Stimulatory Short-Term Effects of Free Fatty Acids on Glucagon Secretion at Low to Normal Glucose Concentrations

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While free fatty acids (FFA) are well known as insulin secretagogues, their effects on pancreatic α cells have been mostly neglected. In the present study we therefore systematically analyzed the glucagon metabolism of rat pancreatic islets under the influence of FFA. Primary islets were incubated in the presence or absence of 200 μmol/L albumin-complexed palmitate or oleate at 2.8 mmol/L versus 16.7 mmol/L glucose and glucagon secretion was monitored over 8 hours. In addition to these time-course experiments, dose dependency of palmitate-induced effects was tested by a 2-hour incubation with 50 to 300 μmol/L albumin-complexed palmitate at 2.8 mmol/L and 5.6 mmol/L glucose. Apart from glucagon secretion, intracellular immunoreactive glucagon and cellular preproglucagon-mRNA (PPG-mRNA) content were determined from the remaining cell lysates. FFA, especially palmitate, induced a significant and dose-dependent increase of glucagon secretion (in average 2-fold above control) during the first 120 minutes of incubation at low to normal glucose (2.8 and 5.6 mmol/L). There was no significant glucagonotropic effect of FFA at concomitant 16.7 mmol/L glucose. Intracellular glucagon as well as cellular PPG-mRNA content were found to be dose-dependently diminished by palmitate when compared with untreated controls at 5.6 mmol/L glucose. The present analysis therefore points to a new role for FFA as a nutritient secretagogue and a modulator of α-cellular glucagon metabolism.

IN THE LAST DECADE the interest in interactions between free fatty acids (FFA) and endocrine islet function has been stimulated by the theory of lipotoxicity in obesity-related type 2 diabetes.[1] Numerous β-cell studies employing new methods and approaches of fatty acid research have shown a complexity of fatty acid–induced effects.[2,3] In contrast to β cells, the influence of FFA on the glucagon producing α cell has received less attention.

More than 30 years ago, Madison et al,[4] followed by Luyckx and Lefebvre,[5] observed an increase of plasma insulin and a decrease of plasma glucagon when plasma FFA were artificially elevated in starved dogs. Edwards and Taylor subsequently described a glucagon-supressing effect of FFA in isolated guinea pig islets.[6] These early data provided the basis for the working hypothesis that a fatty acid–induced inhibitory feedback on pancreatic glucagon secretion (together with a stimulatory feedback on insulin secretion) may prevent overwhelming lipolysis during fasting.[7,8]

In contrast to these reports, more recent studies suggest a stimulatory effect of FFA on glucagon secretion: Gross and Mialhe[9] observed a fatty acid–induced increase of glucagon secretion in the isolated perfused pancreas of ducks and Gremlich et al[10] and Dumonteil et al[11] found elevated amounts of glucagon in cellular supernatants of rat pancreatic islets that had been incubated for more than 24 hours with high amounts of palmitate.

Taken together, only a few data concerning the influence of FFA on the secretion of glucagon from isolated rat pancreatic islets have been published. Here, we systematically examined the influence of FFA on (1) pancreatic glucagon secretion, (2) intracellular content of glucagon, and (3) preproglucagon-mRNA (PPG-mRNA) expression. Short-term fatty acid exposure ($\leq$8 hours) was chosen to avoid interfering cytotoxic and/or proapoptotic effects of palmitate known to occur with long-term exposure.[10,11]

MATERIALS AND METHODS

Materials

Radioimmunoassays for pancreatic glucagon (crossreactivity to oxyntomodulin $<$0.1%) and for immunoreactive insulin (IRI) were performed with kits from Linco Research (St Louis, MO). Fatty acid ultra-free bovine serum albumin (BSA, Fraction V), protease inhibitor cocktail (Complete), Collagenase P (1.5 U $\cdot$ mg^{-1}), as well as all technical and chemical equipment for semiquantitative Light Cycler Polymerase Chain Reaction (PCR), were from Roche Diagnostics (Mannheim, Germany). Reverse transcription was performed using a kit from Promega (Madison, WI). Palmitic (hexadecanoic) acid and oleic (cis-Δ9-octadecenoic) acid and all other analytical-grade biochemicals were purchased either from Sigma-Aldrich (Taufkirchen, Germany) or from Merck Eurolab (Darmstadt, Germany).

