

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 β -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 in vitro and in vivo effects of saturated and polyunsaturated acids on insulin secretion, insulin signaling, and expression of genes involved in β -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 caused inhibition of both glucose- and potassium-stimulated insulin secretion, but addition of eicosapentaenoate (EPA) restored both inhibitions. Concomitantly, palmitate activated and EPA abolished both mRNA and nuclear protein of SREBP-1c, accompanied by reciprocal changes of SREBP-1c target genes such as insulin receptor substrate-2 (IRS-2) and granuphilin. These palmitate-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 constitutively active Akt compensated it. Uncoupling protein-2 (UCP-2) also plays a crucial role in the palmitate inhibition of insulin secretion, as confirmed by knockdown experiments, but SREBP-1c contribution to UCP-2 regulation was partial. The palmitate-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 palmitate-mediated insulin secretion defect, and EPA prevents it through SREBP-1c inhibition, implicating a therapeutic potential for treating diabetes related to lipotoxicity. *Diabetes* 57:2382–2392, 2008

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Molecular mechanisms of pancreatic islet β -cell failure, a crucial pathological contributor to the development of diabetes, have been extensively explored (1–3). Impairment of glucose-stimulated insulin secretion (GSIS) is an early feature of type 2 diabetes, and influx of fatty acids into β -cells (β -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 α -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). Although β -cells have traditionally been thought to simply produce insulin in response to glucose, more recent studies have highlighted the role of insulin signaling in β -cells. Studies on insulin signaling in β -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 β -cell mass and insulin secretion.

Sterol regulatory element-binding protein (SREBP)-1c is a membrane-bound transcription factor of the basic helix loop helix 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 (PUFAs), 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 (A. Takahashi, H. Shimano, 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 β -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 PUFAs, and inhibition of IRS-2 were reproducibly observed in β -cells. As the major downstream insulin signaling pathway, the IRS-2/PI3K/Akt pathway has links to cell growth and survival and to glucose metabolism leading to ATP production (29). Insulin signaling in β -cells has been thought to be important for β -cell mass based on 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 has also been noticed in the 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 palmitate, a typical saturated fatty acid, on GSIS in isolated islets and found that palmitate impairs GSIS and that addition of EPA protects against these effects. Analyses of palmitate-EPA on gene expression, including SREBP-1c and their target genes, led to clarification of the molecular mechanisms of palmitate-induced β -cell lipotoxicity and protective effects of EPA.

RESEARCH DESIGN AND METHODS

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

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

Isolation of mouse pancreatic islets. Isolation of islets from mice was carried out according to the Ficoll-Conray protocol as described previously (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% BSA and 4 mg/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 manually under stereomicroscope. Isolated islets were put in culture medium (RPMI 1640 supplemented with 10% FCS, 0.5% BSA, 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin as antibiotics) for 16–18 h at 37°C in a humidified atmosphere containing 5% CO_2 before the experiments.

Analyses of insulin secretion and insulin contents of islets. Insulin release from islets was measured as described previously (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 mmol/l glucose for 30 min. Islets medium was replaced with KRBH containing 20 mmol/l glucose or alternatively 30 mmol/l KCl plus 2.8 mmol/l 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 enzyme-linked immunosorbent assay kit. Hoechst-33258 was used to determine the DNA contents of sonicated islets.

Determination of ATP-to-ADP ratio and triglyceride contents of islets. ATP and ADP contents in isolated islets were as described previously (26,38,41). ATP and ADP were extracted from islets with 5% 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 (20 mmol/l HEPES and 3 mmol/l MgCl_2 , pH 7.75) containing 2.3 units/ml pyruvate kinase and 1.5 mmol/l phosphoenolpyruvate at room temperature for 15 min.

Triglycerides (TGs) of islets were measured after extracting lipids with Folch's method. After 1–2 min of sonication, islets were mixed with chloroform and methanol (2:1) for lipid extraction, dried up by evaporation, and resuspended in isopropanol. TG 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 manufacturers' 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) 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 supplemental Table 3, which is available in the online appendix at <http://dx.doi.org/10.2337/db06-1806>.

Immunoblot analyses were performed as described previously (26,38). Cell extracts from isolated islets were probed with rabbit polyclonal anti-SREBP-1 (sc-8984; Santa Cruz Biotechnology, Santa Cruz, CA), anti-IRS-2 (06–506; Upstate Technology, Bedford, MA), anti-Akt (no. 9272), anti-phospho Akt (S473; no. 9271), anti-phospho Akt (T308; no. 9275; Cell Signaling, Beverly, MA), anti-UCP-2 (Research Diagnostic, San Antonio, TX), and anti- α -tubulin (sc-8035; Santa Cruz Biotechnology). 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 islets with palmitate and EPA. Palmitate and EPA were dissolved to 100 mmol/l in methanol to make stock solutions for later dilution in RPMI 1640 supplemented with 0.5% BSA to a final concentration of 400 $\mu\text{mol}/\text{l}$ (palmitate) and 50 $\mu\text{mol}/\text{l}$ (EPA), respectively. Islets were treated for 48 h before indicated experiments.

Cellular uptake of [^{14}C]palmitate. [^{14}C]palmitate uptake of islets was measured as described previously (42). Briefly, the isolated islets were incubated for 60 min in culture medium containing 400 $\mu\text{mol}/\text{l}$ palmitate, 0.3 $\mu\text{Ci}/\text{ml}$ radiolabeled [^{14}C]palmitate with or without 50 $\mu\text{mol}/\text{l}$ EPA, and 0.06 $\mu\text{Ci}/\text{ml}$ [^3H]mannitol. The latter was used to calculate correction for nonspecific 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 $12,000 \times g$ for 1 min, the residual radioactivity was determined.

