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The short-term effect of fatty acids on glucagon secretion is influenced by their chain length, spatial configuration, and degree of unsaturation: studies in vitro

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Abstract

The influence of fatty acids on beta cell function has been well established whereas little is known about the role of fatty acids on alpha cell function. The aim of our study was to investigate the short-term effects of chain length, spatial configuration, and degree of unsaturation of fatty acids on glucagon secretion from isolated mouse islets and alpha tumor cell 1 clone 6 cells (alpha TC1-6 cells). Glucagon release was measured with different saturated and unsaturated fatty acids as well as *cis* and *trans* isomers of fatty acids at low and high glucose. Palmitate (0.1-0.5 mmol/L) immediately stimulated glucagon release in a dose-dependent manner from both isolated islets and alpha TC 1-6 cells. The longer chain length of saturated fatty acids, the higher glucagon responses were obtained. The average fold increase in glucagon to saturated fatty acids (0.3 mmol/L) compared to control was octanoate 1.5, laurate 2.0, myristate 2.9, palmitate 5.4, and stearate 6.2, respectively. Saturated fatty acids were more effective than unsaturated fatty acids in stimulating glucagon secretion. At an equimolar concentration, *trans*-fatty acids were more potent than their *cis* isomers. Fatty acids immediately stimulate glucagon secretion from isolated mouse islets pancreatic alpha cells. The chain length, spatial configuration, and degree of unsaturation of fatty acids influence the glucagonotropic effect.

1. Introduction

Fatty acids are believed to be a major endogenous energy source for islets and to play an important role in stimulus response coupling in pancreatic beta cells [1]. Short-term exposure to fatty acids powerfully potentiates glucose-stimulated insulin secretion, whereas long-term exposure results in elevated basal insulin secretion and a blunted response to glucose [2]. The dichotomy in response is seen in vivo and in vitro. Fatty acids change messenger RNA expression of genes encoding key proteins in the insulin signaling cascade, that is, insulin receptor (IR), IR substrate-1, and IR substrate-2. The changes in insulin secretion and gene expressions in beta cells are both dose- and time-dependent [3,4].

Although a number of studies have revealed the importance of the chain length, spatial configuration, and degree of unsaturation of fatty acids for insulin secretion [5-9], little is known about the role of fatty acids on glucagon secretion. Conflicting data have been reported from in vivo and in vitro studies on the influence of fatty acids on glucagon release [10-24]. In early reports, fatty acids inhibited glucagon release from isolated rat pancreatic islets [17,20], whereas a more recent study demonstrated that 48-hour exposure to 0.6 mmol/L palmitate potently stimulates glucagon release [13]. Mixtures of linoleic and linolenic acids possessed a stimulatory effect on glucagon release [10]. It remains, however, to be elucidated how individual fatty acids influence glucagon secretion from pancreatic alpha cells.

Here we have studied the short-term effects of fatty acids on glucagon secretion from isolated mouse islets. We wished to establish the dose dependency of palmitate on glucagon secretion; to assess the influence on glucagon secretion of the chain length of saturated fatty acids (octanoate [C8:0], laurate [C12:0], myristate [C14:0], palmitate [C16:0], and stearate [C18:0]); to compare the effects of *cis* and *trans* forms of C18:1 fatty acids (oleate [C18:1 *cis*], elaidate [C18:1 *trans*],

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cis-vaccenic acid [C18:1], and *trans*-vaccenic acid [C18:1]); and to test the importance of the degree of unsaturation for the glucagon responses (stearate [C18:0], oleate [C18:1], linoleate [C18:2], and linolenate [C18:3]) at low and high glucose concentrations, respectively.

2. Materials and methods

2.1. Isolation of islets

Female Naval Medical Research Institute mice (Bomholtgaard Breeding and Research Center, Ry, Denmark) were maintained on a 12-hour light/dark cycle and had free access to water and an ordinary laboratory chow diet. At the time of experiments, they weighed 20 to 30 g and were handled in accordance with Danish law, with the approval of the Animal Experiments Inspectorate under the Ministry of Justice.

We used the collagenase digestion technique to obtain pancreatic islets as previously described [25,26]. Islets were handpicked under a stereo-microscope and kept in modified RPMI 1640 overnight in an atmosphere of 95% humidified air/5% CO_2 (37°C). The modified RPMI 1640 was supplemented with 10% (vol/vol) fetal calf serum (FCS), penicillin G (100 U/mL), and streptomycin (100 μg/mL) (all from GIBCO BRL, Paisley, UK). In each experiment, the islets were obtained from 8 to 16 mice.

