Fatty acids induce amylin expression and secretion by pancreatic β-cells

Qi D, Cai K, Wang O, Li Z, Chen J, Deng B, Qian L, Le Y. Fatty acids induce amylin expression and secretion by pancreatic β-cells. *Am J Physiol Endocrinol Metab* 298: E99–E107, 2010. First published October 20, 2009; doi:10.1152/ajpendo.00242.2009.—Amylin is the major component of pancreatic amyloid, which is implicated in the development of type 2 diabetes. It is costored with insulin in the secretory granules of pancreatic β-cells and cosecreted with insulin following stimulation with glucose. Here, we investigate the effect of fatty acids (FAs) on amylin expression and secretion by β-cells and explore the underlying mechanisms. Palmitate and oleate dose-dependently induced amylin mRNA accumulation in murine pancreatic β-cell line MIN6 and primary pancreatic islets. The inductive effect of FAs on amylin expression is independent of glucose concentration. FAs upregulated amylin expression at the transcriptional level, and FAs must be metabolized to induce amylin expression. FAs also significantly induced human amylin promoter activation. Pretreatment of MIN6 cells with Ca2⁺ chelator (EGTA, BAPTA-AM) PKC inhibitor G0-6976 or protein synthesis inhibitor cycloheximide significantly inhibited FA-induced amylin mRNA expression. Transcription factors cAMP-responsive element-binding protein, pancreatic and duodenal homeobox factor-1, and peroxisome proliferator-activated receptor were not involved in FA-induced amylin expression. Palmitate and oleate both increased amylin and insulin release from MIN6 cells and stimulated amylin expression but had no effect on insulin expression. Mice refed with Intralipid had significantly higher levels of plasma FFA, amylin, and insulin than those refed with saline. These data demonstrate that FAs differently regulate amylin and insulin expression and induce both amylin and insulin release. Ca2⁺ and PKC signaling pathways and de novo-synthesized protein(s) were involved in FA-induced amylin expression. Induction of amylin production and release by FAs may contribute to its biological functions under physiological conditions.

In pancreatic β-cells, amylin is costored with insulin in the secretory granules and cosecreted in a regulated manner following stimulation with glucose and a variety of other secretagogues (5, 22). Glucose stimulates amylin mRNA expression and protein release by rodent and human pancreatic islets (10–12, 35). The plasma level of amylin increased in normal mice fed a high-fat diet for long term (34, 48). In human amylin transgenic mice, amyloid does not appear spontaneously but appears after long-term, high-fat dietary intake (21, 47). These results suggest that fatty acids (FAs) may stimulate amylin release and enhance islet amyloid formation. But the effect of FAs on amylin expression and release by pancreatic β-cells is not clear.

In the present study, we show that FAs upregulate amylin expression at both mRNA and protein levels in murine pancreatic β-cells but have no effect on insulin expression. The inductive effect of FA on amylin expression is mediated by the Ca2⁺-PKC signaling pathway and de novo-synthesized protein(s). FAs induce both amylin and insulin secretion from β-cells in vitro. Lipid intake sequentially increases plasma FFA and amylin/insulin levels in mice, suggesting that FFA may induce amylin and insulin release in vivo.

**MATERIALS AND METHODS**

Pancreatic islet preparation and cell culture. Female and male C57BL/6 mice at the age of 10 wk were obtained from Shanghai SLAC Laboratory Animal. Pancreatic islets were isolated from C57/BL6 mice by type V collagenase (Sigma, St. Louis, MO) digestion followed by Ficoll 400 (Amersham Pharmacia Biotech, Piscataway, NJ) gradient separation, as described previously (32). About 150 islets can be isolated from the pancreas of a mouse. Islets were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO-BRL, Burlington, ON, Canada) containing 5.6 mM glucose, 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Batches of 100 islets were used for RNA extraction, and 200 islets were used for cell lysate preparation and Western blot assay. Murine pancreatic β-cell line MIN6 cells were cultured in DMEM containing 5.6 mM glucose, 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere at 37°C with 5% CO₂. Palmitate or oleate (sodium salt; Sigma) was conjugated with FA-free BSA (Sigma) in a molar ratio of 2.5:1 as described (33). The BSA-coupled FA, with a final concentration of 2.0 mM, was stored in aliquots at −20°C.