Small interfering RNA for UCP-2. The small interfering RNA (siRNA) construct for mouse UCP-2 was generated within the coding region of UCP-2: 5'-GTCGAAGCCTACAAGACCA-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 constitutively active Akt and siRNA of UCP2. Infection of constitutively active Akt (43) and siRNA of UCP-2 adenovirus studies were performed as described previously (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. The in vivo palmitate-rich diet study and KK-Ay mice study were described in RESEARCH DESIGN AND METHODS in the online appendix. Briefly, in the palmitate-rich diet study, C57BL6 mice were fed with control diet (fish oil-free diet), tripalmitin diet (20% tripalmitin), and tripalmitin plus 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 arabic) or EPA-E at a dose of $1 \text{ g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ for 28 days. **Statistical analysis.** Results are reported as means \pm SE. Statistical analyses were performed using one-way ANOVA followed by Dunnett's procedure or two-way ANOVA followed by Tukey's procedure.

RESULTS

Palmitate impairs and EPA restores insulin secretion in murine islets. To investigate pancreatic lipotoxicity, we evaluated effects of palmitate (C16:0) on the insulin

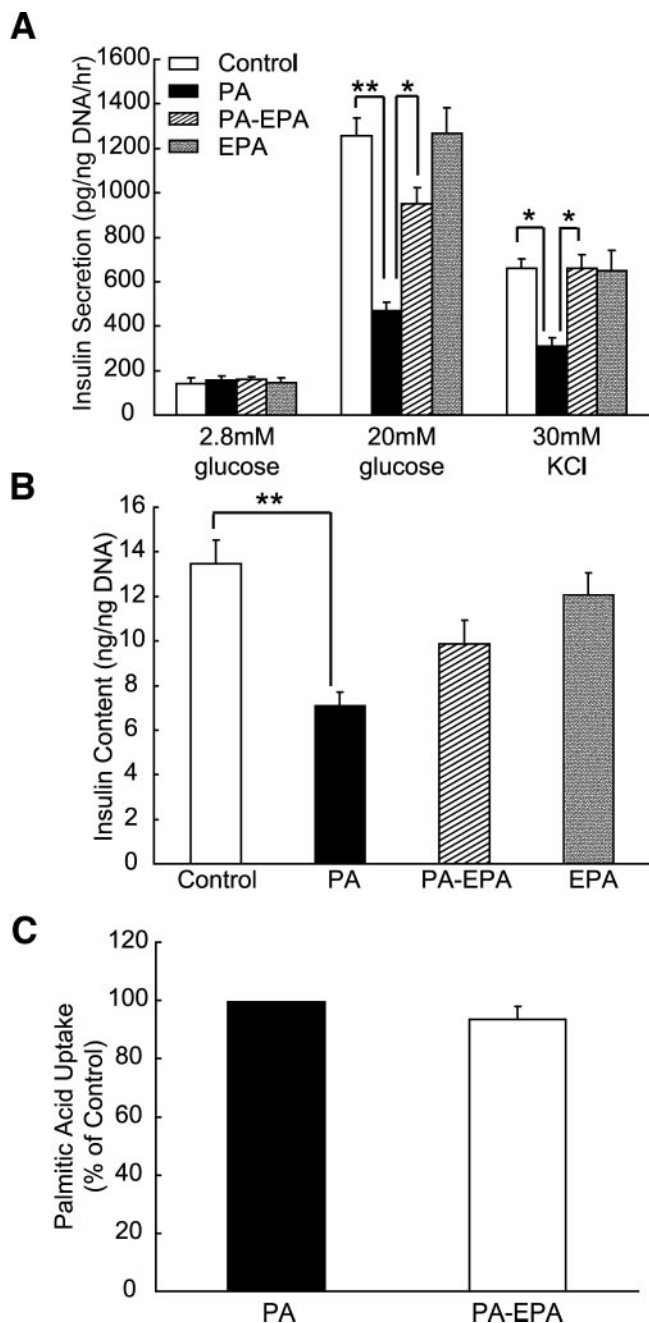


FIG. 1. Lipotoxic effects of palmitate and protective effects of EPA on insulin secretion in murine-isolated islets. **A:** Low GSIS (2.8 mmol/l), high GSIS (20 mmol/l), and KSIS from murine-isolated islets incubated without (control, white bars) or with palmitate (black bars), palmitate-EPA (bold hatched bars), or EPA (regular hatched bars). **B:** Insulin content of islets incubated without (control) or with palmitate, palmitate-EPA, or EPA. **C:** Palmitate uptake in islets isolated from C57BL/6 mice. Three independent experiments were performed using four sets of islets for each repetition, and results are expressed as means \pm SE. Statistical analysis was performed using one-way ANOVA followed by Dunnett's procedure. $**P < 0.01$ and $*P < 0.05$ vs. palmitate group, respectively.

secretion of isolated mouse pancreatic islets. Although palmitate (400 μ mol/l) had no effect on basal insulin secretion (low glucose concentrations), stimulation with high glucose concentrations, i.e., GSIS, was inhibited by the addition of palmitate (Fig. 1A). When 50 μ mol/l EPA (C20:5, n-3) was combined with palmitate-treated islets (hereafter referred to as palmitate-EPA), the suppressed insulin secretion was restored to near-normal levels (Fig.

1A). Palmitate 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 palmitate and EPA on insulin secretion were dose dependent (supplemental 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 palmitate-suppressed insulin secretion. The slight changes of insulin content compared with GSIS and KSIS by palmitate and EPA indicate 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 palmitate-induced lipotoxicity could be inhibition of cellular uptake of palmitate. To exclude this possibility, uptake of labeled palmitate was measured and was not affected by additional EPA (Fig. 1C). These data indicate that the EPA does not interfere with palmitate uptake but rather directly competes with palmitate in intracellular events.

Palmitate and EPA regulate SREBP-1c and its target genes. Gene expression in palmitate- and palmitate-EPA-treated islets was investigated using real-time PCR. SREBP-1c mRNA was highly induced by palmitate 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 fatty acid synthase, stearoyl-CoA desaturase 1, and elongation of long-chain fatty acids family number 6 showed similar patterns of regulation by palmitate and palmitate-EPA (Fig. 2B). TG content, as an indication of SREBP-1c effect and lipotoxicity, was increased by palmitate and repressed by palmitate-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 palmitate and was partially restored by addition of EPA, implicating a role for SREBP-1c-mediated IRS-2 repression in the palmitate-EPA-mediated changes in β -cell physiology (Fig. 2D; supplemental Fig. 2A).