2.2. Incubation studies

After overnight culture, islets were rinsed and preincubated in modified Krebs ringer buffer (KRB) containing 125 mmol/L NaCl, 5.9 mmol/L KCl, 1.2 mmol/L $MgCl_2$, 1.28 mmol/L $CaCl_2$, 5.0 mmol/L $NaHCO_3$, 25 mmol/L HEPES (pH 7.4), 0.5% bovine serum albumin [BSA] (fatty acid–free), and 16.7 mmol/L glucose (BSA from Roche, Mannheim, Germany; the rest from GIBCO BRL) in an atmosphere of 95% humidified air/5% CO_2 for 60 minutes. For glucagon and insulin secretion studies, batches of 5 islets were incubated in a 37°C water bath with 250 μL KRB containing 0.5% BSA, different concentrations of glucose, and various amounts of fatty acids for 2 hours. As controls,

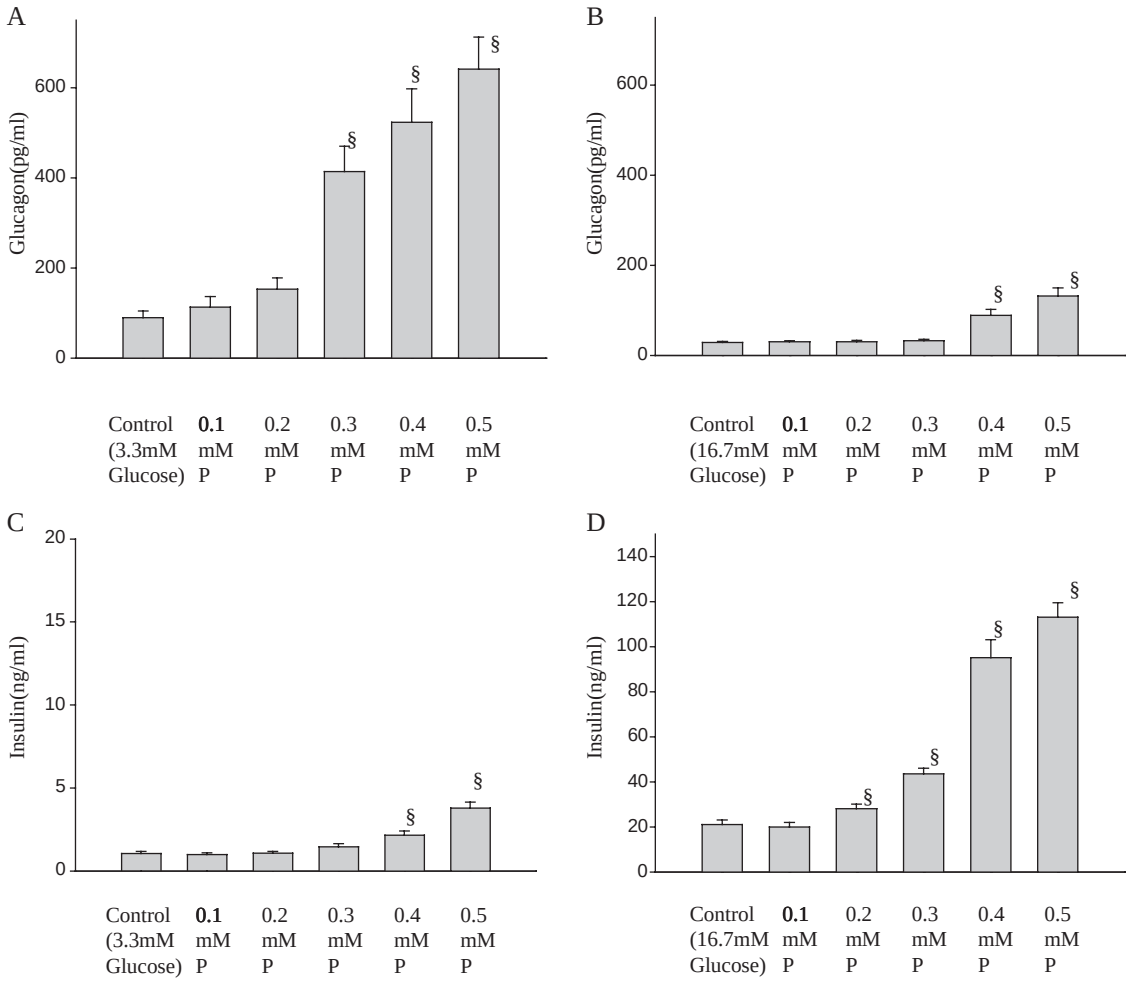

Fig. 1. Effects of 2-hour incubation with 0.1 to 0.5 mmol/L palmitate on glucagon (A and B) and insulin secretion (C and D) from isolated mouse islets in the presence of 3.3 mmol/L (A and C) or 16.7 mmol/L glucose (B and D). Values are mean ± SEM for 15 to 24 batches of islets. $^{\S}P < .05$ indicates a significant difference compared to control.

