Palmitate impairs and eicosapentaenoate restores insulin secretion through regulation of SREBP-1c in pancreatic islets.

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Objective: Chronic exposure to fatty acids causes \(\beta\)-cell failure, often referred to as lipotoxicity. We investigated its mechanisms focusing on contribution of SREBP-1c, a key transcription factor for lipogenesis.

Research Design and Methods: We studied \textit{in vitro} and \textit{in vivo} effects of saturated and polyunsaturated acids on insulin secretion, insulin-signaling and expression of genes involved in \(\beta\)-cell functions. Pancreatic islets isolated from C57BL/6 control and SREBP-1-null mice, and adenoviral gene-delivery or -knockdown systems of related genes were used.

Results: Incubation of C57BL/6 islets with palmitate (PA) caused inhibition of both glucose- and potassium-stimulated insulin secretion, but addition of eicosapentaenoate (EPA) restored both inhibitions. Concomitantly, PA activated, and EPA abolished both mRNA and nuclear protein of SREBP-1c, accompanied by reciprocal changes of SREBP-1c-target genes such as IRS-2 and granuphilin. These PA-EPA effects on insulin secretion were abolished in SREBP-1-null islets. Suppression of IRS-2/Akt pathway could be a part of the downstream mechanism for the SREBP-1c-mediated insulin secretion defect because adenoviral constitutive-active Akt compensated it. UCP-2 also plays a crucial role in the PA inhibition of insulin secretion as confirmed by knockdown experiments, but SREBP-1c-contribution to UCP-2-regulation was partial. The PA-EPA regulation of insulin secretion was similarly observed in islets from C57BL/6 mice pretreated with dietary manipulations. Furthermore, administration of EPA to diabetic KK.Ay mice ameliorated impairment of insulin secretion in their islets.

Conclusions: SREBP-1c plays a dominant role in PA-mediated insulin secretion defect, and EPA prevents it through SREBP-1c inhibition, implicating a therapeutic potential for diabetes related to lipotoxicity.
Molecular mechanisms of pancreatic islet \( \beta \)-cell failure, a crucial pathological contributor to the development for diabetes mellitus have been extensively explored (1-3). Impairment of glucose-stimulated insulin secretion (GSIS) is an early feature of type2 diabetes, and influx of fatty acids into \( \beta \)-cells, \( \beta \)-cell lipotoxicity, has been thought to be involved in its pathogenesis (4; 5). The intracellular events leading to GSIS include glucose metabolism for ATP production, closure of ATP-dependent K channels, membrane voltage-dependent calcium influx, calcium-dependent vesicle transport, and exocytosis of \( \alpha \)-granules containing insulin (6; 7). Lipotoxicity has been implicated in reducing GSIS via many of these steps (8). For example, uncoupling protein-2 (UCP-2), a mitochondrial membrane protein involved in energy production, plays an important role in fatty acid-induced lipotoxic effects (9-12). While \( \beta \)-cells have been traditionally thought to simply produce insulin in response to glucose, more recent studies have highlighted the role of insulin-signaling in \( \beta \)-cells. Studies on insulin-signaling in \( \beta \)-cells such as targeted disruption of the insulin receptor (13) and insulin receptor substrate-2 (IRS-2) (14; 15) have shown that this pathway can influence both \( \beta \)-cell mass and insulin secretion.

Sterol regulatory element-binding protein (SREBP)-1c is a membrane-bound transcription factor of the basic HLH leucine zipper family, and has been established as a regulator of lipogenic enzymes in the liver (16; 17). Expression of SREBP-1c is highly upregulated by dietary intake of carbohydrates and sugars (18-21). Conversely, polyunsaturated fatty acids, such as eicosapentaenoate (EPA), have been shown to inhibit hepatic SREBP-1c through multiple mechanisms (22; 23). Recent data suggested that hepatic SREBP-1c is also induced by dietary saturated fatty acids (24). The data from SREBP-1c-transgenic and LDL receptor knockout doubly mutant mice provide evidence that activation of this nutritionally regulated lipid transcription factor could be involved in formation of components of metabolic syndrome such as hyperlipidemia and atherosclerosis (Takahashi.A and Shimano.H unpublished data). Furthermore, SREBP-1c directly represses IRS-2 expression and leads to hepatic insulin resistance as a part of underlying pathogenesis for metabolic syndrome (25). In pancreatic \( \beta \)-cells, activation of SREBP-1c has been shown to be involved in impaired insulin secretion and glucose intolerance (26-28). Features of hepatic SREBP-1c induction by saturated fatty acids, repression by PUFA, and inhibition of IRS-2 were reproducibly observed in \( \beta \)-cells. As the major downstream insulin-signaling, the IRS-2/PI3K/Akt pathway has links to cell growth and survival as well as to glucose metabolism leading to ATP production (29). Insulin-signaling in \( \beta \)-cells has been thought to
be important for β-cell mass based upon analyses of β-cell-specific transgenic mice of Akt (30-32), and tissue-specific knockout mice of insulin receptor (13) and IRS-2 (14; 15). More recently, importance of insulin-signaling in β-cell function is also noticed in the aspect of glucose/insulin-signaling/Foxo1 pathway (29; 33; 34). Nuclear Foxo1 has a negative effect on β-cell mass and insulin secretion in vivo (34). Insulin-signaling phosphorylates nuclear Foxo1 for nuclear exclusion and contributes to β-cell protection.

