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# Chronic Effects of Different Non-esterified Fatty Acids on Pancreatic Islets of Rats

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***Aims*: The aim of this study was to examine the chronic effects of different non-esterified fatty acids (NEFA) on insulin secretion by pancreatic islets of normal Wistar rats in vitro. *Methods*: Pancreatic islets were isolated from normal Wistar rats, and were incubated with 0.2, 0.4, or 0.8 mmol/L palmitate (C16:0), stearate (C18:0), oleate (C18:1), or linoleate (C18:2) for 24 h, then the insulin secretion and pyruvate dehydrogenase (PDH) activity were examined. *Results*: Neither islet insulin content nor islet DNA content differed among islets incubated with each kind of NEFA. Compared with control, linoleate significantly inhibited glucose-stimulated insulin secretion (GSIS) and PDH activity at each concentration ($p < 0.05$), while others inhibited GSIS and PDH activity significantly only at 0.4 and 0.8 mmol/L ($p < 0.05$). There was no significant difference in GSIS and PDH activity among islets pretreated by palmitate, stearate, and oleate at the same concentration ($p > 0.05$). However, linoleate decreased GSIS more than others at the same concentration ($p < 0.05$), while linoleate (0.4 or 0.8 mmol/L) inhibited PDH activity more than others at the same concentration ($p < 0.05$). *Conclusions:* Elevation of palmitate, stearate, oleate or linoleate decreases the β-cell secretory response to glucose, through inhibiting PDH activity. Linoleate exerts more negative effect on GSIS than other NEFA.**

**Key Words:** Non-esterified fatty acids; pancreatic islet; insulin secretion; glucose intolerance; pyruvate dehydrogenase.

## Introduction

The prevalence of type 2 diabetes mellitus and glucose intolerance are increasing rapidly worldwide *(1)*. Type 2 diabetes mellitus is characterized by insulin resistance and by a defect in insulin secretion. More and more studies have focused on the effects of non-esterified fatty acids (NEFA) on insulin secretion *(2–5)*. NEFA acutely stimulate insulin secretion *(6,7)*, but they also induce a long-term impairment of glucose-induced insulin secretion *(3–5)*.

Whether different long-chain fatty acids exert distinct influences on insulin secretion remains disputed. The acute, direct, effects of saturated, monounsaturated, and polyunsaturated fatty acids on cells have been compared. Similar effects of palmitate and oleate in incubated rat islets have also been reported *(8)*. In contrast, palmitate was found not to affect, and oleate, linoleate to stimulate insulin secretion from perifused mouse islets *(9)*. Exactly opposite findings were obtained with the perfused rat pancreas in which the insulinotropic effect of the fatty acids increased with the chain length, but decreased with the degree of unsaturation *(10)*. However, the chronic, direct, effects of saturated, monounsaturated, and polyunsaturated long-chain fatty acids on pancreatic islets have rarely been compared. It is unclear whether the inhibition of glucose-stimulated insulin secretion (GSIS) after long time pre-incubation depends on the type of NEFA and which one exerts more negative effects than others on β-cell function in vitro.

In our previous study *(11)*, we have reported that 24 h preculture with palmitate decreases insulin secretion from pancreatic islets to the response of glucose. In the present study, we evaluated the insulin secretory response to glucose after exposing pancreatic islets to palmitate (C16:0, saturated NEFA , the dominating NEFA in plasma), stearate (C18:0), oleate (C18:1, monounsaturated NEFA, one of the major NEFA in plasma), or linoleate (C18:2, polyunsaturated NEFA, predominant in fat emulsion) for 24 h; and assessed the effect of different kinds of NEFA on PDH activity in islets.

## Results

### *Islet Insulin Content*

There was no significant difference in islet insulin content among control, palmitate, stearate, oleate, and linoleate-treated pancreatic islets (Table 1, $p > 0.05$).