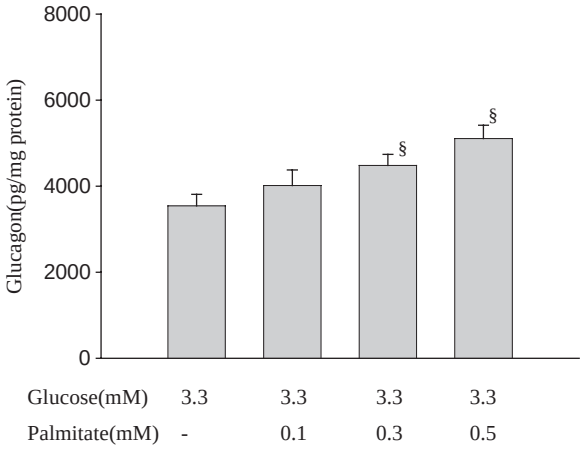

Fig. 2. Effects of 2-hour incubation with 0.1 to 0.5 mmol/L palmitate on glucagon secretion after adjusting for protein concentration from alpha TC1-6 cells in the presence of 3.3 mmol/L glucose. Values are mean ± SEM (n = 18). $^{\S}P < .05$ indicates a significant difference compared to control.

islets were cultured without fatty acids. At the end of incubation, 200-μL incubation medium was collected on ice for glucagon and insulin assay.

The alpha tumor cell 1 (alpha TC1) line was derived from an adenoma in transgenic mice expressing the SV40 large T-antigen oncogene. The alpha TC1 clone 6 cells (alpha TC1-6) were cloned by a limiting dilution method and only secrete glucagon [27]. They (passage number 17-19) were cultured in DMEM containing 18 mmol/L glucose and 10% FCS under an atmosphere of 95% humidified air/5% CO_2 at 37°C, passaged once a week and fed with new medium twice a week. After preincubation in 1 mL KRB supplemented 3.3 mmol/L glucose in 24-well plates for 30 minutes, cells were incubated with 1 mL KRB containing 3.3 mmol/L glucose; 0.5% BSA; and 0.1, 0.3, or 0.5 mmol/L palmitate, respectively. At the end of the 2-hour exposure time, supernatants were collected, centrifuged, and kept for glucagon measurements.

2.3. Materials

Oleate, linoleate, linolenate, and *cis*- and *trans*-vaccenic acid were purchased in their oily form. Octanoate, laurate, myristate, palmitate, stearate, and elaidate were obtained as solid powders. All the fatty acids were purchased from Sigma (St Louis, Mo). Collagenase was from Roche (Mannheim, Germany).

2.4. Solutions

All fatty acids (sodium salts) were prepared by dissolving and heating to equal molar amounts of NaOH and fatty acids supplemented with distilled water to a concentration of 500 mmol/L. They were further diluted with 5% BSA (fatty acid–free) to 50 mmol/L fatty acids. The stock solutions were filter sterilized and stored at −20°C. Each fatty acid solution was freshly prepared before each experiment.

2.5. Glucagon assay

Glucagon was analyzed by radioimmunoassay kit (Linco Research Inc, St Charles, Mo). The glucagon antibody is specific for pancreatic glucagon and has no cross-reaction with other islet polypeptides. The sensitivity of glucagon assay is 20 pg/mL.

2.6. Insulin assay

Insulin was measured by radioimmunoassay with the guinea pig, antiporcine insulin antibody PNILGP4, mono-^{125}I-(Tyr-A14)–labeled human insulin as tracer, and rat insulin (Novo Nordisk, Bagsvaerd, Denmark) as a standard. Free and bound radioactivity was separated using ethanol. Both inter- and intra-assay coefficients of variation are between 5% and 10%.

2.7. Protein assay

After secretion study, cells were lysed in 0.1mol/L NaOH. The total protein was measured by a Bio-Rad detergent compatible protein kit (Bio-Rad laboratories, Hercules, Calif).

2.8. Statistics

Student unpaired *t* test and 1-way analysis of variance were used for the statistical analysis. Significant differences were considered for *P* values less than .05. Data are expressed as the mean ± SEM.

3. Results

3.1. Dose-dependent effects of palmitate on glucagon and insulin secretion

The effect of palmitate (0.1-0.5 mmol/L) on glucagon secretion was evaluated at 3.3 or 16.7 mmol/L glucose. As seen in Fig. 1, palmitate caused a dose-dependent stimulation of glucagon secretion at 3.3 mmol/L glucose. The threshold was detected at a palmitate concentration between 0.2 and 0.3 mmol/L ($P < .001$) in the presence of 0.5% BSA, whereas a 6-fold increase in glucagon secretion occurred to 0.5 mmol/L palmitate. At high glucose (16.7 mmol/L), glucagon secretion was reduced and a higher palmitate concentration was needed to elicit a glucagon response.