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

SREBP-1c plays a dominant role in palmitate-EPA effects on insulin secretion. The contribution of SREBP-1c to palmitate-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

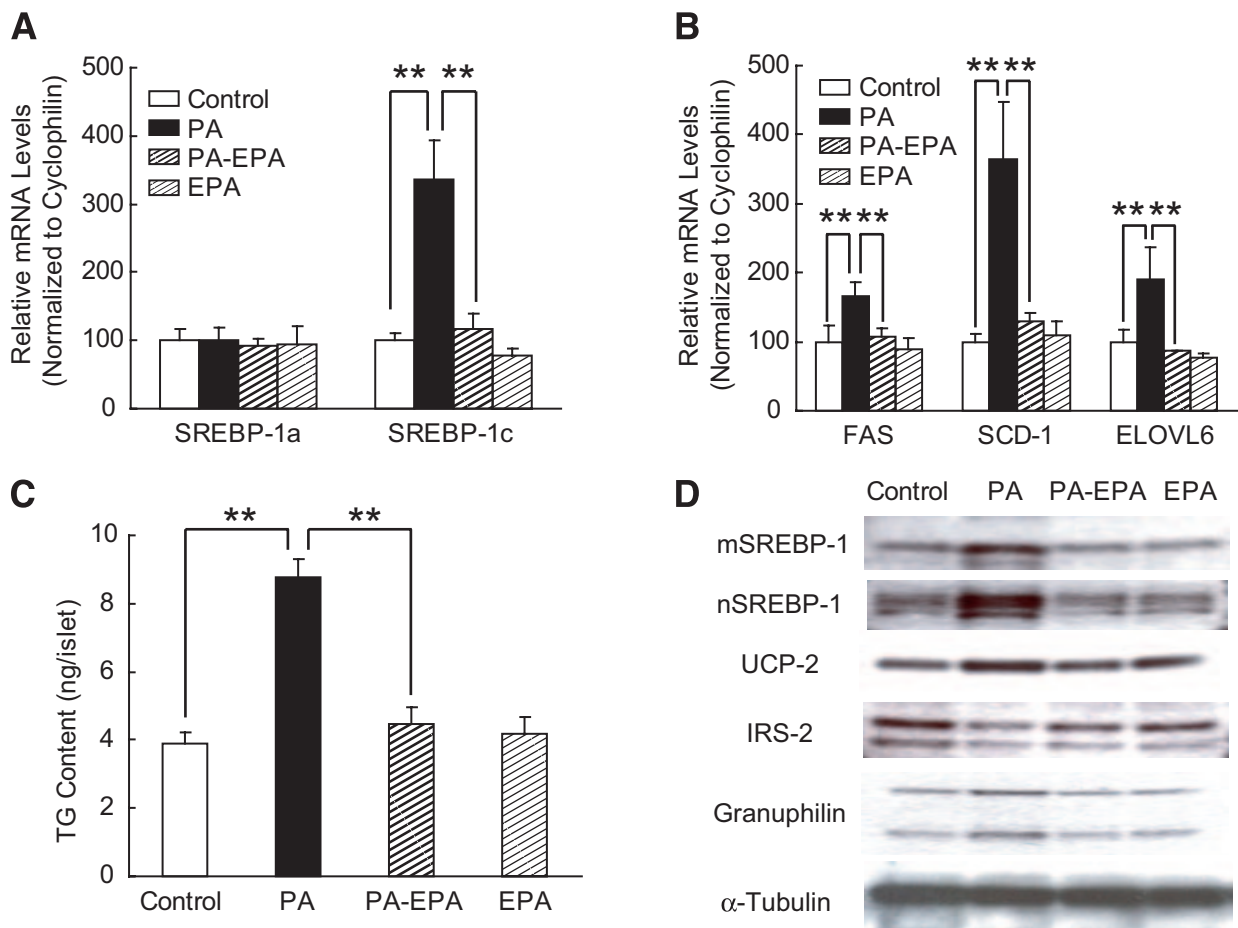


FIG. 2. Gene expression and protein profiles in murine-isolated islets treated with palmitate or palmitate-EPA. **A** and **B**: Levels of mRNA of various genes in pancreatic islets isolated from C57BL/6 mice without (control, white bars) or with palmitate (black bars), palmitate-EPA (hatched bars), 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 TG levels of islets incubated with palmitate, palmitate-EPA, or EPA. **D**: Immunoblot analysis of indicated proteins in the islets. Cont., control; mSREBP-1, membrane form of SREBP-1; nSREBP-1, nuclear form of SREBP-1. α -Tubulin protein was used as a loading control. Three independent experiments were performed using four sets of islets, and results are expressed as means \pm SE. Statistical analyses were performed using one-way ANOVA followed by Dunnett's procedure. ** $P < 0.01$ vs. palmitate group.

(Fig. 3A). The absence of SREBP-1 abolished palmitate-induced inhibition of GSIS and KSIS (Fig. 3A). Because of 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 palmitate-induced pancreatic lipotoxicity and amelioration of that by EPA depend on SREBP-1c. Predictably, from the primary role of SREBP-1c in lipogenesis, the elevation and repression of TG content in wild-type islets by palmitate and EPA, respectively, were absent in SREBP-1-null islets (Fig. 3B). Suppression of IRS-2 and stimulation of granuphilin mRNA expressions caused by palmitate in wild-type islets were both blunted in SREBP-1-null islets (Fig. 4A). Accordingly, the reversal of palmitate 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 palmitate was observed even in SREBP-1-null islets, but EPA treatment reversed the palmitate effect in both genotypes (Fig. 4A).