### *Islet DNA Content*

There was no significant difference in islet DNA content among control (31 ± 5.6 ng/islet), palmitate (0.2 mmol/L vs

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**Table 1**
Effect of 24 h Non-esterified Fatty Acids Exposure on Insulin Secretion and Islet Insulin Content from Islets Isolated from Wistar Rats

| Culture conditions | *n* | Insulin secretion (nmol/μg DNA) | | Insulin content (nmol/μg DNA) |
|---|---|---|---|---|
| | | Glucose (mmol/L) | | |
| | | 3 | 15 | |
| Control | 16 | 1.43 ± 0.42 | 5.81 ± 0.69 | 686 ± 127 |
| Palmitate | | | | |
| 0.2 mmol/L | 8 | 1.52 ± 0.39 | 5.43 ± 0.79 | 663 ± 145 |
| 0.4 mmol/L | 8 | 1.59 ± 0.40 | 5.09 ± 0.88[a] | 673 ± 120 |
| 0.8 mmol/L | 8 | 1.58 ± 0.47 | 4.43 ± 0.71[a] | 665 ± 138 |
| Stearate | | | | |
| 0.2 mmol/L | 9 | 1.53 ± 0.41 | 5.39 ± 0.91 | 680 ± 138 |
| 0.4 mmol/L | 9 | 1.62 ± 0.50 | 4.92 ± 0.78[a] | 665 ± 140 |
| 0.8 mmol/L | 9 | 1.57 ± 0.43 | 4.27 ± 0.68[a] | 673 ± 144 |
| Oleate | | | | |
| 0.2 mmol/L | 8 | 1.60 ± 0.51 | 5.30 ± 0.87 | 670 ± 132 |
| 0.4 mmol/L | 8 | 1.54 ± 0.42 | 4.87 ± 0.96[a] | 678 ± 147 |
| 0.8 mmol/L | 9 | 1.66 ± 0.40 | 4.08 ± 0.89[a] | 663 ± 135 |
| Linoleate | | | | |
| 0.2 mmol/L | 8 | 1.51 ± 0.50 | 4.42 ± 0.96[a,b,c,d] | 683 ± 151 |
| 0.4 mmol/L | 8 | 1.43 ± 0.43 | 3.73 ± 1.26[a,b,c,d] | 674 ± 139 |
| 0.8 mmol/L | 8 | 1.61 ± 0.51 | 3.13 ± 0.93[a,b,c,d] | 668 ± 148 |

Islets were cultured for 24 h in DMEM containing both 10% fetal bovine serum and 5.5 mmol/L glucose with or without 0.2, 0.4, or 0.8 mmol/L palmitate, stearate, oleate, and linoleate. The islets were then preincubated in KRB (pH 7.4) containing 3.0 mmol/L glucose for 60 min at 37°C. Three size-matched islets in each culture tube were then incubated for 60 min in 1.0 mL KRB containing glucose (3.0 or 15 mmol/L). Values are expressed as means ± SD. [a]$p < 0.05$ compared with control. [b]$p < 0.05$ compared with palmitate at the same concentration. [c]$p < 0.05$ compared with stearate at the same concentration. [d]$p < 0.05$ compared with oleate at the same concentration.

0.4 mmol/L vs 0.8 mmol/L: 30 ± 4.8 ng/islet, 31 ± 4.4 ng/islet, 32 ± 5.9 ng/islet), stearate (31 ± 4.7 ng/islet, 31 ± 5.0 ng/islet, 30 ± 5.1 ng/islet), oleate (31 ± 4.9 ng/islet, 30 ± 4.5 ng/islet, 31 ± 5.3 ng/islet), and linoleate (30 ± 5.1 ng/islet, 32 ± 6.1 ng/islet, 31 ± 4.7 ng/islet)-treated pancreatic islets ($n$ = 6–9, $p > 0.05$).

### *Effect of NEFA on Insulin Secretion In Vitro*

There was no significant difference in GSIS between islets cultured with and without ethanol (Fig. 1, $p > 0.05$).

