUSSION

Modulation of glucose homeostasis and insulin sensitivity has long been ascribed to CLAs and proposed to involve, at least in part, activation of the peroxisome proliferator-activated receptor γ (PPAR- γ), which is a target for the potent insulin-sensitizing thiazolidinedione antidiabetic drugs (5, 8, 33). Increased insulin-releasing capacity of pancreatic islets from CLA-fed mice has been disclosed (34–36); however, a molecular explanation for the acute insulinotropic action of CLAs is still lacking. In this study, we present sev-

eral lines of evidence that acute insulinotropic effects of CLAs are mediated via activation of the β -cell-specific G protein-coupled receptor FFA1, an emerging therapeutic target to treat type 2 diabetes (15, 16, 21, 27, 37, 38). First, increase of $[Ca^{2+}]_i$ and inositol phosphate production in response to CLAs was consistently observed in FFA1-expressing cells regardless of the cellular background, whereas it was not observed in cells lacking FFA1. Second, Ca^{2+} mobilization was completely prevented by prior desensitization with the small molecule FFA1 agonist TUG424 or by pretreatment of cells with a specific FFA1 antagonist. Third, real time non-invasive holistic DMR measurements showed specific activation of FFA1 by CLAs. Together, these findings demonstrate a direct interaction between FFA1 and CLAs *in vitro*. Importantly, insulin secretion by CLAs could also be detected in isolated islets from wild type but was abolished or markedly reduced in islets from FFA1^{-/-} mice, suggesting that FFA1 is implicated in CLA potentiation of GSIS also *in vivo*.

At present, the clinical significance of the CLA-mediated FFA1 activation is unclear. In humans, basal plasma levels of CLAs are in the μ M range (~ 7 – 70μ M) (8, 39). Daily supplementation with CLA in healthy human volunteers resulted in 3–4-fold increases in plasma CLA levels (40). Given the 3–4-fold increase of plasma CLA with supplementation (39–41) and the fact that local tissue concentrations can easily reach levels 10-fold higher than plasma concentrations (42, 43), the experimental CLA concentrations used herein should be both physiologically attainable and relevant. It is therefore conceivable that therapeutic concentrations of CLAs may likely be sufficient to activate FFA1 *in vivo*. This in turn raises the possibility that FFA1 is responsible for both acute insulinotropic effects but also development of diabetes after long term ingestion of CLAs, two observations that have been made in various clinical studies with oral CLA supplementation (8, 13).

So far, FFA1 has been consistently involved in mediating the acute stimulatory effects of various long chain fatty acids on insulin secretion (15, 16, 21, 32, 44, 45), but it has also been implicated in potential deleterious effects of fatty acids on β -cell function (27, 38, 44), albeit the latter aspect is considered as rather controversial (27, 38). Nevertheless, these aspects are of prime importance to antidiabetic drug discovery because a potential contribution of FFA1 to β -cell dysfunction would disqualify FFA1 agonists as novel type 2 diabetes drugs. Because it is completely unresolved at present whether inhibition or activation of FFA1 constitutes the desired therapeutic strategy to treat type 2 diabetes (19, 38), it is impossible to predict whether long term ingestion of CLAs will constitute a new avenue to combat this metabolic disorder or rather be detrimental to β -cell health. The clinical consequences of FFA1 stimulation by CLAs in humans remain largely to be elucidated, but our study presents unambiguous evidence that CLAs are highly efficacious agonists for FFA1 both *in vitro* and *in vivo*. We also provide novel mechanistic insights into the complex signaling behaviors of these pleiotropic lipid mediators and add, for the first time, a specific cell surface receptor to the list of molecular CLA targets, a finding with both clinical significance and great implication for the development of FFA1 modulators as a novel treatment for type 2 diabetes.

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