Islet Isolation and Culture

Pancreatic islets were isolated from male 200-g Wistar-rats (Charles River, Sulzfeld, Germany) by collagenase digestion and Histopaque-Ficoll density gradient centrifugation as described previously.[12] The newly isolated islets were then conditioned for 16 hours in RPMI-1640/11.2 mmol/L glucose/10% (wt/vol) fetal calf serum (FCS) at 37°C in a 5% CO_2 atmosphere.[13] Afterwards islets were used for the experiments as described below. All animal procedures complied with the *UFAW Handbook on the Care and Management of Laboratory Animals* and the *German Law on the Protection of Animals* and were approved by the local animal's rights committee.

Preparation of the FFA/BSA Complex Solution

Stock solutions of oleic and palmitic acid were prepared as previously described[14] and employed in the experiments at a 1:50 dilution. Final FFA concentrations ranged between 50 μmol/L and 300 μmol/L with a concomitant fix concentration of 0.2 % (wt/vol) BSA. These

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concentrations were chosen according to the fasting plasma FFA levels published previously for rats.[15,16] Controls were incubated with 0.2 % (wt/vol) BSA alone.

Time-Course Experiments

Batches of 40 islets were washed and transferred into 1,000 μL (V_0) of Krebs-Ringer bicarbonate buffer/16 mmol/L HEPES (pH 7.4) containing either 2.8 mmol/L or 16.7 mmol/L glucose with or without FFA. Two FFA compounds were tested: (1) 200 μmol/L palmitate/0.2% BSA and (2) 200 μmol/L oleate/0.2 % BSA. At different time points, t_x (where t_1= 30 minutes, t_2= 60 minutes, t_3 = 120 minutes, t_4 = 240 minutes, and t_5 = 480 minutes), aliquots of a certain volume, V_p (V_1 − V_5) were taken from the supernatants, centrifuged, and kept frozen pending analysis by radioimmunoassay for concentration (c_1 − c_5) of glucagon and insulin, respectively. Cumulative secretion of glucagon or insulin at time t_x were calculated according to:

$$\mathrm{Secr}_{t_x} = \sum_{n=1}^{x}[(c_n - c_{n-1}) \times (V_0 - \sum_{p=1}^{n-1} V_p)]$$

After 8 hours, islets were washed and lysed in 250 μL ice-cold, detergent-containing HEPES-buffer (50 mmol/L HEPES [pH 8.0], 0.1% [vol/vol] Triton X-100 plus protease inhibitor cocktail). Following sonication and centrifugation at 10,000 × *g* for 2 minutes to remove debris, aliquots were taken for the assessment of cellular glucagon by radioimmunoassay.

Dose-Dependency Experiments

Batches of 40 islets were washed and transferred into Krebs-Ringer bicarbonate buffer/16 mmol/L HEPES (pH 7.4) containing either 2.8 mmol/L or 5.6 mmol/L glucose with or without additional palmitate supplementation. Five different palmitate concentrations were tested: (1) 50 μmol/L palmitate/0.2 % BSA ($r_{palmitate:albumin}$ = 1.7), (2) 100 μmol/L palmitate/0.2% BSA ($r_{palmitate:albumin}$ = 3.4), (3) 150 μmol/L palmitate/0.2% BSA ($r_{palmitate:albumin}$ = 5.1), (4) 200 μmol/L palmitate/0.2% BSA ($r_{palmitate:albumin}$ = 6.7), and (5) 300 μmol/L palmitate/0.2% BSA ($r_{palmitate:albumin}$ = 10.1). After 2 hours of incubation, supernatants were collected, centrifuged, and kept frozen. Glucagon levels of the supernatants as well as of the corresponding intracellular lysates (see above) were determined by radioimmunoassay.