Palmitate also elicited a dose-dependent insulin secretion at both low and high glucose. A stimulatory effect was found at ≥0.4 and ≥0.2 mmol/L palmitate in the presence of 3.3 and 16.7 mmol/L glucose, respectively.

To determine whether the palmitate effect on glucagon secretion was a direct effect on alpha cell, clonal alpha TC1-6 cells were exposed to 3 different concentrations of palmitate (0.1, 0.3 and 0.5 mmol/L) for 2 hours in the presence of 3.3 mmol/L glucose. A dose-dependent stimulation of glucagon release was detected in Fig. 2 after adjusting for protein concentration.

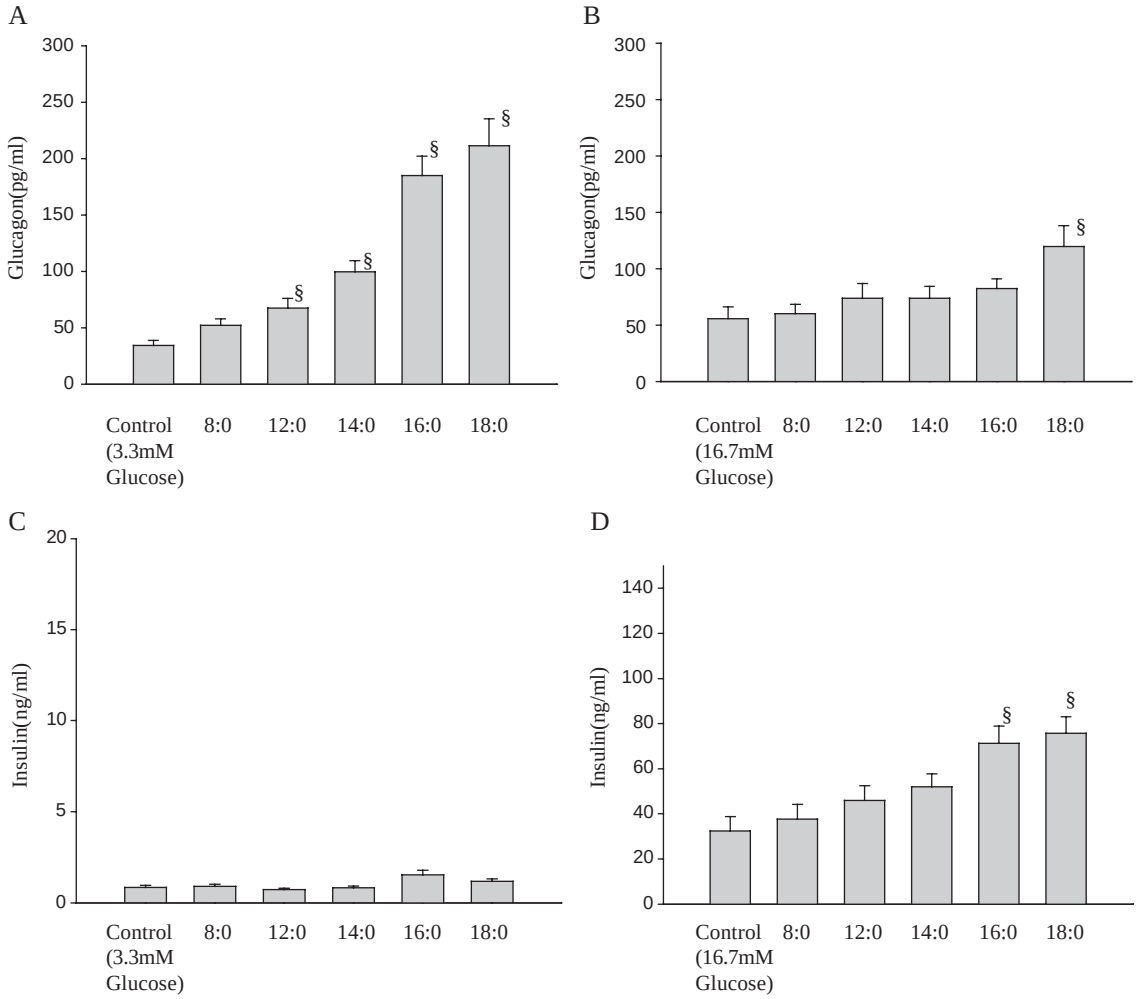

Fig. 3. Influence of 2-hour addition of an equimolar concentration (0.3 mmol/L) of octanoate (C8:0), laurate (C12:0), myristate (C14:0), palmitate (C16:0), and stearate (C18:0) on glucagon (A and B) and insulin secretion (C and D) from isolated mouse islets in the presence of low (3.3 mmol/L) (A and C) and high glucose (16.7 mmol/L) (B and D). Values are mean ± SEM for 13 to 24 batches of islets. $^{\S}P < .05$ indicates a significant difference compared to control.