IRS-2 suppression by SREBP-1c contributes to palmitate-EPA effects on GSIS. Based on recent cumulative evidence of the importance of insulin signaling in β -cell function and our observation of reciprocal changes in SREBP-1c and IRS-2 by addition of palmitate and/or EPA in islets (Fig. 2D; supplemental Fig. 2A), we hypothesized

that palmitate 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 (supplemental Fig. 2A), suppression and restoration of IRS-2 protein by palmitate and palmitate-EPA, respectively, in wild-type islets was not apparent in SREBP-1-null islets (Fig. 4B). Consequently, Akt phosphorylation impaired by palmitate 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 palmitate-mediated inhibition of insulin signaling and insulin secretion.

To explore impacts of insulin signaling on palmitate-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 overexpression significantly improved both GSIS and KSIS, which were impaired by palmitate, but did not further enhance restoration by EPA, indicating that insulin signaling and insulin secretion were linked in palmitate-EPA effects (Fig. 5A). Akt-CA overexpression only slightly

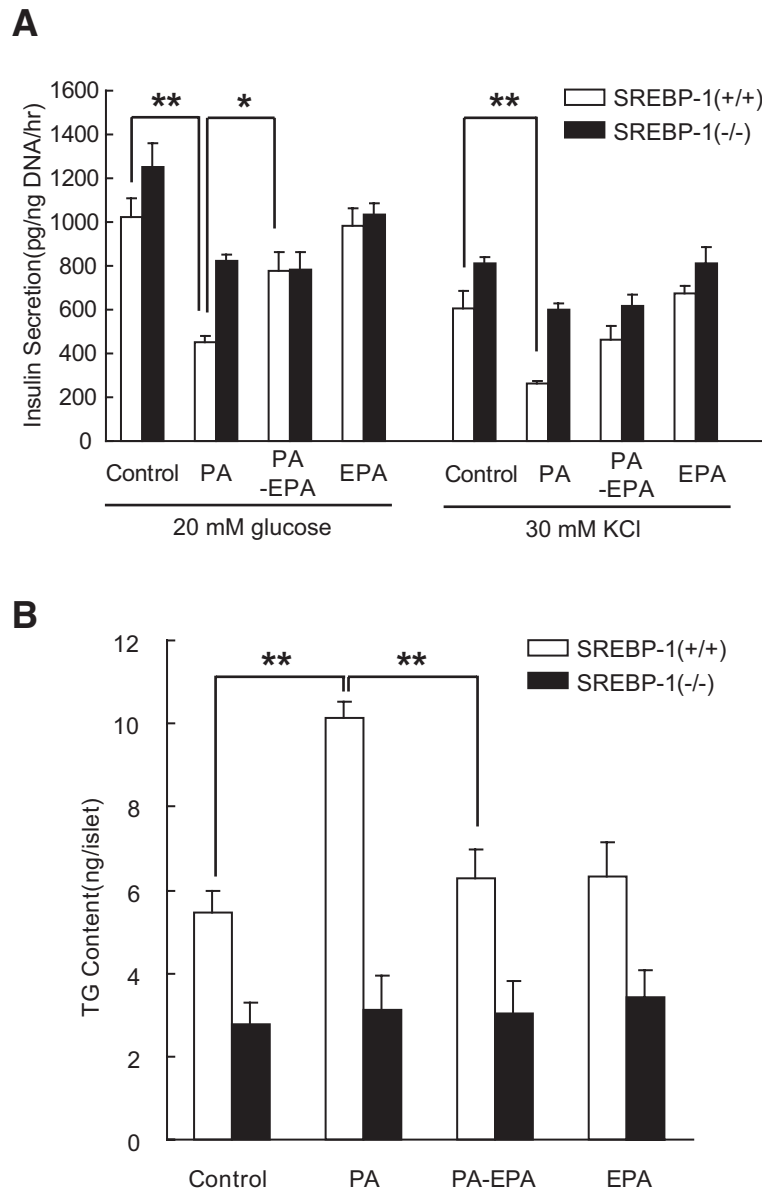


FIG. 3. Protection from palmitate-induced lipotoxicity in islets isolated from SREBP-1-null mice. Islets were isolated from wild-type littermates (white bars) and SREBP-1-null mice (black bars) and incubated without (control) or with palmitate, palmitate-EPA, or EPA for 48 h. GSIS and KSIS (A) and cellular TG contents (B) were measured. Three independent experiments were performed using four sets of islets, and results are expressed as means \pm SE. Statistical analyses were performed using two-way ANOVA followed by Tukey's procedure. $**P < 0.01$ and $*P < 0.05$, respectively.

enhanced phosphorylation of Akt in untreated islets but completely restored suppressed pAkt in palmitate-treated islets (Fig. 5B). Insulin signaling downstream of Akt, such as pAkt, was also consistently suppressed by palmitate. These signaling molecules were all restored by Akt-CA overexpression. Islets treated with palmitate-EPA exhibited signals similar to control islets regardless of Akt-CA overexpression (Fig. 5B). Both SREBP-1 deficiency (Figs. 3A and 4B) and constitutive activation of insulin signaling by Akt-CA (Fig. 5A and B) cancelled the protective effects of EPA against palmitate-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 palmitate-EPA effects on GSIS. The contribution of UCP-2 to the effects of palmitate-EPA on GSIS was estimated in knockdown experiments using adenoviral siRNA of UCP-2. A robust

inhibition of mRNA and protein levels of UCP-2 was obtained (Fig. 6A; supplemental Fig. 3). Gene silencing of UCP-2 did not effect basal insulin secretion or GSIS. In contrast, UCP-2 knockdown significantly protected palmitate-mediated impaired GSIS and canceled the EPA protection (Fig. 6B). Palmitate-mediated reduction in ATP-to-ADP ratio was significantly restored by UCP-2 suppression, and the protective effect of EPA was also canceled (Fig. 6C). Changes in ATP-to-ADP ratio and GSIS by modulation of UCP-2 expression were very similar, confirming that the palmitate-EPA effects on GSIS depend on the UCP-2/ATP system, as was previously suggested by knockout mice studies. Palmitate 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 partially connected.