Light Cycler Analysis of Cellular PPG-mRNA Content

Batches of 10 islets were incubated under the same conditions as described in the previous section. After 120 minutes of exposure, cytoplasmic RNA was prepared from islet lysates according to the method of Gough.[17] Real-time PCR for PPG-mRNA and glyceraldehyde-3-phosphate-dehydrogenase-mRNA (GAPDH-mRNA) was performed as described in detail elsewhere.[18] In brief, first-strand complementary cDNA was synthesized from the RNA templates by priming with arbitrary hexamers. For subsequent PCR amplification (Roche LightCycler system) the following primer pairs (1 μmol/L) were employed: (1) 5′-TCGTGGCTGGATTGTTTGTA-3′ (sense, 17-36) / 5′-CAATGTTGTTCCGGTTCCTC-3′ (antisense, 246-265) for PPG-cDNA; and (2) 5′-TTAGCACCCCTGGCCAAGC-3′ (sense, 495-511) / 5′-CTTACTCCTTGGAGGCCATG-3′ (antisense, 1014-1033) for GAPDH-cDNA. First-strand cDNA-samples were then amplified for 40 cycles of denaturation (95°C for 5 seconds), annealing (57°C [PPG-cDNA] / 56°C [GAPDH-cDNA] for 5 seconds) and extension (72°C for 22 seconds). Efficiency of amplification was 1.97 (E_{PPG} at 5 mmol/L $MgCl_2$) and 1.96 (E_{GAPDH} at 3 mmol/L $MgCl_2$), respectively. The distinct melting points of 86°C for PPG and 88°C for GAPDH, respectively, guaranteed a specific amplification under the given conditions and ruled out interfering primer dimerization. The quantification of the relative amount of the PPG-cDNA and GAPDH-cDNA in each sample was based on the crossing point values obtained by the Light-Cycler software. Results are expressed as PPG/GAPDH-cDNA ratio of the sample divided by PPG/GAPDH-cDNA ratio of a standard cDNA pool used as calibrator.

Statistical Analysis

Unless otherwise mentioned, data are presented as means ± SE of at least 3 independent experiments. Statistically significant differences were analyzed using unpaired Student's *t* test where $P \leq .05$ was considered significant.

RESULTS

Time- and Glucose-Dependent Effects of Palmitate on Glucagon and Insulin Secretion

At 2.8 mmol/L glucose, palmitate elicited a 1.7- to 2.7-fold (ie, in average a 2.0 [±0.3]-fold) increase in glucagon secretion over 30 minutes (P = .041), 60 minutes (P = .0003), 120 minutes (P = .001), and 240 minutes (P = .0003). The glucagonotropic effect was fading after 480 minutes (Fig 1A). At concomitant 16.7 mmol/L glucose, no relevant glucagonotropic palmitate effect was observed (Fig 1B). Comparing glucagon secretion at 16.7 mmol/L glucose with that at 2.8 mmol/L glucose, we could not find a glucose-induced suppression of glucagon secretion in the palmitate-free controls, which is in accordance to previous findings.[6,11,19] However, in the additional presence of 200 μmol/L palmitate there was a glucose-dependent inhibition of glucagon release. The decrease amounted to 37% ± 9 % at 30 minutes (P = .017), 44% ± 11% at 60 minutes (P = .03), and 27% ± 10 % at 120 minutes (P = .02) when comparing the secretory output of islets incubated with 16.7 mmol/L glucose and palmitate with concurrent palmitate-treated islets at 2.8 mmol/L glucose.

To validate proper islet cell function, insulin release was measured under the same experimental conditions (Figs 1C and D). In the presence of palmitate, basal IRI secretion at 2.8 mmol/L glucose was increased 2.0 (±0.1)-fold ($P \leq .01$ for 30, 60, 120, and 240 minutes) as already described.[13,20] IRI secretion in FFA-exposed islets at 16.7 mmol/L glucose was not significantly different from that in control islets, which is also in accordance with previous results.[20]

To finally rule out relevant fatty acid–induced detergent and/or cytotoxic effects on the pancreatic α cell, the percentage of final cumulative glucagon secretion relative to final intracellular glucagon content was assessed after 8 hours when the glucagonotropic effect had declined. In untreated control islets, the ratio amounted to 6% ± 2% at 2.8 mmol/L glucose and to 5% ± 2% at 16.7 mmol/L glucose. In palmitate-exposed islets the ratio was similar with 7% ± 1% at 2.8 mmol/L glucose and 4% ± 1% at 16.7 mmol/L glucose.