3.2. Short-term effects of chain length of saturated fatty acids on glucagon and insulin secretion

To evaluate the influence of chain length on glucagon secretion, islets were incubated in 0.3 mmol/L of 5 different medium- or long-chain saturated fatty acids for 2 hours in the presence of 3.3 or 16.7 mmol/L glucose. The chain length of the saturated fatty acids ranged from C8:0 to C18:0. The fatty acids with a chain length from C12:0 (laurate) to C18:0 (stearate) stimulated glucagon secretion at 3.3 mmol/L glucose (Fig. 3). The longer chain length of fatty acids, the higher glucagon response was observed. Octanoate (C8:0) only tended to enhance glucagon release ($P = .055$). The fold stimulation of glucagon in response to fatty acids compared to control (34.4 ± 4.5 pg/mL) attained, in average, octanoate (C8:0) 1.5, laurate (C12:0) 2.0, myristate (C14:0) 2.9, palmitate (C16:0) 5.4, and stearate (C18:0) 6.2. However, glucagon responses to palmitate and stearate did not differ significantly. The stimulatory effect of fatty acids on glucagon secretion was subdued at high glucose (16.7 mmol/L) and only stearate markedly increased the release of glucagon.

At a low glucose concentration, there was no significant effect of fatty acids on insulin secretion. In contrast, at higher glucose, a marked stimulation of insulin release was seen to palmitate and stearate. The effect of palmitate and stearate did not reach statistical significance.

3.3. Effects of the spatial configuration of fatty acids on glucagon and insulin secretion

The effects on glucagon secretion of *cis* and *trans* isomers of mono-unsaturated fatty acid (C 18:1) were investigated in batch incubations at low and high glucose. As shown in Fig. 4, 0.3 mmol/L *trans*-fatty acids were more potent than their *cis* isomers. Thus, the glucagon responses to oleate (*cis* C18:1δ9), elaidate (*trans* C18:1δ9), *cis*-vaccenic acid (*cis* C18:1δ11), and *trans*-vaccenic acid (*trans* C18:1δ11) were 107 ± 17, 178 ± 29, 83 ± 7 and 153 ± 13 pg/mL, respectively, compared to the control of 52 ± 6 pg/mL. At high glucose (16.7 mmol/L), glucagon