Recently, we reported that granuphilin, a crucial component of the docking machinery of insulin-containing vesicles to the plasma membrane (35-37), is regulated by SREBP-1c in β-cell (38). Thus, taken together with clinical implication of fatty acids as causative factors for β-cell lipotoxicity, it is conceivable that SREBP-1c is involved in β-cell-lipotoxicity-mediated insulin secretion defects in GSIS.

In the current studies, we investigated the effects of PA, a typical saturated fatty acid, on GSIS in isolated islets and found that PA impairs GSIS and addition of EPA protects against these effects. Analyses of PA-EPA on gene expression, including SREBP-1c and their target genes led to clarification of the molecular mechanisms of PA induced β-cell lipotoxicity and protective effects of EPA.

**RESEARCH DESIGN AND METHODS**

**Materials.**

Palmitate (PA) and eicosapentaenate (EPA) were purchased from Sigma (St.Louis, MO). Enhanced chemiluminescence Western blot detection kit, [1-14C] palmitate, and [3H] mannitol were purchased from Amersham Biosciences.

**Animals.**

This project was approved by Animal Care Committee of University of Tsukuba. Male C57BL/6 wild type and KK-Ay mice at 8-weeks of age were purchased from Clea Japan, Inc. (Tokyo, Japan.). SREBP-1-null mice at 14-15-weeks of age were as described (39). The mice were housed in colony cages, maintained on a 12-h light/12-h dark cycle, and given free access to water and a standard chow diet (MF, Oriental yeast, Tokyo, Japan) and adapted to their new environment for 1 week prior to experiments.

**Isolation of mouse pancreatic islets.**

Isolation of islets from mice was carried according to the Ficoll-Conray protocol as described (26; 38; 40). In brief, after clamping the common bile duct at a point close to the duodenum outlet, 2.5 ml Krebs-Ringer bicarbonate buffer (KRBH, pH 7.4) containing 0.5% bovine serum albumin (BSA) and 4mg/ml collagenase (Sigma) was injected into the duct. The swollen pancreas was removed and incubated at 37°C for 20 min. The pancreas was then dispersed by pipetting, and after washing twice with KRBH, the islets were collected manuallys under stereo-microscope. Isolated islets were put in culture medium (RPMI-1640 medium supplemented with 10% fetal calf serum, 0.5% BSA, 100 units/mL penicillin and 100 μg/mL streptomycin as
antibiotics) for 16-18 hr at 37 °C in a humidified atmosphere containing 5 % CO₂ prior to the experiments.

**Analyses of insulin secretion and insulin contents of islets.**

Insulin release from islets was measured as described (26; 38). Batches of 10 islets were incubated for 30 min in 1 mL KRBH pH 7.4 containing 0.5% BSA at 2.8 mM glucose for 30 min. Islets medium was replaced with KRBH containing 20 mM glucose or alternatively 30 mM KCl + 2.8 mM glucose to estimate insulin secretion, and were incubated for 30 min. At the end of each incubation period, the medium was collected and islets were subjected to insulin extraction with acidic ethanol (0.2 mol/L HCl in 75% ethanol) for insulin measurement with an insulin ELISA kit. Hoechst-33258 was used to determine the DNA contents of sonicated islets.