**RNA extraction and real-time PCR**. Total RNA was extracted from MIN6 cells or mouse pancreatic islets using the TRIzol reagent (Invitrogen, Carlsbad, CA) and depleated of contaminating DNA with RNase-free DNase according to manufacturers’ instructions. cDNA was synthesized from 2 μg of RNA with M-MuLV reverse transcriptase and random hexamer according to the manufacturer’s instructions (Fermentas, Burlington, ON, Canada). Quantitative real-time PCR was performed using an ABI Prism 7500 sequence detector (Applied Biosystems, Foster City, CA). Briefly, reverse-transcribed cDNA in triplicate samples were checked for amylin mRNA level with SYBR Green PCR master kit (TOYOBO Biotech, Osaka, Japan) according to the manufacturer’s instructions. Primers for murine amylin were 5’-CAGCTGTCCTCCTCATCCTC (sense) and 5’-GCACCTCCG-
TTGTGCCATCT (antisense). Primers for murine preproinsulin were: 5′-AGACCATAGCAAGAGGCTCA (sense) and 5′-AAGTGCCAC
CACAAGGGCC (antisense). Mouse β-actin primers were 5′-
CAAGGGCTTGCTT (sense) and 5′-GCCAGGGATTCC-
ATACCCA (antisense). The assays were initiated with 5 min at
95°C and then 40 cycles of 15 s at 94°C and 1 min at 60°C. Threshold
cycle (Ct, cycle of gene amplification that exceeds the minimum level
of fluorescent detection) was calculated for both amylin and β-actin.
Amplification of the amylin or preproinsulin cDNA was normalized to β-
actin expression. Relative levels of amylin or preproinsulin mRNA
expression were calculated using the 2(-ΔΔCt) method (24).

Plasmid construction, transient transfection, and luciferase report
assay. Human genomic DNA was utilized as a PCR template to create
amylin promoter luciferase constructs. The amylin promoter spanning
from 222 bp upstream to 450 bp downstream of the transcriptional
start site (28) was amplified by PCR and cloned into the pGL3-basic
luciferase reporter plasmid (Promega) between the MluI and XhoI
sites. Primers for PCR were (sense) 5′-TGCTCTCCTAATTTTCTGC
and (antisense) 5′-CTGTGTAGCACCATTACC. The construc-
tion was named pGL3-hAP (human amylin promoter) and confirmed
by restriction enzyme digestion as well as sequencing. MIN6 cells
(2 × 10^5/well) were plated in a 24-well tissue culture plate. pGL3-
hAP was cotransfected with the pRL-TK Renilla plasmid with an 80:1
ratio using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Transfec-
tion of the plasmids was performed in triplicate in at least three
independent experiments. Twenty-four hours after the transfection,
cells were cultured in DMEM containing different concentrations of
palmitate or oleate for another 24 h. Luciferase activity of the
promoter construct and the pRL-TK construct was measured sequen-
tially using the Dual-Luciferase Reporter Assay System (Promega).
Variation in transfection efficiency was normalized by dividing the
promoter construct activity by the respective cotransfected pRL-TK
luciferase activity.

Animals and treatment. C57/BL6 mice age 6 wk were maintained
on a 12:12-h light-dark cycle in a temperature- and humidity-con-
trolled room and fed a standard laboratory diet ad libitum. Mice were
randomly divided into control group, fasting group, (fasted for 5 h),
and refeeding groups (6 mice per group). Mice in the refeeding groups
were fasted for 5 h followed by refeeding with 0.5 ml of 30%
 Intralipid (Sino-Swed Pharmaceutical, China) or the same volume of
saline via oral gavage and then killed 2, 4, 6, or 8 h after refeeding.
Blood samples were collected in EDTA-containing tubes. All exper-
iments using animals were in accordance with the National Institutes
of Heath Guide for the Care and Use of Laboratory Animals and
the Regulations of Animal Care and Use at the Institute for Nutritional
Sciences, Chinese Academy of Sciences, and were approved by the
Biological Research Ethics Committee, Institute for Nutritional
Sciences, Chinese Academy of Sciences.