Comparison Between Palmitate and Oleate in Terms of Glucagon Secretion

Using the same experimental setting we additionally tested oleate in terms of glucagon secretion (Fig 2). At 2.8 mmol/L glucose, both palmitate and oleate exerted significant glucagonotropic effects during the first 120 minutes of exposure. However, the glucagonotropic effect of palmitate was more pronounced. Thus, palmitate induced during the first 30 min-

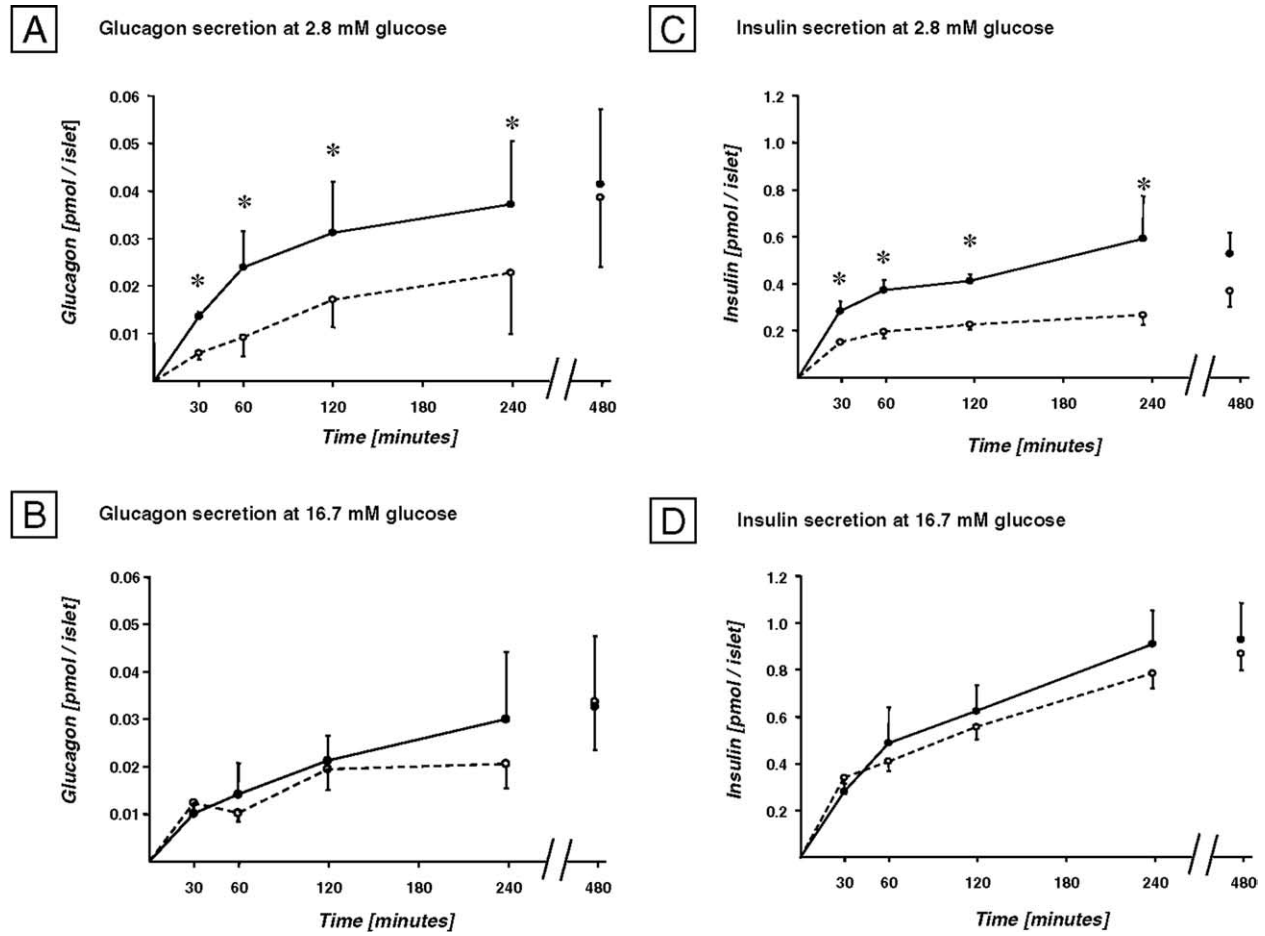

Fig 1. Time- and glucose-dependent effects of palmitate on glucagon and insulin secretion. Forty rat pancreatic islets were cultured at 2.8 mmol/L glucose (A and C) or at 16.7 mmol/L glucose (B and D) in the presence or absence of 200 μmol/L palmitate pre-complexed to 0.2% albumin over 8 hours. (●) Palmitate; (○) control. The curves depict the cumulative secretion of glucagon (A and B) and IRI (C and D) measured in 4 independent experiments (mean ± SE). *Significant changes ($P \leq .05$) when standardized for the corresponding control.