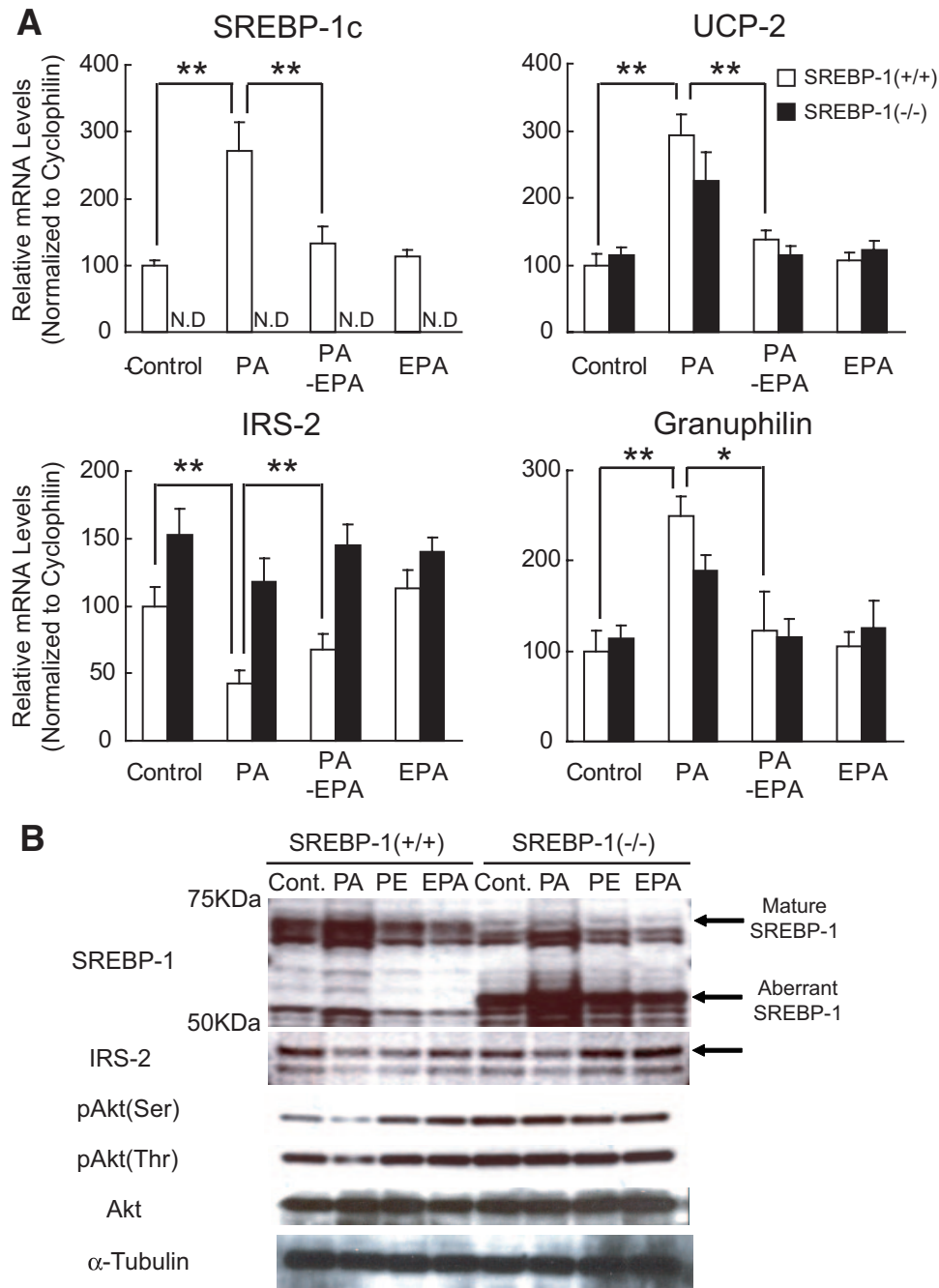


FIG. 4. Gene expression and protein profiles in islets isolated from SREBP-1-null mice treated with palmitate or palmitate-EPA. Islets were isolated from SREBP-1-null mice and wild-type littermates and incubated without (control) or with palmitate, palmitate-EPA (PE), or EPA for 48 h. **A:** mRNA levels 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 as means \pm SE. Statistical analyses were performed using two-way ANOVA followed by Tukey's procedure. ** $P < 0.01$ and * $P < 0.05$, respectively.

EPA in vivo exhibits a protective role against palmitate lipotoxicity in islets. To determine whether the effects of palmitate-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 GSISs in freshly isolated islets from these animals were measured. Palmitate feeding impaired and EPA restored GSIS in conjunction with changes in islet SREBP-1c expression (Fig. 7A and B). These data demonstrate that dietary palmitate and EPA influence insulin secretion in vivo in a similar manner to palmitate-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 $1 \text{ g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ for 28 days restored GSIS and suppressed SREBP-1c expression (Fig. 7C and D), 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 (supplemental Tables 1 and 2; Fig. 4).