Immunocytochemistry and images. To examine the expressions
of amylin and insulin by MIN6 cells, cells grown on glass coverslips
were serum starved for 12 h, treated with control medium or fatty acid
for 12 h, rinsed in PBS, and fixed with 4% paraformaldehyde for
15 min at room temperature. The cells were then washed with PBS
and incubated with rabbit anti-rat amylin antiserum (1:1,000, T-4145;
Peninsula Laboratory, Belmont, CA) and mouse anti-insulin antibody
(1:300; Sigma, St. Louis, MO) overnight at 4°C. After being washed
and incubated with FITC-conjugated goat anti-rabbit antibody (1:200;
Sigma) for 45 min at 37°C, the cells were washed and incubated with
Alexa fluo 546-conjugated goat anti-mouse antibody (1:200; Invitro-
gen, Carlsbad, CA) for another 45 min at 37°C and then washed and
stained with Hoechst 33342 and mounted. Immunofluorescence label-
ing was observed under confocal microscope LSM 510 META (Carl
Zeiss, Germany). The immunostaining conditions and image capture
settings were identical between control and fatty acid treatment
groups.

Electrophoresis and immunoblotting. Murine islets were lysed in
40 μl of lysis buffer as described previously (45). Islet proteins (10 or
15 μg) were electrophoresed on a 15% polyacrylamide gel using
Tris-tricine buffer and transferred onto polyvinylidene difluoro-
membrane (Millipore, Bedford, MA). The membrane was blocked
with 5% nonfat milk and then incubated with anti-rat amylin anti-
serum (1:1,000, T-4145) or anti-β-actin monoclonal antibody (1:3,
Sigma) overnight at 4°C. After incubation with a horseradish
peroxidase-conjugated secondary antibody, the protein bands were
detected with a Supersignal West Pico chemiluminescent substrate
(Pierce, Rockford, IL) and X-Omat BT film (Eastman Kodak, Roch-
ester, NY).

Measurement of blood glucose, free fatty acid, amylin, and insulin.
Plasma was isolated by low-speed centrifugation of blood at 4°C.
Plasma glucose levels were measured with a FreeStyle Blood Glucose
Monitoring System (TheraSense Alameda, CA). Plasma free fatty
acid, amylin, and insulin levels were determined using a NEFA-C kit
(Wako Chemicals, Richmond, VA), an enzyme immunoassay (EIA)
kit (Peninsula Laboratories, San Carlos, CA), and an ELISA kit
(Millipore, Bedford, MA), respectively.

Statistical analysis. Data are presented as means ± SD. Statistical
differences of plasma glucose, free fatty acid, insulin, and amylin
among control, fasting, and refeeding groups were analyzed by
one-way ANOVA. Data of other experiments were analyzed by
unpaired Student’s t-test.

RESULTS

The main dietary FAs palmitate and oleate induce murine
amylin expression and human amylin promoter activation. To
evaluate the effect of FAs on amylin gene expression, murine
pancreatic β-cell line MIN6 was challenged with different
concentrations of the most common saturated and monounsat-
urated long-chain fatty acids palmitate (C16:0) and oleate
(C18:1, ω9) for different lengths of time, and the mRNA levels
of amylin were detected by quantitative real-time PCR. MIN6
cells cultured in DMEM containing 5.6 mM glucose expressed
transcripts for amylin. Both palmitate and oleate enhanced
amylin mRNA level in a time- and dose-dependent manner
(Fig. 1, A–D). The minimum concentration of palmitate or
oleate to significantly induce amylin gene expression was
obtained at 400 μM (Fig. 1, B and D). BSA at concentrations
to conjugate the FAs had no effect on amylin mRNA expres-
sion (data not shown). Palmitate and oleate at tested concen-
trations had no effect on viability of MIN6 cells as examined
by MTT assay (data not shown). Since ingestion of a fatty meal
rich in either saturated or polyunsaturated fat causes significant
increases in the levels of plasma FFA to 1 mm (16), the
concentrations of FAs that induce β-cell amylin expression are
within the physiological range.

Since glucose upregulates amylin expression in β-cells, we
examined the effect of glucose on FA-induced amylin expres-
sion in MIN6 cells. As shown in Fig. 1E, glucose induced
amylin mRNA expression in a dose-dependent manner. The
minimum concentration of glucose to significantly induce
amylin gene expression was obtained at 16 mM. Both palmi-
tate and oleate significantly upregulated amylin mRNA levels
in the presence of glucose at concentrations of 5.6 and 11 mM.
When MIN6 cells were cultured in medium containing higher
levels of glucose, palmitate or oleate had no synergistic effect
with glucose on amylin expression. These results suggest that
the inductive effect of FAs on amylin expression is indepen-
dent of glucose concentration.