utes a 1.6 [±0.3]-fold higher ($P = .043$) and during the first 60 minutes an 1.8 [±0.4]-fold higher ($P = .006$) secretory output than the concurrent oleate samples (Fig 2A).

At 16.7 mmol/L glucose, there was no FFA-induced glucagonotropic effect during the first 120 minutes of exposure, neither with palmitate nor with oleate (Fig 2B).

Dose-Dependent Effects of Palmitate on Glucagon Secretion and Cellular Content

The glucagonotropic effect of FFA was further characterized in terms of its dose dependency. Based on the time-course experiments, we chose the following experimental conditions: (1) palmitate as model FFA (concentrations 50 to 300 μmol/L), (2) low (2.8 mmol/L) to normal (5.6 mmol/L) glucose concentrations, and (3) short exposure time (120 minutes).

At 2.8 mmol/L glucose, glucagon release in the presence of palmitate was 1.8 (±0.1)-fold increased when compared to concurrent controls (Fig 3A). This reached statistical significance at 50 μmol/L ($P = .001$), 200 μmol/L ($P = .003$), and 300 μmol/L ($P = .0002$). Palmitate also exerted glucagonotropic effects at 5.6 mmol/L glucose with a 1.7 (±0.3)-fold increase above the corresponding controls (Fig 3B). This stimulating effect was statistically significant at 150 μmol/L ($P = .045$), 200 μmol/L ($P = .034$), and 300 μmol/L ($P = .020$) with a dose-dependent increase between 50 μmol/L and 200 μmol/L palmitate.

Palmitate not only influenced glucagon secretion but also intracellular glucagon stores. At 2.8 mmol/L glucose, palmitate induced a dose-dependent depletion of cellular glucagon up to 45% ± 5% when compared to untreated control islets ($P \leq .02$ for 50, 150, 200, and 300 μmol/L palmitate; Fig 3A). At 5.6 mmol/L glucose, palmitate led to a reduction of the intracellular glucagon stores up to 35% ± 15% ($P \leq .02$ for 100, 150, and 200 μmol/L palmitate; Fig 3B).

Notably, the palmitate-induced depletion of intracellular glucagon (compared to control) was more than 10-fold higher than the corresponding palmitate-induced surplus glucagon secretion (Fig 3).

Palmitate-Induced Effects on PPG-mRNA

To investigate the effects of FFA on the cellular PPG-mRNA content, batches of 10 islets were incubated for 120 minutes