**Determination of ATP/ADP ratio and triglyceride contents of islets.**

ATP and ADP contents in isolated islets were as described (26; 38; 41). ATP and ADP were extracted from islets with 5 % of trichloroacetic acid. After centrifugation, the supernatants were neutralized with NaOH. ATP content was measured using the CellTiter-Glo luminescent cell viability assay kit (Promega, Madison, WI). ADP content was estimated after conversion of ADP to ATP in the reaction buffer (20mM HEPES and 3mM MgCl₂, pH 7.75) containing 2.3 U/ml pyruvate kinase and 1.5mM phosphoenolpyruvate at room temperature for 15 min.

Triglycerides of islets were measured after extracting lipids with the Folch’s method. After 1-2min of sonication, islets were mixed with chloroform and methanol (2:1) for lipid extraction, dried up by evaporation and re-suspended in isopropanol. Triglyceride concentration was measured using the GPO-trinder kit (Sigma).

**Real-time PCR and Immunoblot analysis.**

Total RNA extraction with the TRIzol reagent (Invitrogen, Carlsbad, CA) and DNase-I treatment using the RNeasy Micro kit (Qiagen, Hilden, Germany) were performed according to the manufacturer’s instructions. cDNA was synthesized with ThermoScript (Invitrogen) and comparative analysis of mRNA levels was performed with fluorescence-based real-time PCR. Real-time PCR analyses were performed using SYBR-Green Dye (Nippon Gene, Tokyo, Japan) in an ABI 7000 PCR instrument (Applied Biosystems, Foster City, CA). The relative abundance for each transcript was calculated by a standard curve of cycle thresholds for serial dilutions of a cDNA sample and normalized to cyclophilin. Primer sequences are described in Appendix Table 3.

Immunoblot analyses were performed as described (26; 38). Cell extracts from isolated islets were probed with rabbit polyclonal anti-SREBP-1 (sc-8984, Santa Cruz, Santa Cruz, CA), anti-IRS-2 (06-506, Upstate Technology, Bedford, MA), anti-Akt (#9272) anti-phospho Akt (S473, #9271), anti-phospho
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Akt (T308, #9275, Cell Signaling, Beverly, MA), anti-UCP-2 (Research Diagnostic Inc, San Antonio, TX), and anti-α-tubulin (sc-8035, Santa Cruz). Anti-granuphilin a/b antibody was used as previously described (37; 38) Detection was performed using an ECL advance Western blotting detection kit and ECL Hyperfilm (Amersham Biosciences).

**Treatment of islet with PA and EPA.**

PA and EPA were dissolved to 100mM in methanol to make stock solutions for later dilution in RPMI-1640 supplemented with 0.5 % BSA to a final concentration of 400 μM (PA) and 50 μM (EPA) respectively. Islets were treated for 48 hr before indicated experiments.

**Cellular uptake of [1-14C]palmitate**

[1-14C]palmitate uptake of islets was measured as described (42). Briefly, the isolated islets were incubated for 60 min in culture medium containing 400μM palmitate, radiolabeled [1-14C]palmitate (0.3 μCi/ml) with or without 50 μM EPA, and [3H]mannitol (0.06 μCi/ml). The latter was used to calculated correction for non-specific uptake. Ice-cold 0.5 N NaOH was added to the islets to terminate the uptake reaction and neutralized by 0.5 N HCl. After the removal of the supernatant by centrifugation at 12,000 g for 1 min the residual radioactivity was determined.

**Small interfering RNA (siRNA) for UCP-2.**

The siRNA construct for mouse UCP-2 was generated within the coding region of UCP-2; 5’-GTCGAAGCCTACAAGCCAA-3’ (Ad-UCP-2 RNAi). The siRNA for LacZ (Ad-LacZ RNAi) from Invitrogen (BLOCK-iT U6 RNAi Entry Vector Kit, K4944-00) was used as a control according to manufacturer’s instructions. Oligonucleotide containing this sequence was subcloned into U6/RNAi Entry vector (Invitrogen). UCP-2 RNAi adenoviruses were generated using BLOCK-iT Adenoviral RNAi Expression System (Invitrogen).

**Adenovirus infections for constitutive active form Akt and siRNA of UCP2.**

Infection of constitutive-Active Akt (43) and siRNA of UCP-2 adenovirus studies were performed as described (25; 38; 44). In brief, generation of recombinant adenoviral plasmid was produced by homologous recombination with the pAdEasy-1 plasmid (Invitrogen). Production of recombinant adenoviruses was performed by CsCl gradient centrifugation as previously described (25; 38; 44).