To determine whether palmitate metabolism is necessary for
the inductive effect on amylin mRNA expression, we next
examined the effect of 2-bromopalmitate (a form of palmitate that cannot be metabolized) (7) on amylin gene expression. As shown in Fig. 1F, exposure of MIN6 cells to 400 μM 2-bromopalmitate for 8 h had no effect on amylin mRNA level. Coincubation with 2-bromopalmitate had no effect on amylin mRNA upregulation by palmitate. These results suggest that palmitate must be metabolized to have its effect on amylin expression.

To demonstrate the physiological relevance of FA-simulated amylin gene expression, we used isolated mouse pancreatic islets. Consistent with the results obtained from MIN6 cells, mRNA levels of amylin in murine pancreatic islets were significantly enhanced by palmitate and oleate after 8 and 6 h of stimulation, respectively (Fig. 1G). Interestingly, the increase of amylin expression in response to FAs is more robust in islets than in MIN6 cells, suggesting that primary islets are more sensitive to FFA than transformed -cells.

To determine whether the FA-induced increase of amylin mRNA levels was due to the increase of amylin mRNA stability, MIN6 cells pretreated with or without FA (400 μM palmitate for 8 h or 400 μM oleate for 6 h) were cultured with 10 μg/ml actinomycin D for different periods of time before amylin mRNA levels were examined by real-time PCR. Although the levels of amylin mRNA were markedly increased in the presence of palmitate (Fig. 1B) or oleate (Fig. 1D), there was no significant difference in the curves of mRNA decay between FA-treated and control groups (Fig. 2), suggesting that the main effect of palmitate and oleate on expression of amylin is at transcription level.

To determine whether human amylin gene expression is also upregulated by FAs, we examined FA-induced human amylin promoter activation by luciferase reporter assay. Compared with MIN6 cells transfected with pGL3-basic vector, cells transfected with the human amylin promoter construct pGL3-hAP responded to palmitate and oleate (Fig. 3). These results demonstrate that both human and murine amylin gene expression is enhanced by FAs. Thus, we can use murine MIN6 cells and pancreas as models to study the effect of FAs on amylin gene expression and explore the mechanisms involved.

Involvement of Ca2⁺ and PKC in palmitate- and oleate-induced amylin gene expression. FAs cause the entry of extracellular Ca2⁺ into the -cell (7). To directly test the role of extracellular Ca2⁺ in the induction of amylin expression by FAs, MIN6 cells were pretreated with EGTA (Sigma), the
extracellular calcium chelator, or BAPTA-AM (Calbiochem, La Jolla, CA), an intracellular calcium chelator, before FAs stimulation. EGTA (0.1–1 mM) significantly attenuated the induction of amylin in response to palmitate (Fig. 4A) or oleate (Fig. 4B). BAPTA-AM (1–10 μM) inhibited palmitate- and oleate-induced amylin gene expression in a dose-dependent manner (Fig. 4, C and D). EGTA and BAPTA-AM at tested concentrations had no effect on cell viability as examined by MTT assay (data not shown). These results suggest that cytosolic Ca\(^{2+}\) elevation consequent to FA-promoted Ca\(^{2+}\) influx and release from internal stores is crucial for FA-induced amylin gene expression.

Previous studies have shown that FAs and their CoA derivatives activate certain PKC isoforms (1, 41). We therefore examined whether PKC was involved in FA-induced amylin gene expression. Gö-6976 (Calbiochem), an inhibitor of Ca\(^{2+}\)-dependent PKC\(\alpha\) and PKC\(\beta1\), significantly inhibited 400 μM palmitate- or oleate-induced amylin mRNA expression (Fig. 5). Gö-6976 at tested concentrations had no effect on cell viability as examined by MTT assay (data not shown). These results suggested that Ca\(^{2+}\)-dependent PKC\(\alpha\) and PKC\(\beta1\) were involved in palmitate- or oleate-stimulated amylin gene expression.

Requirement of de novo protein synthesis for FA-induced amylin expression. FAs regulate gene expression by activating a number of transcription factors (7, 37), among which pancreatic and duodenal homeobox factor-1 (PDX-1) and peroxi-

Fig. 2. Effect of palmitate and oleate on amylin mRNA stability. MIN6 cells pretreated without or with 400 μM palmitate for 8 h (A) or 400 μM oleate for 6 h (B) were cultured with 10 μg/ml actinomycin D for indicated time intervals. Amylin mRNA levels were then examined by real-time PCR. Results represent means ± SD of 3 independent experiments.