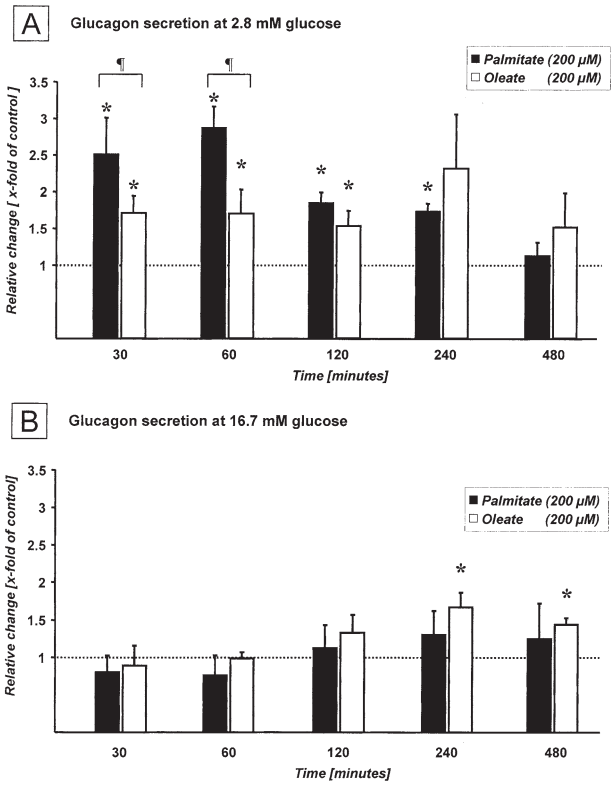

Fig 2. Type-specific effects of palmitate and oleate on glucagon secretion. Fourty rat pancreatic islets were cultured at 2.8 mmol/L glucose (A) or at 16.7 mmol/L glucose (B) in the presence or absence of either 200 µmol/L palmitate (see also Fig 1) or 200 µmol/L oleate. Secretion of glucagon was monitored over 8 hours. The bars depict the type-specific FFA effects as a relative change over the concurrent untreated control (mean ± SE, n = 4). The absolute levels of glucagon secretion in the controls were as follows: 2.8 mmol/L glucose–30 minutes: 0.006 ± 0.001 pmol/islet, —60 minutes: 0.009 ± 0.004 pmol/islet, —120 minutes: 0.017 ± 0.006 pmol/islet, —240 minutes: 0.022 ± 0.013 pmol/islet, ±480 minutes: 0.038 ± 0.014 pmol/islet; 16.7 mmol/L glucose—30 minutes: 0.012 ± 0.001 pmol/islet, —60 minutes: 0.010 ± 0.002 pmol/islet, —120 minutes: 0.020 ± 0.004 pmol/islet, —240 minutes: 0.021 ± 0.005 pmol/islet, —480 minutes: 0.034 ± 0.010 pmol/islet. *Significant changes above control ($P \leq .05$); ¶significant differences between palmitate and oleate ($P \leq .05$).

with different concentrations of palmitate as described for the dose-dependency experiments. In the absence of palmitate, there was a trend towards a reduction of the PPG-mRNA content at 2.8 mmol/L glucose when compared to the PPG-mRNA content at 5.6 mmol/L glucose (by 40% ± 20%), which was not statistically significant ($P = .09$). In the presence of palmitate, increasing concentrations of palmitate tended to gradually reduce the intracellular content of PPG-mRNA. This was most pronounced at 5.6 mmol/L glucose, reaching a maximum reduction of 40% ± 10% ($P = .003$) with 300 µmol/L palmitate (Fig 4).

DISCUSSION

In the present study, palmitate and oleate induced a relevant increase of glucagon secretion from isolated rat pancreatic islets during short-term exposure (ie, ≤4 hours). This glucagonotropic effect was only observed at low to normal glucose levels (2.8 to 5.6 mmol/L) but not at high glucose (16.7 mmol/L). A decrease of the glucose concentration per se (ie, in the absence of FFA) exerted no relevant glucagono-