**Palmitate rich diet study and KK-Ay mice study.**

In vivo palmitate rich diet study and KK-Ay mice study were described in Appendix RESEARCH DESIGN and METHODS. Briefly, in palmitate rich diet study, C57BL6 mice were fed with control diet (Fish oil free diet), Triplamitin diet (20% Tripalmitin), and Tripalmitin + EPA-E diet (20% Tripalmitin and 5% EPA-E) for 28 days. In KK-Ay mice study, KK-Ay mice were administered vehicle (5 % gum aravic) or EPA-E at a dose of 1g/kg/day for 28 days.

**Statistical analysis.**

Results are reported as mean ± S.E.M.
Statistical analyses were performed using one-way ANOVA followed by Dunnett’s procedure or two-way ANOVA followed by Turkey’s procedure.

RESULTS

Palmitate (PA) impairs and eicosapentaenoate (EPA) restores insulin secretion in murine islets.

To investigate pancreatic lipotoxicity, we evaluated effects of palmitate (PA, C16:0) on the insulin secretion of isolated mouse pancreatic islets. While PA (400 μM) had no effect on basal insulin secretion (low glucose concentrations), stimulation with high glucose concentrations, i.e. glucose-stimulated insulin secretion (GSIS), was inhibited by addition of PA (Fig. 1A). When eicosapentaenoate (EPA C20:5, n-3, 50 μM) was combined with PA-treated islets (hereafter referred to as PA-EPA), the suppressed insulin secretion was restored to near-normal levels (Fig. 1A). PA inhibition and EPA restoration of insulin secretion was also observed after addition of KCl, which bypasses ATP-sensitive channels to stimulate insulin secretion (KCl-stimulated insulin secretion, KSIS) (Fig. 1A). PA inhibition and EPA restoration of insulin secretion was also observed after addition of KCl, which bypasses ATP-sensitive channels to stimulate insulin secretion (KCl-stimulated insulin secretion, KSIS) (Fig. 1A). These effects of PA and EPA on insulin secretion were dose-dependent (Appendix Fig. 1A and B). EPA by itself did not have any effect on GSIS or KSIS, indicating that EPA does not intrinsically increase, but cancels PA-suppressed insulin secretion. The slight changes of insulin content compared with GSIS and KSIS by PA and EPA indicates that the phenomenon in insulin content was only a part of the mechanism (Fig. 1B). Considering the experimental setting, the protective effect of EPA against PA-induced lipotoxicity could be inhibition of cellular uptake of PA. To exclude this possibility, uptake of labeled PA was measured and was not affected by additional EPA (Fig. 1C). These data indicate that the EPA does not interfere with PA uptake, but rather, directly competes with PA in intracellular events.

PA and EPA regulate SREBP-1c and its target genes.

Gene expression in PA- and PA-EPA-treated islets was investigated using real-time PCR. SREBP-1c mRNA, was highly induced by PA and completely suppressed by EPA, but not SREBP-1a mRNA (Fig. 2A). These changes in SREBP-1c mRNA were associated with those in both membrane and nuclear forms of SREBP-1c protein (Fig. 2D). In accordance, its target genes such as FAS (Fatty acid synthase), SCD1 (Stearoy-CoA desaturase 1), and ELOVL6 (the elongation of long-chain fatty acids family number 6) showed similar patterns of regulation by PA and PA-EPA (Fig. 2B). Triglyceride (TG) content, as an indication of SREBP-1c effect and lipotoxicity, was increased by PA and repressed by PA-EPA (Fig. 2C).

We recently reported that SREBP directly suppressed hepatic IRS-2 expression and caused insulin resistance in the liver (25). In accordance with changes in SREBP-1c in
islets, IRS-2 was strongly suppressed by PA and was partially restored by addition of EPA, implicating a role for SREBP-1c-mediated IRS-2 repression in the PA-EPA-mediated changes in β-cell physiology (Fig. 2D and Appendix Fig. 2A).

UCP-2 has been established to play a key role in lipotoxicity of pancreatic β-cells through dissociation of fatty acid oxidation and ATP production (Appendix Fig. 2B) (9-12). UCP-2 promoter was also reported as an SREBP target (11; 12). This key regulator of lipotoxicity was modulated by PA and PA-EPA in a similar manner at both mRNA and protein levels (Appendix Fig. 2A and Fig. 2D).