Fig. 3. Effect of palmitate and oleate on human amylin promoter activity in MIN6 cells. pGL3-basic vector (pGL3) or human amylin gene promoter luciferase construct (pGL3-hAP) was cotransfected with pRL-TK Renilla luciferase reporter plasmid into MIN6 cells in medium containing 5.6 mM glucose. After 24 h, medium containing 400 μM palmitate or oleate were added, and luciferase activity was measured 24 h later. All data are shown as means ± SD of 3 independent experiments with each condition tested in triplicate. *P < 0.05, **P < 0.01, normalized luciferase activity in pGL3-hAP-transfected cells in the presence of palmitate or oleate vs. medium control.

Fig. 4. Ca\(^{2+}\) is involved in palmitate-induced amylin gene expression. MIN6 cells were incubated with different concentrations of EGTA (A and B) or BAPTA-AM (BAP) (C and D) for 1 h and then stimulated with 400 μM palmitate (Pal; A and C) for 8 h or 400 μM oleate (Ole; B and D) for 6 h and examined for amylin expression by real-time PCR. *P < 0.05, **P < 0.01 vs. cells cultured with control medium; #P < 0.05 vs. cells treated with 400 μM palmitate or oleate. Results represent means ± SD of 3 independent experiments.

Fig. 5. Involvement of PKC in palmitate- and oleate-induced amylin expression. MIN6 cells were incubated with different concentrations of Gö-6976 (Gö.) for 1 h and then stimulated with 400 μM palmitate for 8 h (A) or 400 μM oleate for 6 h (B) and examined for amylin expression by real-time PCR. *P < 0.05, **P < 0.01 vs. cells cultured with control medium. #P < 0.05 vs. cells treated with 400 μM palmitate or oleate. Results represent means ± SD of 3 independent experiments.
some proliferator-activated receptor (PPAR) have binding sites in the promoter region of murine amylin gene as analyzed with the AliBaba 2.1 program. Thus, we examined the involvement of PDX-1 and PPAR in FA-induced amylin upregulation by using dominant negative PDX-1 expression vector and PPARα antagonist MK-886, respectively. The results indicated that none of these transcription factors was involved in amylin upregulation by FA (data not shown). Then we examined the effect of cycloheximide, a protein synthesis inhibitor, on amylin upregulation by FAs. Pretreatment of MIN6 cells with cycloheximide dose-dependently inhibited amylin mRNA upregulation induced by palmitate or oleate (Fig. 6), suggesting that de novo-synthesized protein(s) mediate FA-induced amylin expression.

**FAs induce amylin protein expression and release.** The effects of FAs on amylin and insulin protein expression were examined in MIN6 cells. Immunocytochemistry assay showed that MIN6 cells expressed basal levels of amylin and insulin, with a lower level of amylin than insulin. Amylin was colocalized with insulin in MIN6 cells. Palmitate or oleate (both at 400 μM) induced amylin protein expression after 12 h of stimulation but had no effect on insulin expression (Fig. 7A). Western blot assay showed that stimulation of murine islets with 400 μM palmitate, 400 μM oleate, or 16 mM glucose all significantly increased the levels of proamylin and the intermediate forms of amylin (Fig. 7B). We also checked the effect of FAs on preproinsulin expression at the mRNA level. Although 400 μM palmitate and oleate induced amylin expression in a time-dependent manner (Fig. 1, A and C), neither of these two FAs affected insulin mRNA level in MIN6 cells during the same period of stimulation (Fig. 7C). These results indicate that FAs differently regulate amylin and insulin expression in β-cells.

We next examined the effect of FAs on amylin and insulin secretion in vitro by enzyme immunoassay and ELISA, respectively. As shown in Fig. 8A, 400 μM palmitate or oleate induced amylin released from MIN6 cells in a time-dependent manner. Palmitate or oleate (400 μM) also induced insulin release from MIN6 cells during the same period of time (Fig. 8B). The basal secretion and FA-induced secretion of insulin were higher than those of amylin. These results suggest that amylin and insulin are cosecreted from β-cells in response to FAs. The inductive effect of FAs on insulin secretion is more potent than on amylin secretion.