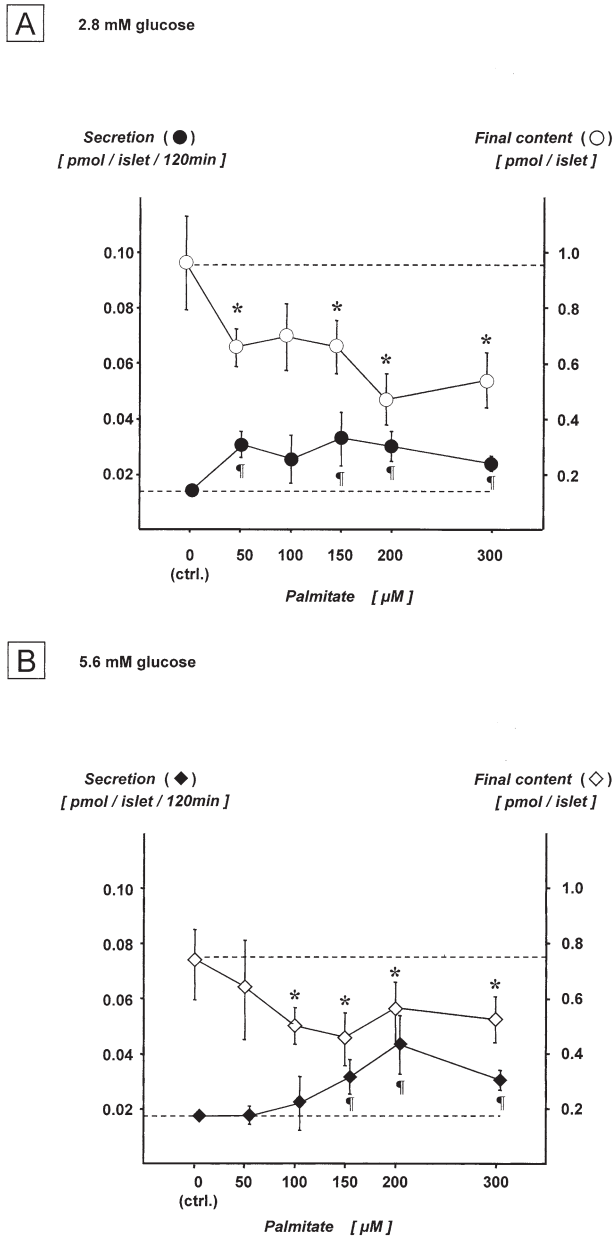

Fig 3. Dose-dependent effects of palmitate on α-cellular glucagon secretion and intracellular content. Fourty rat pancreatic islets were cultured at 2.8 mmol/L glucose (A) or at 5.6 mmol/L glucose (B) for 2 hours with 0 to 300 µmol/L palmitate (pre-complexed to 0.2 % albumin). Glucagon secretion (●, ◆, left ordinate) was determined from the supernatants, and corresponding glucagon content (○, ◇, right ordinate) from the cellular lysates. Values are means ± SE of 4 independent experiments. Significant changes *v* control (ie, 0 µmol/L palmitate) are indicated by ¶ for glucagon secretion and by * for glucagon content, respectively ($P \leq .05$). Note that the scale of the right ordinate (glucagon content) exceeds that of the left ordinate by 1 magnitude.

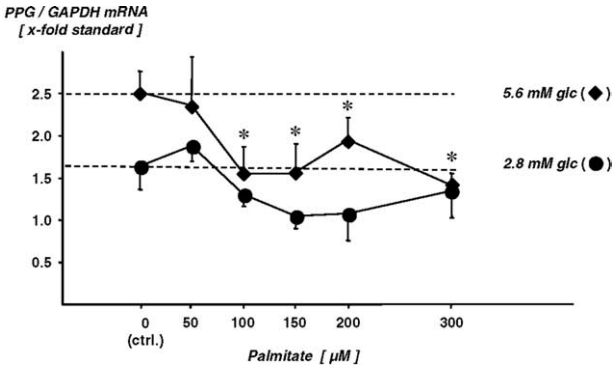

Fig 4. Dose-dependent effects of nonesterified fatty acids on islet PPG-mRNA content. Ten rat pancreatic islets were cultured at 2.8 mmol/L glucose (●) or at 5.6 mmol/L glucose (◆) for 2 hours with 0 to 300 µmol/L palmitate (pre-complexed to 0.2% albumin). PPG-mRNA content was determined by real-time reverse transcriptase-PCR analysis from the cellular lysates. Values are means ± SE of 4 independent experiments. *Significant changes above the palmitate-free controls at 2.8 or 5.6 mmol/L (dashed lines), $P \leq .05$.

tropic effect, which is in accordance to previous in vitro studies.[6,11,19] Ishishara et al recently described a moderate decrease of glucagon secretion in rat pancreatic islets solely induced by glucose. In addition, they also observed a paradoxical stimulation of glucagon secretion by the glycolysis intermediate pyruvate possibly through the closure of α-cellular K_{ATP} channels.[21] In contrast to our present results, the study of Ishishara et al might indicate an active inhibitory effect of glucose on glucagon secretion. Nonetheless, the stimulating effect of pyruvate at low glucose together with our present findings point to acetyl-coenzyme A (CoA) and the rapid generation of adenosine triphosphate (ATP) as possible coupling factors for nutritient-stimulated glucagon secretion. Islets (which naturally comprise both α and β cells) characteristically metabolize mitochondrial pyruvate in a remarkably high degree through pyruvate carboxylase.[22,23] At low glucose levels, it can therefore be speculated that the 4-carbon intermediates of the Krebs cycle preponderate the production of acetyl-CoA from pyruvate.[3] Long-chain-acyl-CoA esters from exogenously administered FFA would be an alternative source for mitochondrial acetyl-CoA and for efficient energy production. High glucose levels might act as a preventive factor by providing cataplerotic intermediates (eg, malonyl-CoA), which then negatively regulate mitochondrial β-oxidation through inhibition of carnitine palmitoyltransferase I (CPT-I).[2] However, to definitively prove whether the FFA-induced stimulation of glucagon secretion we describe here is dependent on such mechanisms involving mitochondrial fatty acid oxidation, further studies, eg, measuring the glucagon response after inhibition of CPT-I by etomoxir or bromopalmitate, would be required.