Granuphilin was an effector of Rab27a and its overexpression was reported to inhibit exocytosis of insulin granules (35-37). We recently reported that granuphilin promoter was a direct target of SREBP-1c and SREBP-1c/granuphilin pathway was a potential mechanism for impairment GSIS in diabetes, leading to β-cell lipotoxicity (38). This key molecule of lipotoxicity was up-regulated by PA and suppressed by PA-EPA in both mRNA and protein levels (Appendix Fig. 2A and Fig. 2D).

**SREBP-1c plays a dominant role in PA-EPA effects on insulin secretion.**

The contribution of SREBP-1c in PA-EPA effects on both GSIS and KSIS was estimated using islets from SREBP-1-null mice. Basal insulin secretion was not affected by SREBP-1 deficiency (data not shown); however, GSIS was modestly increased (Fig. 3A). The absence of SREBP-1 abolished PA-induced inhibition of GSIS and KSIS (Fig. 3A). Due to this, EPA protection from impairment of GSIS and KSIS as observed in wild type islets, was not detected in SREBP-1-null islets (Fig. 3A). These data suggest that PA-induced pancreatic lipotoxicity and amelioration of that by EPA depend on SREBP-1c. Predictably from primary role of SREBP-1c in lipogenesis, the elevation and repression of TG content in wild type islets by PA and EPA, respectively, were absent in SREBP-1-null islets (Fig. 3B). Suppression of IRS-2 and stimulation of granuphilin mRNA expressions caused by PA in wild type islets were both blunted in SREBP-1 null islets (Fig. 4A). Accordingly, the reversal of PA effects on IRS-2 and granuphilin expression by EPA was not observed in SREBP-1-null islets. On the other hand, induction of UCP-2 expression by PA was observed even in SREBP-1-null islets, but EPA treatment reversed the PA effect in both genotypes (Fig. 4A).

**IRS-2 suppression by SREBP-1c contributes to PA-EPA effects on GSIS.**

Based upon recent cumulative evidence on importance of insulin-signaling in β-cell function and our observation of reciprocal changes in SREBP-1c and IRS-2 by addition of PA and/or EPA in islets (Fig. 2D and Appendix Fig. 2A), we hypothesized that PA suppression of insulin secretion might be due to impaired insulin-signaling caused by induction of SREBP-1c leading to decreased
IRS-2 expression. To test this hypothesis, insulin-signaling was estimated in wild type and SREBP-1-null islets by analysis of Akt phosphorylation status. Consistent with changes at the mRNA level (Appendix Fig. 2A), suppression and restoration of IRS-2 protein by PA and PA-EPA, respectively, in wild type islets was not apparent in SREBP-1 null islets (Fig. 4B). Consequently, Akt phosphorylation impaired by PA in wild type islets was completely abolished by the absence of SREBP-1 (Fig. 4B). These data suggest that SREBP-1c could be highly involved in PA-mediated inhibition of insulin-signaling and insulin secretion.

To explore impacts of insulin-signaling on PA-EPA-regulated insulin secretion, forced activation of insulin-signaling downstream of IRS-2, was induced in mouse isolated islets by adenoviral gene transfer of constitutively active (dominant positive) Akt (Akt-CA). Akt-CA over-expression significantly improved both GSIS and KSIS that were impaired by PA, but did not further enhance restoration by EPA, indicating that insulin-signaling and insulin secretion were linked in PA-EPA effects (Fig. 5A). Akt-CA over-expression only slightly enhanced phosphorylation of Akt in untreated islets, but completely restored suppressed pAkt in PA-treated islets (Fig. 5B). Insulin-signaling downstream of Akt such as pAkt was also consistently suppressed by PA. These signaling molecules were all restored by Akt-CA over-expression. Islets treated with PA-EPA exhibited signals similar to control islets regardless Akt-CA over-expression (Fig. 5B). Both SREBP-1 deficiency (Fig. 3A and 4B) and constitutive activation of insulin-signaling by Akt-CA (Fig. 5A and 5B) cancelled the protective effects of EPA against PA induced impaired insulin secretion and insulin-signaling. Activation of Akt did not change either SREBP-1c or UCP-2 (Fig. 5C).