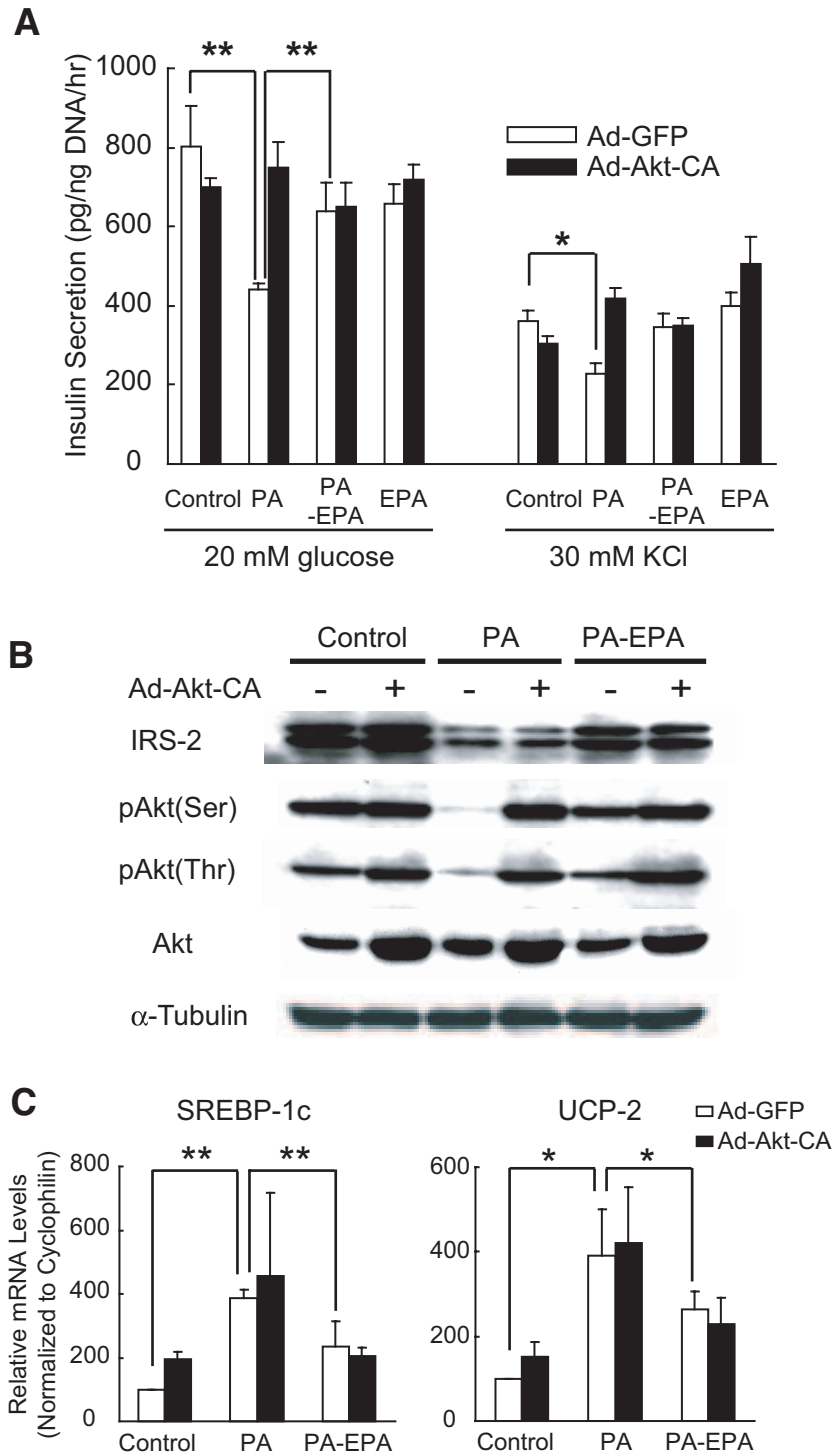


FIG. 5. Effects of overexpression of constitutively active Akt in islets treated with palmitate or palmitate-EPA. Islets were isolated from C57BL/6 mice and incubated without (control) or with palmitate or palmitate-EPA for 48 h. Islets were infected (100 multiplicity of infection, respectively) with adenoviral-GFP (Ad-GFP) or adenoviral-constitutively active Akt (Ad-Akt-CA) for 48 h before incubation with palmitate or palmitate-EPA. GSIS and KSIS (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 are shown. Three independent experiments were performed using four sets of islets, and results are expressed as means \pm SE. Statistical analyses were performed using two-way ANOVA followed by Tukey's procedure. $**P < 0.01$ and $*P < 0.05$, respectively.

DISCUSSION

It has long been known that chronic exposure of palmitate to islets or β -cell lines causes lipotoxicity leading to blunted GSIS (1,3–5). Our current studies clearly demonstrate that this palmitate-induced impairment of insulin

secretion is restored by supplement of EPA. The results also indicated that this palmitate-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