To examine the effect of FAs on amylin and insulin release in vivo, mice were fasted for 5 h followed by refeeding with 0.5 ml of 30% Intralipid or the same volume of saline via gastric gavage. Plasma levels of glucose, FFA, amylin, and insulin were measured at 2, 4, 6, and 8 h after the refeeding. Compared with the control mice, which were fed a standard laboratory diet ad libitum, fasting for 5 h or fasting for 5 h followed by refeeding with lipid/saline had no significant effect on plasma glucose levels (Fig. 9A). No significant difference of plasma FFA, amylin, or insulin levels were observed between the control and fasting group (fig. 9, B–D). Compared with those in control mice, the plasma FFA level had a tendency to increase, with statistical significance presented at 8 h after saline refeeding (Fig. 9B). The plasma amylin levels did not change significantly (Fig. 9C), and the plasma insulin levels decreased significantly at 6–8 h after saline refeeding (Fig. 9D). However, in mice refed with Intralipid, plasma FFA concentrations significantly increased at 2–4 h, amylin levels were elevated at 4–6 h, and insulin levels were elevated at 4 h compared with those in mice refed with saline at corresponding time points (Fig. 9, B–D). The increase of plasma amylin or insulin was right after the increase of plasma FFA, suggesting that high-lipid diet induced pancreatic amylin and insulin release, maybe through the increase of plasma FFA levels.

**DISCUSSION**

In the present study, we studied the effect of FAs on pancreatic amylin expression and release. Our data show that FAs at physiological concentrations induce amylin expression at mRNA and protein levels but have no effect on amylin mRNA stability in pancreatic β-cells. We demonstrated that the upregulation of amylin gene transcription by FAs is Ca2+ and PKC dependent and requires de novo protein synthesis. FAs differently regulate amylin and insulin expression but induce both amylin and insulin secretion from β-cells. High-lipid diet sequentially increases plasma FFA and amylin/insulin concentrations in mice.

Amylin is the major component of pancreatic amyloid deposited in excess in pancreatic islets in most patients with type 2 diabetes (6, 30, 49). This 37-amino acid peptide is synthesized primarily in islet β-cells, where it is colocalized (25) and normally cosecreted with insulin (9, 22). Both insulin and amylin gene expression and release by pancreatic islets are regulated by glucose (9, 11, 12, 22). Glucose stimulates insulin and amylin gene expression in a PDX-1-dependent manner (28, 29). Acute exposure of the pancreatic β-cell to FAs results in an increase of insulin release but have no effect on insulin gene expression (2, 15); however, the effect of FAs on the expression and release of amylin by β-cells is not clear. Our present study shows that palmitate and oleate, the physiologically predominant FAs in serum (40), induce amylin gene expression in both MIN6 cells and murine primary islets, and the maximum effect of FAs was reached at 6–8 h (Fig. 1, A–D and G). The inductive effect of FAs on amylin expression is independent of glucose concentration. FA metabolism is required for the induction of amylin gene, since the nonmetabo-
lizable 2-bromopalmitate is ineffective (Fig. 1F). Busch et al. (4) used oligonucleotide arrays to define global alterations in gene expression in MIN6 cells by 48-h exposure to palmitate or oleate. Amylin expression was not reported to be altered by FAs. The discrepancy may be due to the difference in experimental conditions such as time course of FA exposure and absolute FA concentrations used.