**Contribution of UCP-2 to PA-EPA effects on GSIS**

The contribution of UCP-2 to the effects of PA-EPA on GSIS was estimated in knock-down experiments using adenoviral siRNA of UCP-2. A robust inhibition of mRNA and protein levels of UCP-2 was obtained (Fig. 6A and Appendix Fig. 3). Gene silencing of UCP-2 did not effect basal insulin secretion or GSIS. In contrast, UCP-2 knockdown significantly protected PA-mediated impaired GSIS and canceled the EPA-protection (Fig. 6B). PA-mediated reduction in ATP/ADP ratio was significantly restored by UCP-2 suppression, and protective effect of EPA was also canceled (Fig. 6C). Changes in ATP/ADP ratio and GSIS by modulation of UCP-2 expression were very similar, confirming that the PA-EPA effects on GSIS depend upon the UCP-2/ATP system, as was previously suggested by knockout mice studies. PA-induction of SREBP-1c was not affected by UCP-2 knockdown (Fig. 6D). Taken together with partial regulation of UCP-2 in SREBP-1 null islets (Fig. 4A), effects of UCP-2 and SREBP-1c on GSIS are
EPA in vivo exhibits a protective role against PA-lipotoxicity in islets.
To determine whether the effects of PA-EPA on GSIS in isolated islets could be extended in vivo, mice were fed a fish oil-free diet with or without 20% tripalmitin or tripalmitin plus 5% EPA ethyl ester for 28 days and GSIS in freshly isolated islets from these animals were measured. PA feeding impaired and EPA restored GSIS in conjunction with changes in islet SREBP-1c expression (Fig. 7A and 7B). These data demonstrate that dietary PA and EPA influence insulin secretion in vivo in a similar manner to PA-EPA effects observed in isolated islets.

The effect of EPA on GSIS in vivo was further investigated in isolated islets from KK-Ay mice, a model of obesity and type-2 diabetes (45). In islets from KK-Ay mice, GSIS was impaired and SREBP-1c expression was increased. Administration of EPA ethyl ester at a dose of 1 g/kg/day for 28 days restored GSIS and suppressed SREBP-1c expression (Fig. 7C and 7D), leading to restoration of GSIS and KSIS. In both in vivo experiments, these data did not accompany changes in food intake, or gross morphological changes in pancreatic islets (shown in appendix Table 1, 2 and Fig.4).

DISCUSSION
It has long been known that chronic exposure of PA to islets or β-cell lines causes lipotoxicity leading to blunted GSIS (1; 3-5). Our current studies clearly demonstrate that this PA-induced impairment of insulin secretion is restored by supplement of EPA. The results also indicated that this PA-EPA regulation is not due to cell toxicity or apoptosis (data not shown), but mediated through two major key molecules: SREBP-1c and UCP-2. Several factors are known to be important for function of β-cell such as ATP/ADP ratio, IRS-2/Akt insulin-signaling, and granuphilin. These factors are all consistently disturbed by PA, and improved by additional EPA, through up- and down-regulation of SREBP-1c, respectively. Taken together with over-expression and knockout experiments of SREBP-1, it can be concluded that SREBP-1c plays a crucial role in for β-cell lipotoxicity as a causative upstream factor.

Contribution of UCP-2 to ATP depletion and impaired insulin secretion has been well established (9-12; 26). Our current studies also confirm this in PA-mediated suppression of GSIS. PA led to up-regulation of UCP-2 and reduction of intracellular ATP. Knockdown of UCP-2 by siRNA restored PA induced impairment of GSIS. SREBP has been reported to directly bind to and activate the UCP-2 promoter (11; 12). Supportively, we observed that β-cell-specific over-expression of SREBP-1c elevated UCP-2 expression contributing to the β-cell lipotoxicity in transgenic mice (26). However, based upon the current results from
SREBP-1-null islets, SREBP-1c only partially participated in PA-induced expression of UCP-2. Conversely, UCP-2 knockdown did not affect SREBP-1c expression in islets. Thus, although both key molecules play a dominant role in β-cell lipotoxicity, there might not be a definite causative relationship between SREBP-1c, an indicator of lipogenesis, and UCP-2, an indicator of energy consumption (Fig. 8).

Our data on Akt-CA over-expression experiments provide another evidence for importance of insulin-signaling in β-cell function. PA inhibited and EPA restored insulin-signaling in an opposite manner to SREBP-1c expression. Based upon the potential of SREBP-1c on insulin-signaling through regulation of IRS-2 (25; 26), we explored involvement of insulin-signaling in PA-EPA regulation of insulin secretion. Activation of Akt did not change normal insulin secretion, but markedly ameliorated PA-impaired insulin secretion in isolated islets. Thus, insulin-signaling could be a prerequisite for insulin secretion, and the importance of its role in insulin secretion becomes overt only upon its impairment. PA-suppression of insulin-signaling was cancelled in SREBP-1-null islets. Based upon these data, we conclude that SREBP-1c in β-cells plays a crucial role in the inhibition of insulin-signaling via suppression of IRS-2 and contributes to impaired insulin secretion (26). In contrast to established effects of insulin-signaling on β-cell mass (13-15; 29-34), our data indicated that activation of Akt could restore insulin secretion impaired by PA in a short term. Precise molecular mechanism for this is currently unknown, although phosphorylation of Foxo-1 and anti-apoptosis could be involved (29; 34).