Neither 0.2, 0.4, nor 0.8 mmol/L palmitate, stearate, oleate, linoleate affected the insulin secretion in response to 3.0 mmol/L glucose (Table 1, $p > 0.05$). However, when the islets were stimulated with 15 mmol/L glucose, 0.2, 0.4, and 0.8 mmol/L palmitate, stearate, oleate, linoleate decreased GSIS by 7%, 12%, and 25%; 7%, 15%, and 26%; 8%, 17%, and 30%; 23%, 35%, and 47%, respectively. The decrease in GSIS by NEFA related to the concentration of NEFA ($p < 0.05$), which was 0.2 vs 0.8 mmol/L. Compared with control, linoleate significantly inhibited GSIS at each concentration, while others used in our study significantly inhibited GSIS only at 0.4 and 0.8 mmol/L (Table 1, $p < 0.05$). There was no significant difference in GSIS among islets pre-cultured with palmitate, stearate and oleate at the same concentration ($p > 0.05$). However, linoleate exerted more negative effect on GSIS than others at the same concentration (Table 1, $p < 0.05$).

### *Effect of NEFA on PDH Activity*

Compared with control, linoleate significantly inhibited PDH activity at each concentration, while others significantly inhibited PDH activity only at 0.4 and 0.8 mmol/L (Fig. 2, $p < 0.05$). Linoleate (0.4 and 0.8 mmol/L) inhibited PDH activity more than other NEFA when they were compared at the same concentration ($p < 0.05$). There was no significant difference in inhibiting PDH activity among palmitate, stearate, and oleate ($p > 0.05$). Palmitate, stearate, oleate, and linoleate significantly inhibited PDH activity at 0.8 rather than 0.2 mmol/L ($p < 0.05$).

## Discussion

In previous studies, feeding rats or mice a high-fat/low-carbohydrate diet results in the impairment of glucose toler-

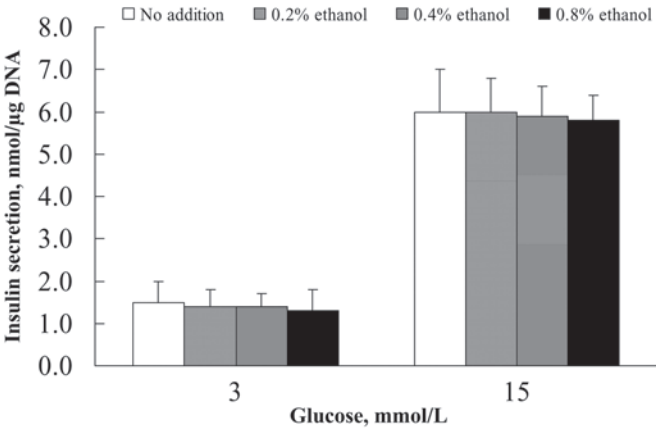


**Fig. 1.** Effect of ethanol exposure on insulin secretion from islets isolated from Wistar rats. Islets were cultured for 24 h in DMEM containing both 10% fetal bovine serum and 5.5 mmol/L glucose with or without ethanol. The islets were then preincubated in KRB (pH 7.4) containing 3.0 mmol/L glucose for 60 min at 37°C. Three size-matched islets in each culture tube were then incubated for 60 min in 1.0 mL KRB containing glucose (3.0 or 15 mmol/L). Values are expressed as means ± SD. Six separate experiments were performed with a total of 10–12 observations at each point. Each observation was derived from three size-matched islets. There was no difference in glucose-induced insulin secretion of islets cultured in DMEM with or without ethanol.