Palmitate has been reported to induce gene expression in /H9252/Ca2+/- and PKC-dependent manner (39, 41). Our results indicate that Ca2+ and PKC are also implicated in FA-induced amylin expression. Chelation of extracellular and intracellular Ca2+ with EGTA and BAPTA-AM, respectively, suppressed the induction of amylin gene expression by palmitate and oleate. It has been documented that palmitate promotes Ca2+ influx via L-type Ca2+ channels in rat islets (46). Hence, the available evidence suggests the possibility that FAs would promote a rise in cytosolic Ca2+ by opening of voltage-gated Ca2+ channels and mobilizing Ca2+ from internal stores. Palmitate was shown to promote the translocation of PKC to a particular fraction in islet tissue, an event requiring metabolism of the FA (1). Our results showed that the upregulation of amylin expression was significantly inhibited by Go6-6976, an inhibitor of Ca2+-dependent PKCα and PKCβ1 (Fig. 5). These results suggest that FAs induce amylin gene expression through a Ca2+-PKC-related signaling pathway. cAMP-responsive element-binding protein (CREB) has been reported to be a downstream transcription factor of Ca2+-PKC signaling in pancreatic -cells (45), and the binding site for CREB exists in the promoter region of murine amylin gene, as analyzed with the AliBaba 2.1 program. Our experiments using dominant negative CREB expression vector revealed that CREB was not involved in amylin upregulation by FA (data not shown). We also examined the involvement of PDX-1 and PPAR in FA-induced amylin upregulation by using dominant negative PDX-1 expression vector and PPARα antagonist MK-886, respectively. None of these transcription factors was found to be involved in amylin upregulation by FA (data not shown). However, the expression of amylin induced by FAs was inhibited by cycloheximide, suggesting that the upregulation of amylin by FAs required de novo synthesis of unidentified protein(s).
The effect of FAs on amylin protein expression and release is not clear. Our study shows that stimulation of MIN6 with palmitate or oleate for 12 h induces amylin protein expression, especially the unprocessed and intermediate forms of amylin (Fig. 7). Stimulation of MIN6 cells with FAs significantly induces amylin and insulin release with a similar time course, suggesting that amylin is cosecreted with insulin in response to FAs. Refeeding mice with high-lipid diet significantly increases plasma amylin level, which happens right after the increase of plasma FFA level (Fig. 9). Glucose has been reported to stimulate amylin expression and release by β-cells (9, 11, 12, 22). Our results showed that plasma glucose levels were comparable between the lipid and saline refeeding groups. These results suggest that high-lipid diet-induced...
plasma FFA elevation may play a significant role in inducing amylin release.

It has been reported that acute exposure of INS-1 cells to palmitate or oleate had no effect on preproinsulin mRNA level (2), but acute presentation of FFA to pancreatic β-cells is a potent stimulus for insulin secretion (15). There is also a growing body of evidence suggesting that high-fat diet-induced elevation of circulating FFA influence insulin secretion in vivo (3, 44). Consistently, our results showed that palmitate and oleate had no effect on insulin expression at both mRNA and protein levels (Fig. 7) but induced insulin secretion from MIN6 cells (Fig. 8). High lipid diet sequentially induced elevation of plasma FFA level and insulin concentration (Fig. 9). Although glucose upregulates both amylin and insulin expression (28, 29), our results demonstrate that FAs upregulate only the expression of amylin. The difference in expression between amylin and insulin in response to FAs may be related to different functions of amylin and insulin under physiological conditions.

Amylin has multiple functions, including inhibition of gastric emptying and glucagon, lipase, and amylase secretion (26, 50). Short- and long-term effects of amylin on food intake and body weight have been observed in rodents (26). Our findings that FAs can significantly induce pancreatic amylin expression and release indicate that FAs are physiological regulators of amylin. Amylin production in response to FAs may be important for its biological functions under physiological conditions.

Studies have shown the association of amylin with type 2 diabetes (30). Amylin has been reported to inhibit β-cell insulin secretion, induce β-cell apoptosis, and cause insulin resistance in both in vitro and in vivo systems (18, 19, 23, 36, 43). Although the effects of chronic FA elevation on amylin expression and release are not clear, high plasma FFA concentrations have been reported to be associated with elevated amylin levels in obesity, one of the major risk factors of type 2 diabetes. Fasting and day-long plasma FFA levels are usually elevated (~600–800 μM) in obese nondiabetic individuals (13, 14), and elevated circulating levels of amylin have been detected in obese subjects and obese subjects with impaired glucose tolerance (8, 38).

Pancreatic amylin mRNA and plasma amylin levels are also elevated in genetically obese, insulin-resistant rats (20). In addition, FAs have been reported to stimulate the polymerization of amylin in vitro and amyloid fibril formation in cultivated isolated islet of transgenic mice overexpressing human amylin (27). Transgenic mice overexpressing human amylin gene have increased amylin concentrations but develop islet amyloid only against a background of obesity and/or high-fat diet (17, 21, 42, 47). These results suggest that a high plasma FFA level in obesity and insulin resistance may contribute to amylin amyloidogenesis during the development of type 2 diabetes.

Taken together, our studies show that palmitate and oleate cause transcriptional induction of amylin gene in β-cells and murine islets. This induction is mediated by the Ca^{2+}-PKC signaling pathway and de novo synthesized protein(s). High-lipid diet sequentially induces plasma FFA elevation and amylin release. Our results and available evidence suggest that acute induction of amylin expression and release by FAs may contribute to its biological functions under physiological conditions.

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