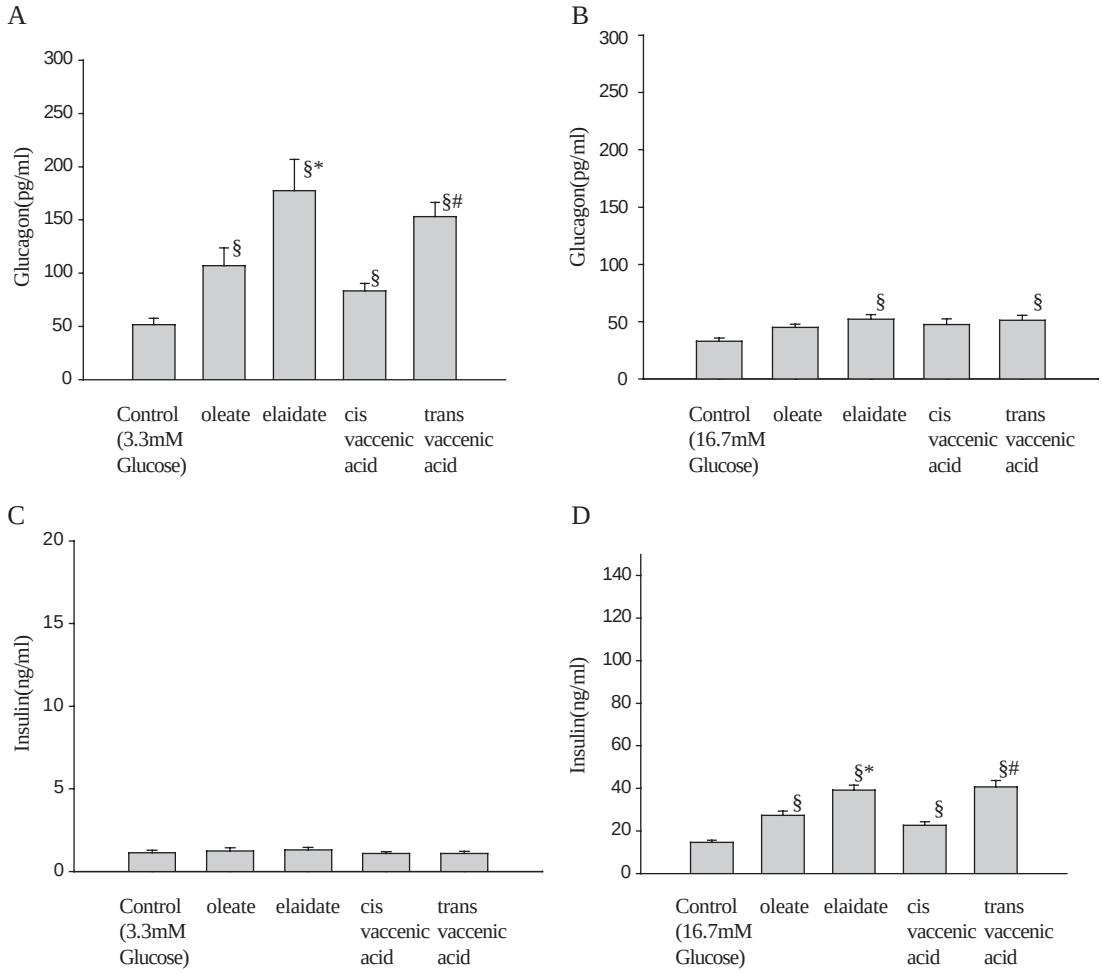

Fig. 4. Effect of 2-hour incubation with 0.3 mmol/L of *cis* and *trans* forms of C18:1 fatty acids (oleate [C18:1 *cis*], elaidate [C18:1 *trans*], *cis*-vaccenic acid [C18:1], and *trans*-vaccenic acid [C18:1]) on glucagon (A and B) and insulin (C and D) secretion from isolated mouse islets in the presence of 3.3 mmol/L (A and C) or 16.7 mmol/L glucose (B and D). Values are mean ± SEM for 17 to 24 batches of islets. $^{\S}P < .05$ indicates a significant difference compared to the control; $^{*}P < .05$, significant difference between elaidate and oleate; $^{\#}P < .05$, significant difference between *cis*- and *trans*-vaccenic acid.

responses to 0.3 mmol/L of the *cis*- and *trans*-fatty acids were suppressed. Even at the high glucose, *trans*-fatty acids tended to be more potent than their *cis* isomers, however, not reaching statistical significance.

Trans-fatty acids were more effective than their corresponding *cis* isomers in potentiating insulin secretion in the presence of 16.7 mmol/L glucose, although they had no effect on insulin release at 3.3 mmol/L glucose.

3.4. Effects of degree of fatty acid unsaturation on glucagon and insulin secretion

Fig. 5 demonstrates the effects of 0.3 mmol/L of stearate (C18:0), oleate (C18:1), linoleate (C18:2), and linolenate (C18:3) on glucagon and insulin secretion at low and high glucose levels. Stearate caused a prominent glucagon response at 3.3 mmol/L glucose, whereas the presence of double bounds in the fatty acid chain markedly reduced the secretory response. Although the glucagon responses tended to be reduced by increasing numbers of double bonds, there was no statistically significant difference. In addition, no change in glucagon secretion was detected to 0.3 mmol/L unsaturated fatty acids at 16.7 mmol/L glucose.

Stearate induced enhanced insulin secretion at both low and high glucose concentrations. Oleate, linoleate, and linolenate produced no change in insulin release at 3.3 mmol/L glucose, whereas in the presence of high glucose, they stimulated insulin secretion. Stearate tended to cause higher insulin responses than unsaturated fatty acids at 16.7 mmol/L glucose.

4. Discussion

The role of fatty acids for the beta cell function has been extensively investigated; however, little is known about the short- and long-term effects of fatty acids on the alpha cell function. Here, we report that short-term exposure to fatty acids increased glucagon secretion. The stimulatory effect of palmitate was dose-dependent and was due to a direct effect on the alpha cell as demonstrated using clonal alpha cells. The glucagonotropic effect of fatty acids was influenced by their