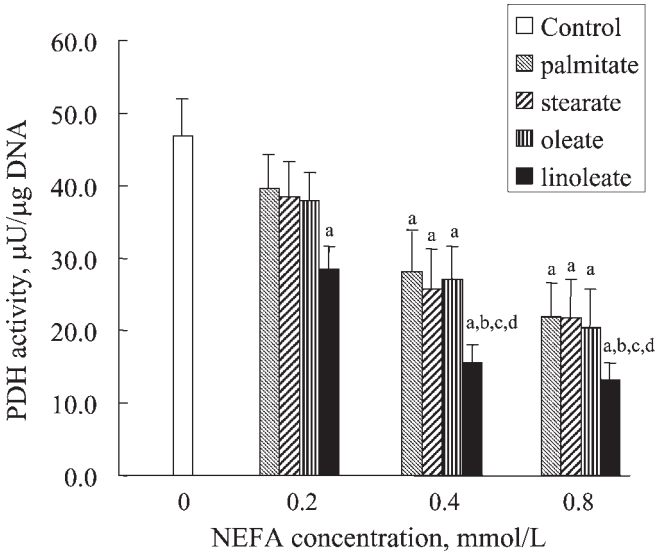


**Fig. 2.** Effect of 24-h non-esterified fatty acid exposure on pyruvate dehydrogenase activity in islets isolated from Wistar rats. Islets were cultured for 24 h in DMEM containing both 10% fetal bovine serum and 5.5 mmol/L glucose with or without 0.2, 0.4, or 0.8 mmol/L of palmitate, stearate, oleate, or linoleate. Values are expressed as means ± SD of five experiments. $^{a}p < 0.05$ compared with control. $^{b}p < 0.05$ compared with palmitate at the same concentration. $^{c}p < 0.05$ compared with stearate at the same concentration. $^{d}p < 0.05$ compared with oleate at the same concentration.

ance *(11,12–14)* or caused diabetes mellitus in normal Wistar rats *(15)*. Randle *(16,17)* and Randle et al. *(18)* proposed that an increase in the NEFA level inhibits glucose oxidation, stimulates hepatic glucose production, and inhibits secretion of insulin by β-cells in the pancreatic islets in response to glucose. Previously, we *(11)* demonstrated that a high-fat/low-carbohydrate diet for 3 d impaired glucose tolerance by inhibiting insulin secretion of pancreatic islets and whole body insulin sensitivity through the Randle cycle, which is the activation of the glucose–fatty acids cycle.

Hu et al. *(19)* proposed that different types of fat play a major role in the development of type 2 diabetes mellitus. Vessby et al. *(20,21)* reported that insulin secretion was not affected by a change of dietary fatty acid composition. However, all of these studies were in vivo researches, a lot of endocrine factors affect the results. It is necessary to turn to an in vitro system to assess the relative effectiveness of individual NEFA.

Stein et al. *(10)* perfused pancreas of rats for 40 min in the presence of different types of fatty acid at the concentration of 0.5 mmol/L, found that palmitate, oleate, and linoleate were potent for the stimulation of insulin secretion. Warnotte et al. *(22)* incubated mice pancreatic islets with NEFA for 60 min and found that palmitate, oleate, and linoleate stimulate GSIS. Both of these researches showed the acute stimulating effect of short time exposure to NEFA on GSIS. However, glucose intolerance and diabetes mellitus are accompanied by prolonged elevation of NEFA. In the present study, we examined the chronic effect of different NEFA on GSIS, and demonstrated that the 24-h exposure of islets to palmitate, stearate, oleate, or linoleate significantly impaired GSIS from pancreatic islets. Moreover, the higher the NEFA's concentration was, the more GSIS was inhibited. This demonstrates that the prolonged elevation of saturated, monounsaturated, or polyunsaturated long-chain NEFA decreases GSIS, i.e., the increase of NEFA impairs insulin secretion. It suggests that short-time culture with NEFA produced more ATP to stimulate GSIS, while long-term incubation with NEFA inhibited glucose oxidation, reduced the cytosolic ATP/ADP ratio, and impaired GSIS.

The present study demonstrated that along with decreased GSIS, PDH activity was inhibited by 24-h exposure to 0.4 or 0.8 mmol/L NEFA (palmitate, stearate, oleate, or linoleate) in pancreatic islets. Zhou and Grill *(23)* showed that 48-h palmitate exposure plays an important role in decreased PDH activity in rat islets. Taken together, these findings indicate that long-term exposure to NEFA, whether it is saturated or unsaturated, decrease GSIS by inhibiting PDH activity in pancreatic islets.

It is not clear why linoleate inhibited GSIS more than others at 0.2 mmol/L, while no significant difference was seen in inhibiting PDH activity among them. Perhaps other pathways are involved in the inhibition of GSIS, such as apoptosis of β-cell by NEFA *(24)*, increasing of uncoupling protein 2 by NEFA *(25)*. Further study is needed.

