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Fatty acid-induced toxicity and neutral lipid accumulation in insulin-producing RINm5F cells

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Abstract

Fatty acids have been shown to cause death of rat and human primary pancreatic beta cells and of insulin-producing cell lines. These studies focused mainly on saturated and monounsaturated FA such as palmitic, stearic and oleic acids. In this study, we have performed a comparison of the toxicity of a wider range of FA. The toxicity of different FA to insulin-producing RINm5F cells was assessed by flow cytometry measuring loss of plasma membrane integrity and increase in DNA fragmentation. Additionally, the FA induced neutral lipid accumulation and the FA composition were determined. Palmitic, linoleic, γ-linolenic, oleic, stearic, and eicosapentaenoic acid caused DNA fragmentation of insulin-producing RINm5F cells. Loss of membrane integrity was mainly caused by linoleic and γ-linolenic acid. There was no correlation between cytotoxicity and the abundance of the FA in the cells as determined by HPLC analysis. Taken as whole, the toxic effect of the FA on insulin-producing RINm5F cells varied irrespective of the chain length and the degree of unsaturation. In these cells PA and LA exhibited the highest toxicity, whereas AA was not toxic. In addition, the toxicity of most tested FA was inversely related to low NLA, except for AA and EPA. The results of this study contribute to the understanding of the role of FA in the impairment of pancreatic beta cell function that occurs in type 2 diabetes and obesity.

Keywords: Fatty acids; Beta cell death; RINm5F cells; Neutral lipid accumulation; Lipotoxicity

1. Introduction

The process of glucose-induced insulin secretion by pancreatic islets and its functioning are well known to be modulated by cytokines (Bloch and Vardi, 2005), reactive oxygen and nitrogen species (Azevedo-Martins et al., 2003), diet composition (Hefetz et al., 2006) and fatty acids (Haber et al., 2003a,b; Poitout and Robertson, 2002; Purrello and Rabuazzo, 2000; Raz et al., 2005). During the process of insulin secretion induced by glucose substantial changes occur in fatty acid composition of pancreatic beta cells (Martins et al., 2004; Rustenbeck et al., 1994). However, long-term exposure to elevated plasma concentrations of fatty acids (FA) plays a key role in the development of type 2 diabetes mellitus (Aarnes et al., 2002; Cnop et al., 2001; Haber et al., 2003). FA have been shown to cause death of rat (Cnop et al., 2001; Maedler et al., 2001) and human (Lupi et al., 2002) primary pancreatic beta cells and of insulin-producing cell lines (Hawkins et al., 1998). These studies focused mainly on saturated and monounsaturated FA such as palmitic, stearic and oleic acids (Cnop et al., 2001; Eitel et al., 2002; Haber et al., 2003; Shimabukuro et al., 1998). However, a comparison of the toxicity of a wider range of FA has not been performed yet.

The mechanisms underlying the cytotoxic effect of FA still remain controversial. There is evidence that free FA

Abbreviations: PA, palmitic acid; SA, stearic acid; OA, oleic acid; LA, linoleic acid; LNA, γ-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; NLA, neutral lipid accumulation; FA, fatty acids

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induce the production of reactive oxygen species by pancreatic beta cells (Evans et al., 2003). Some studies reported that, in islets of pre-diabetic Zucker rats (Shimabukuro et al., 1997, 1998) and in the HIT-T15 cell line (Okuyama et al., 2003), FA-induced cell damage involves activation of iNOS and NO generation, being correlated with ceramide formation. On the other hand, Aarnes and colleagues (2002) observed in the insulin-producing cell line INS-1E that non-toxic concentrations of FA may potentiate interleukin-1β toxicity, apparently via a NO-independent pathway (Okuyama et al., 2003). Recently, the importance of triglyceride deposition for the toxicity of FA to beta cells has been postulated (Butler et al., 2003). The deposition of triglyceride has been considered by some researchers to impair mitochondrial function and to cause cell death (Maestre et al., 2003). This effect has been associated with an increase in ceramide content and activation of the JNK/SAPK and other signaling pathways (Haber et al., 2003). However, Cnop and colleagues (2001) found an inverse relationship between the cytotoxicity of palmitic and oleic acids with triglyceride accumulation in pancreatic islets (Cnop et al., 2001; Maedler et al., 2001). Therefore, the involvement of triglyceride deposition for the effect of FA on pancreatic beta cell death still remains to be elucidated.

In the present study, flow cytometric analyses have been performed to assess cell damage through loss of plasma membrane integrity and DNA fragmentation and thereby the toxicity of FA on insulin-producing RINm5F tissue culture cells. The fatty acid composition of the cells was determined in order to find out whether the cytotoxicity was correlated with the abundance of the fatty acid. In addition, intracellular neutral lipid accumulation was determined. The aim of this study was to compare the toxicity of different FA and to investigate whether the toxicity of LA, LNA, EPA and AA on insulin-producing RINm5F cells was dependent on their capacity to induce neutral lipid accumulation (NLA) as it has been postulated for the toxicity of PA and OA (Cnop et al., 2001; Maedler et al., 2001).