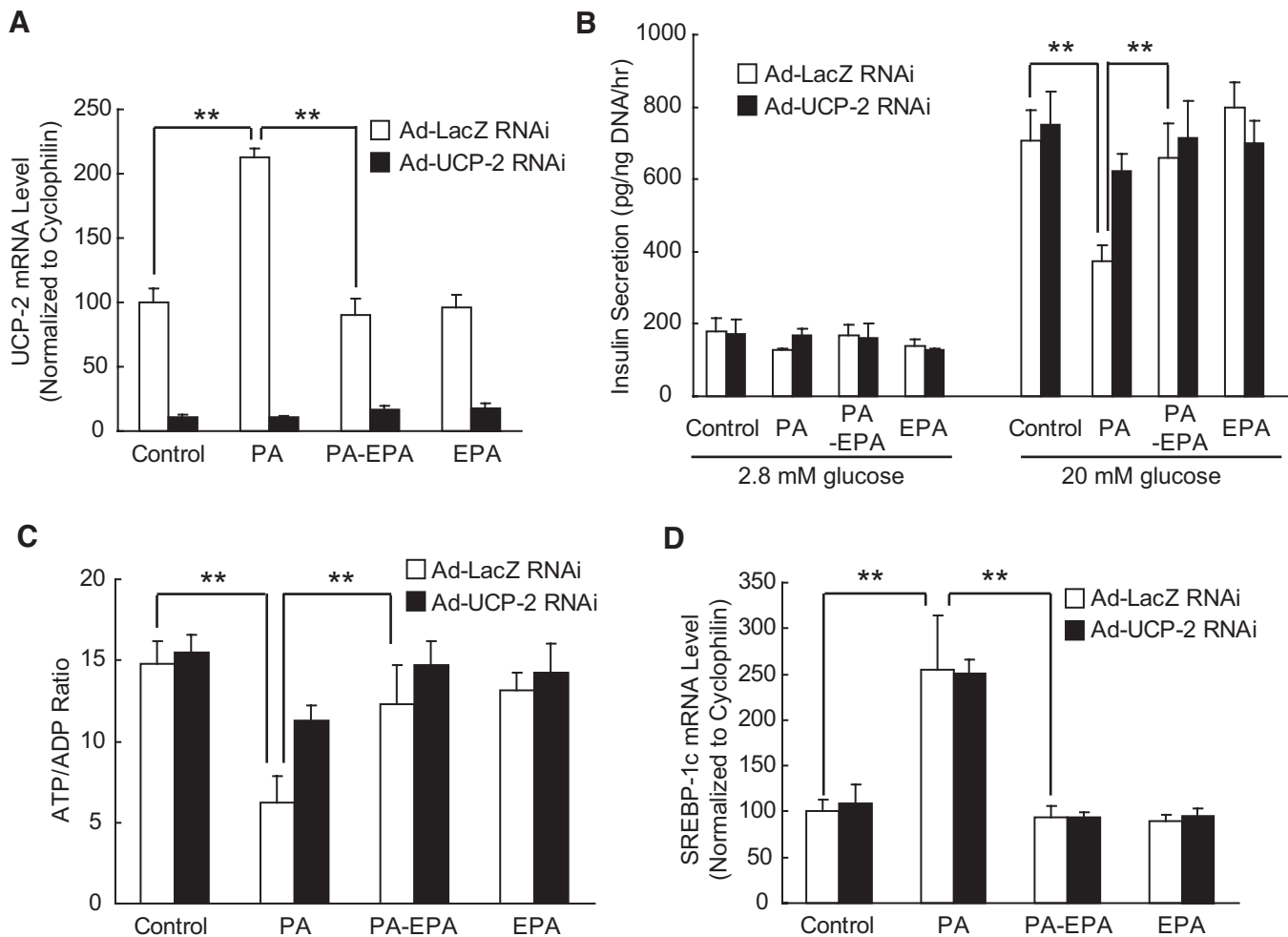


FIG. 6. Effects of UCP-2 gene silencing on murine-isolated islets treated with palmitate or palmitate-EPA. Islets were isolated from C57BL/6 mice and infected (500 multiplicity of infection, respectively) with adenoviral siRNA for LacZ (Ad-LacZ RNAi) or UCP-2 (Ad-UCP-2 RNAi) and cultured without (control) or with palmitate, palmitate-EPA, or EPA for 48 h. **A:** The effects of UCP-2 siRNA on mRNA levels of UCP-2 in pancreatic islets isolated from C57BL/6 mice incubated with palmitate, palmitate-EPA, or EPA were determined by real-time PCR. GSIS (**B**) and ATP-to-ADP ratio (**C**) from the islets after UCP-2 gene silencing and incubation with palmitate, palmitate-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 as means \pm SE. Statistical analyses were performed using two-way ANOVA followed by Tukey's procedure. ** $P < 0.01$.

β -cells, such as ATP-to-ADP ratio, IRS-2/Akt insulin signaling, and granuphilin. These factors are all consistently disturbed by palmitate and improved by additional EPA through up- and downregulation of SREBP-1c, respectively. Taken together with overexpression and knockout experiments of SREBP-1, it can be concluded that SREBP-1c plays a crucial role in 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 palmitate-mediated suppression of GSIS. Palmitate led to upregulation of UCP-2 and reduction of intracellular ATP. Knockdown of UCP-2 by siRNA restored palmitate-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 overexpression of SREBP-1c elevated UCP-2 expression contributing to the β -cell lipotoxicity in transgenic mice (26). However, based on the current results from SREBP-1-null islets, SREBP-1c only partially participated in palmitate-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 overexpression experiments provide further evidence for the importance of insulin signaling in β -cell function. Palmitate inhibited and EPA restored insulin signaling in an opposite manner to SREBP-1c expression. Based on the potential effect of SREBP-1c on insulin signaling through regulation of IRS-2 (25,26), we explored involvement of insulin signaling in palmitate-EPA regulation of insulin secretion. Activation of Akt did not change normal insulin secretion but markedly ameliorated palmitate-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 with regard to its impairment. Palmitate suppression of insulin signaling was cancelled in SREBP-1-null islets. Based on 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