Previous researches *(10,22)* suggested that long-chain saturated NEFA acutely stimulate insulin release more potently than unsaturated ones. The stimulating effect of NEFA on GSIS depends on the degree of saturation of NEFA. In our study, although stearate, oleate, and linoleate have same chain lengths, their inhibiting effect of GSIS were different. Linoleate (C18:2) exerted more negative effect on GSIS than other long-chain NEFA at the same concentration, through inhibiting PDH activity more than other NEFA. There was a tendency that oleate (C18:1) inhibited GSIS more than stearate (C18:0), which did more than palmitate (C16:0); however, no significant difference was seen in the inhibition of GSIS among palmitate, stearate, and oleate, and so was inhibiting PDH activity among these NEFA. From our results, it is impossible to reach the conclusion that the inhibition of NEFA on GSIS depends on the degree of saturation of NEFA. Differences in experimental procedures such as NEFA concentration used and incubation period, may explain the discrepancies among various reports. Further study is needed.

In the present study, the islet insulin content and the islet DNA content (an index of β-cell numbers in the pancreatic islets) were not affected by 24-h exposure of islets to palmitate, stearate, oleate, or linoleate. In contrast, Zhou and Grill *(4)* reported that 48-h culture of pancreatic islets with NEFA reduced the insulin content. Culture of islets with NEFA for less than 48 h may not reduce the content. Further studies are needed.

In conclusion, elevation of palmitate, stearate, oleate, or linoleate decreases the β-cell secretory response to glucose, through inhibiting PDH activity. Linoleate exerted more negative effect on GSIS than other NEFA used in our study at the same concentration.

## Materials and Methods

### *Animals*

Nine-week-old male Wistar rats (Shizuoka Laboratory Animal Center, Shizuoka, Japan) were used throughout the experiments. They were kept individually in stainless-steel wire-bottomed cages in an air-conditioned room (22 ± 2°C, 55 ± 10% relative humidity) with artificial lighting from 06:00 to 18:00. For acclimation, they were maintained on commercial CE-2 powder diet (CE-2, Clea, Japan, carbohydrate 58.9%, protein 29.5%, fat 11.6%) and water *ad libitum* for 1 wk.

The experiments were performed in accordance with the Guidelines for Animal Experiments of the University of Yamanashi, which concur with the United States National Institute of Health Guidelines.

### *Isolation of Pancreatic Islets*

Rats were anesthetized by intraperitoneal injection of 50 mg/kg pentobarbital (sodium salt, Abbott Laboratories, North Chicago, USA). Pancreatic islets were isolated from the pancreas by collagenase digestion *(26)*. In brief, the pancreas was retrogradely filled with 30 mL of Hank's balanced salt solution (HBSS) (Gibco BRL, NY, USA, glucose concentration 5.5 mmol/L), supplemented with 22 mg collagenase (Wako, Osaka, Japan, 183 U/mg). The pancreas was then removed and incubated for 30 min at 37°C. After rinsing, the islets were separated from the remaining exocrine tissue by hand picking under a stereomicroscope.

### *Culture of Pancreatic Islets*

Islets were transferred to Petri dishes containing Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, pH 7.4, glucose concentration 5.5 mmol/L) supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL), 100 U/mL penicillin (Sigma), and 0.1 mg/mL streptomycin (Sigma). Then islets were cultured for 24 h at 37°C in an atmosphere of 5% $CO_2$/95% air with or without any kind of NEFA, such as palmitate, stearate, oleate, and linoleate (sodium salts), which were purchased from Sigma Chemical Co. Each kind of NEFA was dissolved in 99% ethanol. The ethanol solution was mixed with the same volume of 154 mmol/L NaCl, which was then added to the DMEM. The final concentration of NEFA in the medium used was 0, 0.2, 0.4, or 0.8 mmol/L. The final ethanol concentration was 0, 0.2, 0.4, or 0.8% (vol/vol) according to the NEFA concentration used, respectively.