Dietary PUFA such as EPA have been shown to have plasma triglyceride-lowering effects and to improve fatty liver and hepatic insulin resistance (22; 23; 46). We previously reported that PUFA inhibited hepatic SREBP-1c, which contributed to beneficial roles of PUFA against lipotoxicity in the liver (22; 23). Current data provide another beneficial role of EPA; protection from lipotoxicity in pancreatic β-cells. Our data also suggest that this protective action of EPA is mediated mainly through suppression of SREBP-1c. EPA reduced mRNA and nuclear protein levels of SREBP-1c in PA treated islet. In addition, a large portion of EPA protection against PA-induced impaired GSIS was not re-produced in SREBP-1 null islets. Amelioration of impaired GSIS by EPA was also confirmed in vivo with SREBP-1c suppression. EPA also suppressed over-expression of UCP-2 by PA even in SREBP-1 null islets. This suggests that suppression of UCP-2 also may contribute to protective effect by EPA against PA-mediated suppression of GSIS, which is presumably independent of SREBP-1c (Fig. 7).

Our data showing that enhancement of insulin-signaling in β-cells can improve impaired insulin secretion caused by lipotoxic
effects of PA has important clinical relevance. It has been recognized that hyperglycemia exacerbates the impairment of insulin secretion, often referred as glucotoxicity and that short term insulin treatment often effectively improves insulin secretion. This has been thought to be due to reducing blood glucose; however, our current findings implicate that stimulation of insulin-signaling in β-cells could potentially contribute to the improvement of insulin secretion, especially in lipotoxic states. From a long-term standpoint, our findings might relate to the onset of type2 diabetes because intake of excess saturated fatty acids can cause both insulin resistance and impaired insulin secretion in β-cells. Our data also suggest that oral dosing of EPA could contribute to protection from the b-cell lipotoxicity. Since our findings are based mostly upon in vitro studies, further investigations in vivo are needed to test our conclusions.

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**EPA prevents β-cell lipotoxicity through SREBP1c**

Fig. 1 Lipotoxic effects of PA and protective effects of EPA on insulin secretion in murine isolated islets.

(A) Low glucose (2.8 mM)-, high glucose (20 mM)- and KCl- stimulated insulin secretions from murine isolated islets incubated without (Control, white bars) or with PA (black bars), PA-EPA (bold hatched bars) or EPA (regular hatched bars). (B) Insulin content of islets incubated without (Control) or with PA, PA-EPA, or EPA. (C) PA uptake in islets isolated from C57BL/6 mice. Three independent experiments were performed using four sets of islets for each repetition, and results are reexpressed as mean ± S.E.M. Statistical analysis was performed using one-way ANOVA followed by Dunnett’s procedure, **p < 0.01 and * p < 0.05, (versus PA group), respectively. 
EPA prevents β-cell lipotoxicity through SREBP1c

Fig. 2 Gene expression and protein profiles in murine isolated islets treated with PA or PA-EPA.

(A) and (B) Levels of mRNA of various genes in pancreatic islets isolated from C57BL/6 mice without (Control, white bars), or with PA (black bars), PA-EPA (hatched bars), or EPA (regular hatched bars) as determined by real-time PCR. mRNA quantities were calculated as a ratio to the cyclophilin level in the each cDNA sample. Data are shown as the relative expression ratio to control samples. (C) Cellular triglyceride levels of islets incubated with PA, PA-EPA, or EPA. (D) Immunoblot analysis of indicated proteins in the islets. Cont.; Control, mSREBP-1 (membrane form), and nSREBP-1 (nuclear form), respectively. α-tubulin protein was used as a loading control. Three independent experiments were performed using four sets of islets and results are expressed mean ± S.E.M. Statistical analyses were performed using one-way ANOVA followed by Dunnett’s procedure, ** p < 0.01 (versus PA group).
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Fig. 3 Protection from PA-induced lipotoxicity in islets isolated from SREBP-1-null mice.