It has to be noted that the effects we observed cannot be mediated via glucagon-suppressive insulin,[24] since the secretory pattern of fatty acid–induced insulin release paralleled that of the fatty acid–induced glucagon secretion. The somehow blunted insulin secretory response observed at high versus low glucose conditions (16.7 mmol/L *v* 2.8 mmol/L) might be accounted for by the initial preconditioning period at 11.2 mmol/L glucose, which we chose to deactivate any potential glucagonotropic stimulus.

Increased glucagon secretion may be due to an enhanced glucagon synthesis or to a depletion of cellular glucagon stores. Therefore, intracellular content of glucagon was determined. Palmitate dose-dependently induced a reduction of the islet glucagon content by up to 40%, which is reminiscent of the insulin-depleting effect of FFA in the pancreatic β cell.[13,20,25] Notably, the palmitate-induced surplus glucagon secretion (ie, the absolute amount above the secretory release of untreated controls) was found to be an order of magnitude lower than the corresponding amount of palmitate-induced intracellular loss. This indicates that the increase of glucagon secretion is not the primary cause of FFA-induced intracellular glucagon depletion.

In contrast to previous studies,[10,11] interfering cytotoxic and/or apoptotic effects of palmitate can be ruled out in our study since (1) the FFA-induced glucagonotropic effects depended on the concurrent glucose concentration; (2) FFA proved to be a secretagogue at very low concentrations such as 50 µmol/L palmitate/0.2 % BSA, which do not induce relevant β-cell apoptosis for up to 48 hours of incubation[26]; (3) the glucagonotropic effects of FFA were mainly noticed within the short term; and (4) a regulated insulin secretion was retained during the FFA incubation.

To address the question whether the reduced intracellular glucagon content is due to a diminished biosynthesis, PPG-mRNA expression was measured using LightCycler real-time PCR. We found a decrease in preproglucagon-mRNA with increasing palmitate suggesting a reduced biosynthesis of glucagon in the presence of higher concentrations of FFA. In view of the reduced cellular glucagon content it therefore can be speculated that—similar to the the pancreatic β cell[13]—higher FFA concentrations might be able to inhibit the biosynthetic back-up also in the pancreatic α cell. However, the biologic importance of this finding is unclear since we also found a higher content of PPG-mRNA at 5.6 mmol/L glucose when compared to 2.8 mmol/L glucose without having any corresponding effect on the cellular content of the hormone. Also, the fact that the quantitative amount of intracellular glucagon depletion immediately exceeds the secretory loss by far points to a high intracellular turnover rate predominating over the blunted biosynthetic backup proposed above. Whether the trendwise decrease of the FFA-induced stimulation of glucagon secretion observed at higher palmitate levels (>200 µmol/L) is due to the reduction in intracellular content and/or biosynthesis or to a specific regulatory mechanism of the α-cellular stimulus response coupling remains to be determined.

Using palmitate as model FFA, the present study demonstrated (1) a stimulating FFA effect on glucagon secretion at low to normal glucose levels; (2) a FFA-induced depletion of intracellular glucagon content; and (3) a FFA-induced reduction of PPG-mRNA. From these data, a putative physiologic role for FFA and their stimulatory effect on glucagon secretion can be hypothesized, which however challenges conventional paradigms. In the fasting state, glucose and

insulin levels are low, leading to less suppression of lipolysis. Consecutively, higher levels of FFA stimulate secretion of the glucogenic hormone glucagon, which in turn increases hepatic glucose production to sustain fasting blood glucose levels. The lipotoxic effect on cellular glucagon stores and PPG-mRNA needs further characterization in terms of its molecular mechanism and its pathogenic importance. The present study brings FFA into the focus of α-cell research where—similar to the β cell—they might be a physiologically relevant nutritient secretagogue.

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