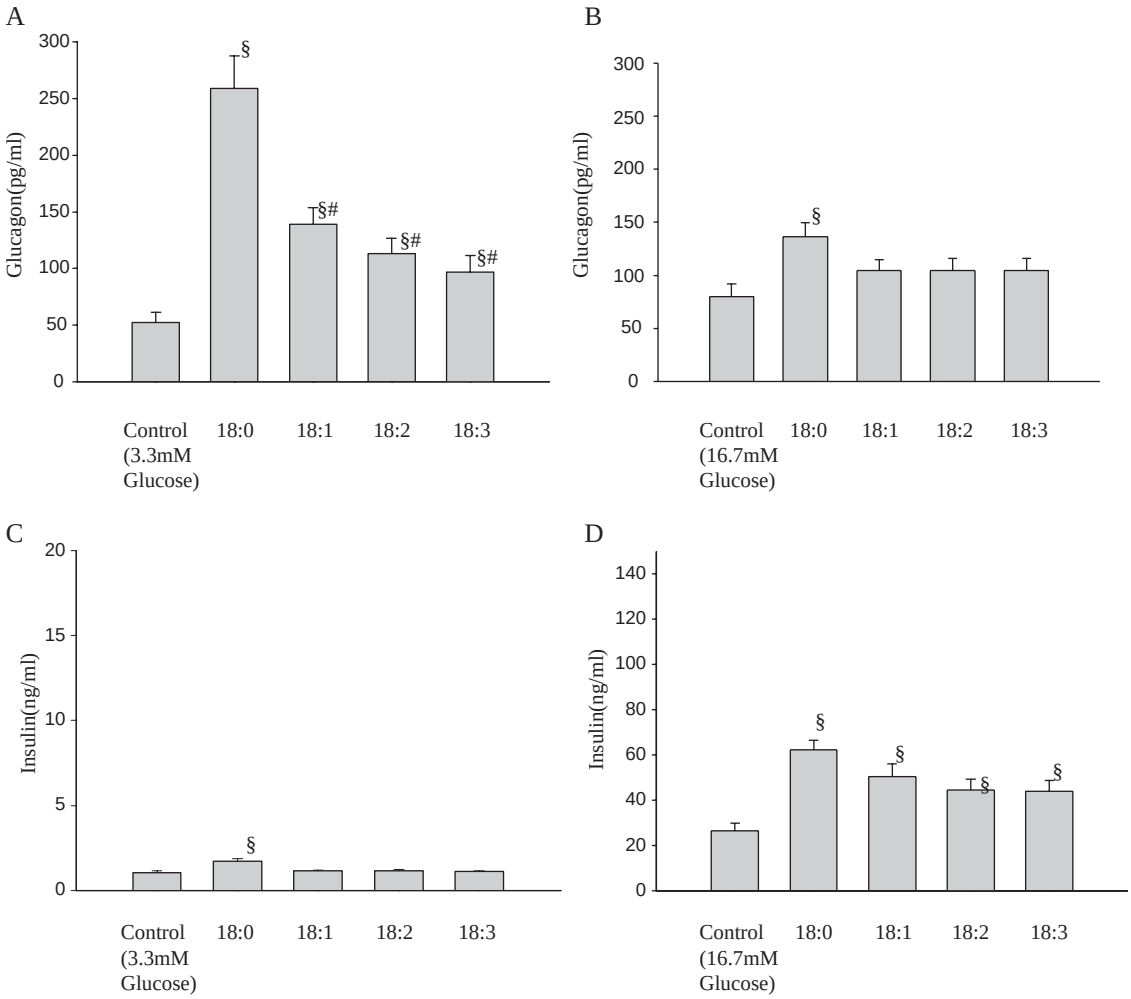

Fig. 5. Comparison of the effects of 2-hour incubation with 0.3 mmol/L of stearate (C18:0), oleate (C18:1), linoleate (C18:2), and linolenate (C18:3) on glucagon (A and B) and insulin (C and D) secretion from isolated mouse islets in the presence of 3.3 mmol/L (A and C) or 16.7 mmol/L glucose (B and D). Values are mean ± SEM for 19 to 24 batches of islets. $^{\S}P < .05$ indicates a significant difference compared to control; $^{\#}P < .05$, significant difference between saturated fatty acids and unsaturated fatty acids.

chain length and spatial configuration. The glucagon response was enhanced with increasing chain length of the saturated fatty acids. Interestingly, *trans*-fatty acids caused larger glucagon responses than their corresponding *cis* isomers. Moreover, saturated fatty acids appear to be more potent than unsaturated fatty acids. Our results corroborate previous studies on the role of fatty acids for insulin secretion [5-9]. Thus, the insulinotropic effect of individual fatty acids increased and decreased dramatically with chain length and degree of unsaturation, respectively. *Trans*-fatty acids stimulate insulin release more potently than their *cis* isomers.

Fatty acids have been found to stimulate [10,11,13-15] and to possess a neutral [14,17] or inhibitory action [12,16-24] on glucagon secretion. Apparent discrepancy between in vivo and in vitro findings might be explained, at least in part, by a role of incretins or the autonomic nervous system. In vitro glucagon responses may depend on the experimental method applied, that is, if the studies were carried out using the perfused pancreas, perifused isolated islets, static incubations of isolated islets, or dispersed islet cells [28]. In addition, species differences may explain some of the different results obtained. The alpha cells in the guinea pig are situated uniformly throughout the islet mass, making them less susceptible to damage during the isolation procedure than in the mouse and rat, in which alpha cells are placed in the periphery [21]. There are species differences in the density and composition of ion channels in alpha cells. Mouse alpha cells express roughly 100 times fewer K_{ATP} channels than do rat alpha cells. The K_{ATP} channels have been found to influence the hormone responses from alpha cells [29-31].