### *Glucose-Stimulated Insulin Secretion Assay In Vitro*

After culture with different kinds of NEFA, islets were preincubated for 60 min at 37°C, in an atmosphere of 95% $O_2$ and 5% $CO_2$, in Krebs–Ringer Buffer (KRB) containing 3.0 mmol/L glucose (basal incubation solution). The buffer consisted of (in mmol/L) 120 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 24 $NaHCO_3$, and 100 mg/mL bovine serum albumin (BSA). After the preincubation, three size-matched islets were transferred into culture tubes containing 1.0 mL KRB supplemented with glucose at different concentrations. After incubating for 60 min, 0.5 mL of the solution was collected from each tube and immediately stored at –20°C until insulin analysis.

### *Islet Insulin Content Assay*

The islet insulin content was measured according to the method described by Ishihara et al. *(27)*.

### *Islet DNA Content Assay*

The islet DNA content, as an indicator of cell number, was measured by the method of Labarca and Paigen *(28)*, which was modified by Hopcroft et al. *(29)*, using calf thymus DNA (Sigma, Type I) as standard.

### *Preparation of Islet Homogenate Extracts*

PDH complex was extracted as Paxton *(30)* described. One hundred islets pretreated with different kinds of NEFA for 24 h, were homogenized in 300 μL homogenization buffer, consisting of (in mmol/L) 50 HEPES (pH 7.5), 0.2 KCl,

3 ethylenediamine tetraacetate, 5 dithiothreitol, 0.1 $N^{\alpha}$-*p*-tosyl-L-lysine chloromethyl ketone, 0.1 mg/mL trypsin inhibitor from egg white, 200 KIE/mL aprotinin (Trasylol, Bayer Leverkusen, Germany), 2% rat serum, and 0.25% (vol/vol) Triton X-100. The extract was kept on ice for 30 min before being used in the assay of PDH activity.

### *Radiochemical Assay of PDH Activity*

Assays were conducted according to the method of Goodwin *(31)* in 1.5 mL uncapped Eppendorf tubes inside a capped 20 mL scintillation vial containing 1.0 mL 1.2 mol/L KOH. The enzyme reaction mixture in a 100 µL volume consisted of (in mmol/L) 50 HEPES (pH 7.5), 1.0 $MgCl_2$, 3 $NAD^+$, 0.4 TPP, 0.4 coenzyme-A, 2 dithiothreitol, 1.0 pyruvate, 0.1% (vol/vol) Triton X-100, 7.5 U/mL lipoamide dehydrogenase (EC 1.8.1.4), and 50 µL islet extract. The reaction was started by adding 0.1 µCi [1-$^{14}$C]pyruvate. Incubations were then performed for 20 min at 37°C. Reactions were stopped by the injection of 100 µL 15% trichloroacetate into the Eppendorf tube. The $^{14}CO_2$ produced was absorbed by KOH in the scintillation vials during a further 2 h incubation at 37°C. After that, the Eppendorf tubes were removed, 5 mL scintillation fluid was added to each vial, and the radioactivity was counted in a scintillation counter. Blanks containing 50 µL homogenization buffer and other reaction components were processed identically with samples containing islet extract. The averaged blank count was subtracted from sample countings. One unit of PDH complex activity was defined as the production of 1 µmol $CO_2$/min at 37°C. Activity was linear with time between 3 and 30 min and with the amount of islet extract derived from 5 to 20 islets (data not shown).

### *Biochemical Analyses*

The insulin concentration was determined using an Insulin ELISA Kit from Morinaga Biochemistry (Yokohama, Japan) using rat insulin as standard with a microplate spectrophotometer system (SPECTRAmax 340 with SOFTmax PRO version 2.1 software, Molecular Devices, Sunnyvale, CA, USA). The intra- and inter-assay coefficients of variation for this insulin assay were each less than 10%, with the minimum detectable concentration being 50 pg/mL.

### *Statistics*

The data were subjected to two-way ANOVA using StatView 5.0 (Abacus Concepts, Berkeley, USA). Fisher's PLSD test was used when there was a significant difference among the groups. The 0.05 level of probability was used as the criterion of significance.

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