Islets were isolated from wild-type littermates (white bars) and SREBP-1-null mice (black bars), and incubated without (Control) or with PA, PA-EPA, or EPA for 48 hr. Glucose- and KCl-stimulated insulin secretions (A) and cellular triglyceride contents (B) were measured. Three independent experiments were performed using four sets of islets and results are expressed mean ± S.E.M. Statistical analyses were performed using two-way ANOVA followed by Turkey’s procedure. ** p < 0.01 and * p < 0.05 respectively.
**Fig. 4 Gene expression and protein profiles in islets isolated from SREBP-1-null mice treated with PA or PA-EPA.**

Islets were isolated from SREBP-1-null mice and wild-type littermates and incubated without (Control) or with PA, PA-EPA (PE), or EPA for 48 hr. (A) mRNA levels of the indicated genes were measured. mRNA levels were determined by real-time PCR, calculated as ratio to cyclophilin expression levels. (B) Immunoblot analysis of SREBP-1 and insulin-signaling proteins. Relative expression ratios to control samples are shown. Three independent experiments were performed using four sets of islets and results are expressed mean ± S.E.M. Statistical analyses were performed using two-way ANOVA followed by Turkey’s procedure. **p < 0.01 and * p < 0.05 respectively.**
Fig. 5 Effects of over-expression of constitutively active Akt in islets treated with PA or PA-EPA.

Islets were isolated from C57BL6 mice and incubated without (Control) or with PA, or PA-EPA for 48 hr. Islets were infected (100 M.O.I. respectively) with adenoviral-GFP (Ad-GFP) or -constitutively active Akt (Ad-Akt-CA) for 48 hr prior to incubation with PA or PA-EPA. Glucose- and KCl- stimulated insulin secretions (A) and protein levels of indicated insulin-signaling molecules (B) were measured. Amounts of insulin-signaling proteins were estimated by immunoblot analysis using indicated antibodies and α–tubulin protein was used as a loading control. Levels of mRNA of SREBP-1c and UCP-2 were determined by real-time PCR (C), calculated as ratio to cyclophilin expression levels. Relative expression ratios to control samples
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are shown. Three independent experiments were performed using four sets of islets and results are expressed mean ± S.E.M. Statistical analyses were performed using two-way ANOVA followed by Turkey’s procedure. ** p < 0.01 and * p < 0.05, respectively.

Fig. 6 Effects of UCP-2 gene silencing on murine isolated islets treated with PA or PA-EPA.

Islets were isolated from C57BL/6 mice and infected (500 M.O.I. respectively) with adenoviral siRNA for LacZ (Ad-LacZ RNAi) or UCP-2 (Ad-UCP-2 RNAi) and cultured without (Control) or with PA, PA-EPA, or EPA for 48 hr. The effects of UCP-2 siRNA on mRNA levels of UCP-2 in pancreatic islets isolated from C57BL/6 mice incubated with PA, PA-EPA, or EPA were determined by real-time PCR (A). Glucose-stimulated insulin secretions (B) and ATP/ADP ratio (C) from the islets after UCP-2 gene silencing and incubation with PA, PA-EPA, or EPA were measured. Level of mRNA of SREBP-1c was determined by real-time PCR (D), calculated as ratio to cyclophilin expression levels. Relative expression ratios to control samples are shown. Three independent experiments were performed using four sets of islets and results are expressed mean ± S.E.M. Statistical analyses were performed using two-way ANOVA followed by Turkey’s procedure. ** p < 0.01.
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**Effect of EPA on insulin secretion in vivo.**

C57BL/6 mice were fed control diet (white bars), 20% Tripalmitin diet (black bars), and Tripalmitin + 5% EPA-E diet (hatched bars) for 28 days. Islets were isolated from individual animals. Glucose- and KCl-stimulated insulin secretion (A) and mRNA levels of SREBP-1c (B) were measured. KK-Ay mice were received administration of vehicle (black bars) or EPA at a dose of 1g/kg/day (hatched bars) for 28 days. Islets were isolated from pool pancreas (3-4 animals). Glucose-stimulated insulin secretion (C) and mRNA levels of SREBP-1c (D) were measured. Three independent experiments were performed using four sets of islets and results are expressed mean ± S.E.M. Statistical analyses between indicated groups were performed using one-way ANOVA followed by Dunnett’s procedure, **p < 0.01 and * p < 0.05, respectively.
Fig. 8 Mechanism by which PA induces and EPA protects impairment of insulin secretion in pancreatic islets.