Quantitative variations in plasma fatty acids of a normal population are present even on the same diet [32]. Blood levels of fatty acids may, however, not be an accurate index of their biologically effective concentrations; thus, the presence of lipoprotein lipase on the endothelial surface of blood capillaries that may cause rapid hydrolysis of triglycerides and increase the local fatty acid concentrations to which cells are exposed [33,34]. Rates of de novo fatty acid synthesis in

islets are low [35]; therefore, fatty acid moieties of triglycerides must be derived from circulating fat fuels. Islets express lipoprotein lipase [36] and may obtain fatty acids from lipoproteins. Moreover, free fatty acids are also taken up, stored, and oxidized by islets [37]. The addition of different concentrations of fatty acids and albumin in vitro may explain to some extent the differential glucagon responses observed in various studies. In plasma, the total fatty acid–albumin ratio is normally below 2 and rarely exceeds 4. Long-chain fatty acids are physiologically bound to albumin, with a maximal fatty acid–albumin molar ratio of 7 [38]. The concentrations of fatty acids ranged between 0.1 and 0.5 mmol/L, whereas the albumin concentration was 0.5% in the present study. Some studies were based on the use of extremely high concentrations of fatty acids, with a fatty acid–albumin ratio of more than 16 considerably exceeding the binding capacity of albumin.

Our study demonstrated that the intrinsic properties of fatty acids critically influenced glucagon responses. Alternatively, chain length and spatial configuration of fatty acids may influence the binding affinity of the individual fatty acid to albumin and consequently result in different amounts of unbound fatty acids and glucagon responses [8]. We have previously proposed that a higher rate of oxidation of *cis* isomers of fatty acids in beta cells would, via the Randle cycle, inhibit glucose oxidation to a greater extent than the corresponding *trans* isomers [8]. Possibly similar conditions are operating in mouse alpha islet cells. Furthermore, uptake rates of individual fatty acids increase with the length of the fatty acid chains and decrease with the number of double bounds. The capacity of triglyceride esterification is greater for unsaturated than for saturated fatty acids [39,40]. These differences in cellular uptake and use of saturated and unsaturated fatty acids would tend to favor the accumulation in islets of saturated fatty acids to higher concentration than unsaturated fatty acids.

The accumulation of triglycerides in islets has been reported to be associated with detrimental islet cell function. In Zucker diabetic fatty rats (ZDF rats), which have mutated leptin receptors, a pronounced increase in islet triglyceride content occurs associated with oxidative damage and beta cell destruction [41]. In contrast, overexpression of leptin receptors prevents the accumulation of triglyceride in islets and reverse diabetes [42].

Most recently, Olofsson et al [43] demonstrated that palmitate increases glucagon secretion by enhanced calcium entry via L-type Ca channels. Concomitantly, they found that palmitate is a strong inhibitor of somatostatin secretion, suggesting that palmitate may enhance the glucagon secretion by relief from paracrine somatostatin-induced inhibition. However, the present study strongly indicates that fatty acids directly influence alpha cells because a modest fatty acid–induced increase in glucagon secretion from clonal alpha cells occurs. The observation that no insulin release could be detected from clonal alpha cells in response to fatty acids and glucose (unpublished results) makes it unlikely that fatty acids act indirectly via beta cells. The palmitate-induced glucagon response from clonal alpha cells occurred considerably smaller than from alpha cells being part of a normal islet architecture. A direct quantitative comparison of the secretory capacity in clonal alpha cells and alpha cells in normal islets seems to be difficult without the exact number of alpha cells in these 2 preparations. It is noteworthy that we have previously demonstrated that alpha cells not longer being part of a normal islet structure respond with a subdued glucagon release [44].

Interestingly, long-chain fatty acids amplify glucose-stimulated insulin secretion from pancreatic beta cells by activating the G protein–coupled receptor, GPR40, which is abundantly expressed in the pancreas. However, there is no evidence of GPR40 messenger RNA expression in pancreatic alpha cell [45]. Further studies are needed to examine the underlying mechanisms involved in fatty acids action in regulation of glucagon release, such as studies in signaling pathways, other receptors, and gene expression.

In conclusion, short-term exposure to fatty acids directly stimulates the glucagon release from pancreatic alpha cells. The stimulatory action on glucagon of individual fatty acids is influenced by chain length, degree of unsaturation, and spatial configuration of fatty acids. Saturated fatty acids stimulate glucagon secretion whereas the presence of double bounds in fatty acids markedly attenuates the glucagon responses. We have only explored the short-term effects on alpha cell secretion of fatty acids. The glucagonotropic action of fatty acids raises the question whether increased levels of circulating fatty acids may in part explain the hyperglucagonemia in type 2 diabetes characterized by a bihormonal abnormality with reduced first-phase insulin secretion and elevated glucagon secretion. To gain further insight into the mechanisms of action of fatty acids, it would be prudent to determine long-term effects of fatty acids on glucagon secretion and changes in gene expressions in alpha cells to see whether there is also a pathophysiologic important lipotoxic action on the alpha cells as has previously been demonstrated in beta cells.

Acknowledgment

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