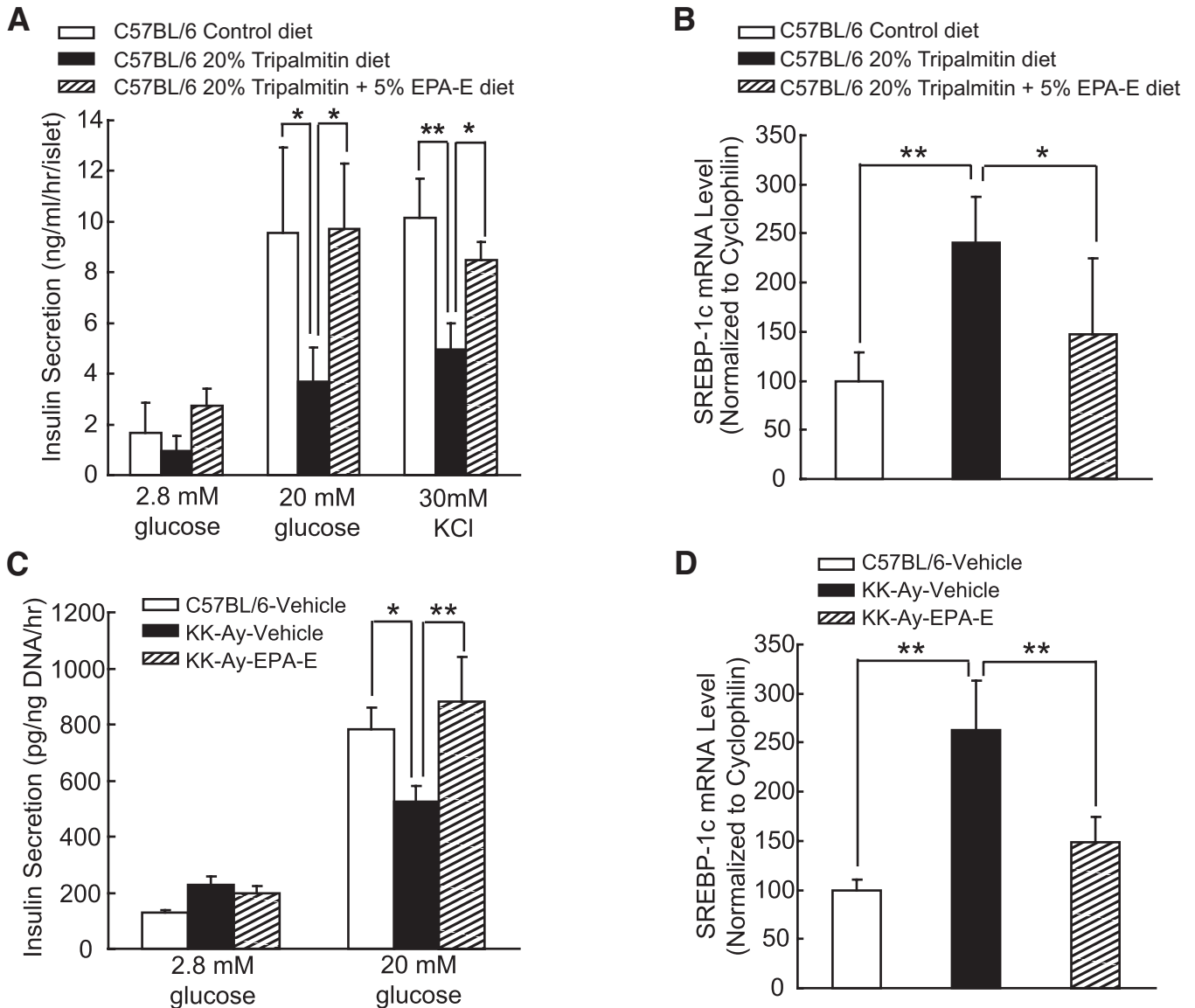


FIG. 7. Effect of EPA on insulin secretion in vivo. C57BL/6 mice were fed control diet (white bars), 20% Tripalmitin diet (black bars), and Tripalmitin + 5% EPA-E diet (hatched bars) for 28 days. Islets were isolated from individual animals. GSIS and KSIIS (A) and mRNA levels of SREBP-1c (B) were measured. KK-Ay mice were administered vehicle (black bars) or EPA at a dose of $1 \text{ g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ (hatched bars) for 28 days. Islets were isolated from pool pancreas (three to four animals). GSIS (C) and mRNA levels of SREBP-1c (D) were measured. Three independent experiments were performed using four sets of islets, and results are expressed as means \pm SE. Statistical analyses between indicated groups were performed using one-way ANOVA followed by Dunnett's procedure. $**P < 0.01$ and $*P < 0.05$, respectively.

mass (13–15,29–34), our data indicated that activation of Akt could restore insulin secretion impaired by palmitate in a short term. The precise molecular mechanism for this is currently unknown, although phosphorylation of Foxo-1 and anti-apoptosis could be involved (29,34).

Dietary PUFAs, such as EPA, have been shown to have plasma TG-lowering effects and to improve fatty liver and hepatic insulin resistance (22,23,46). We previously reported that PUFAs inhibited hepatic SREBP-1c, which contributed to beneficial roles of PUFAs 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 palmitate-treated islet. In addition, a large portion of EPA protection against palmitate-induced impaired GSIS was not reproduced in SREBP-1-null islets.

Amelioration of impaired GSIS by EPA was also confirmed in vivo with SREBP-1c suppression. EPA also suppressed overexpression of UCP-2 by palmitate even in SREBP-1-null islets. This suggests that suppression of UCP-2 also may contribute to protective effect of EPA against palmitate-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 palmitate have important clinical relevance. It has been recognized that hyperglycemia exacerbates the impairment of insulin secretion, often referred to 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 lipo-

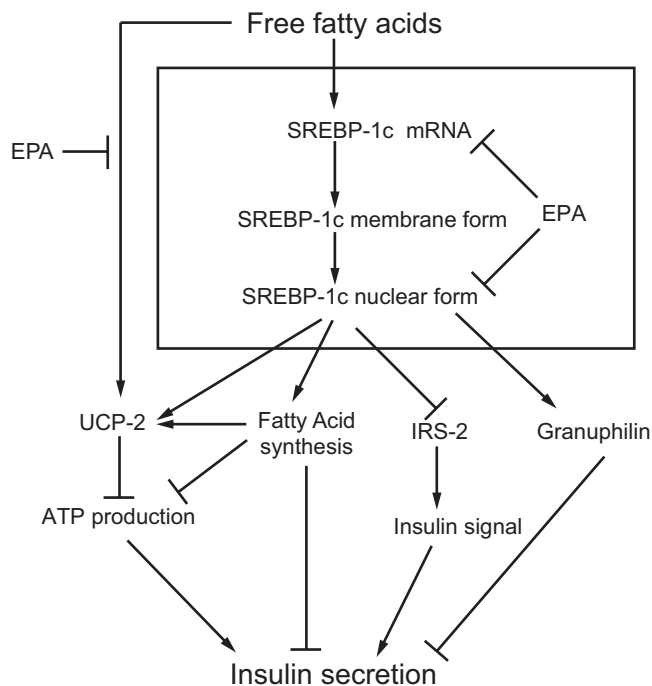


FIG. 8. Mechanism by which palmitate induces and EPA protects impairment of insulin secretion in pancreatic islets.

toxic states. From a long-term standpoint, our findings might relate to the onset of type 2 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 β -cell lipotoxicity. Because our findings are based mostly on in vitro studies, further investigations in vivo are needed to test our conclusions.

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