This experimental approach addressed the following questions: (1) how is the toxicity of unsaturated FA on RINm5F cells compared with palmitic and oleic acids? (2) Is there a relationship between FA toxicity and neutral lipid accumulation for all FA? (3) Is the cytotoxicity related to FA abundance in the cells?

2. Material and methods

2.1. Reagents

RPMI-1640 medium, HEPES, penicillin and streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). Fatty acids and Nile red were from Sigma (St. Louis, MO, USA). All fatty acids presented purity between 98% and 99%. Propidium iodide was purchased from ICN Biomedicals (Costa Mesa, CA, USA), citrate from Merck (Darmstadt, Germany) and sodium bicarbonate from Labsynth Products (Diadema, SP, Brazil). Rhodamine 123 was obtained from Molecular Probes (Eugene, OR, USA) and ethidium bromide from BioRad Laboratories (Hercules, CA, USA). All cell culture material was purchased from Corning (Corning, NY, USA).

The fatty acids used as HPLC standards were obtained from Sigma Chemical Co.: lauric (C12:0; catalogue No L-4250), myristic (C14:0; M-3128), palmitic (C16:0; P-0500), palmitoleic (C16:1-ω6; P-9417); stearic (C18:0; S-475), oleic (C18:1 ω; O-1008), linoleic (C18:2 ω6; L-1376), γ-linolenic (C18:3 ω6; G-2174), arachidonic (C20:4; A-9673), eicosapentaenoic (C20:5 ω3; E-2011), docosahexaenoic (C22:6 ω3; D-2534) and margaric (C17:0; H-3500) acids. 18-Crown-6 and 4-bromomethyl-7-metoxi-coumarin (Sigma Chemical Co.). Acetonitrile (ACN), chloroform, methanol, *n*-hexane, tetra-hydrofuran (THF) were purchased from Merck (Darmstadt, Germany). All solvents were grade HPLC. The standards were solubilized in ACN:THF (1:1) and filtered through Millex-LCR13 (0.45 μm).

2.2. RINm5F cell culture

The RINm5F cell line is a clone derived from the parent RIN-m cells (derived from a rat insulinoma). The cells produce and secrete insulin, but unlike the parental line they do not produce somatostatin (Bhathena et al., 1984). The RINm5F cells were obtained from the Institute of Clinical Biochemistry of the Hannover Medical School (Hannover, Germany). Cells were cultured in RPMI-1640 medium, containing 11.1 mM glucose and 10% (v/v) fetal calf serum (FCS). The medium was supplemented with glutamine (2 mM), HEPES (20 mM), streptomycin (10,000 μg/mL), penicillin (10,000 UI/mL) and sodium bicarbonate (24 mM). Cells were maintained in an incubator (Sanyo, Middlesex, UK) with humidified atmosphere at 37 °C and 5% CO_2.

2.3. Fatty acid treatment

Cultured cells were treated with increasing concentrations (50–400 μM) of different FA for 24 h. Cells were seeded the day before the addition of the FA at 4×10^5 cells per well in six well plates. The FA tested were: (1) saturated: palmitic (C16:0) (PA) and stearic (C18:0) (SA) acids; (2) monounsaturated: oleic acid (C18:1, n-9) (OA); and (3) polyunsaturated: linoleic (C18:2, n-6) (LA), γ-linolenic (C18:3, n-6) (LNA), arachidonic (C20:4, n-6) (AA) and eicosapentaenoic (C20:5, n-3) (EPA) acids. The fatty acids were dissolved in ethanol. The final concentration of ethanol in the culture medium did not exceed 0.05%. This concentration of ethanol was not toxic to the cells, confirming earlier observations (Lima et al., 2002).

2.4. Cell membrane integrity assay

Cells were centrifuged at 1000 rpm for 5 min at 4 °C and the pellet obtained was resuspended in 500 μL phosphate-buffered saline (PBS). Thereafter, 50 μL of propidium iodide solution (50 mg/mL in PBS) were added and then the

cells were analysed using a FACScalibur flow cytometer (Becton Dickinson, San Juan, CA, USA). Propidium iodide is a highly water-soluble fluorescent compound that cannot pass through intact membranes and is generally excluded from viable cells. It binds to DNA by intercalating between the bases with little or no sequence preference (Nicoletti et al., 1991). Fluorescence was measured using the FL2 channel (orange–red fluorescence—585/42 nm). Ten thousand events were analysed per experiment. Cells with propidium iodide fluorescence were then evaluated by using the Cell Quest software (Becton Dickinson).

2.5. DNA fragmentation assay

DNA fragmentation was analysed by flow cytometry after DNA staining with propidium iodide according to the method described before (Nicoletti et al., 1991). The solution used contains detergent (0.1% Triton-X-100) that permeabilizes the cells, allowing the dye to promptly incorporate into DNA. Briefly, after incubation, the cells were centrifuged at 1000 rpm for 5 min at 4 °C. The pellet was gently resuspended in 300 μL hypotonic solution containing 50 μg/mL propidium iodide, 0.1% sodium citrate, and 0.1% Triton-X-100. The cells were then incubated overnight at 4 °C. Fluorescence was measured and analysed as described above (Nicoletti et al., 1991).

2.6. Determination of intracellular neutral lipids

Cells were treated and collected as described above. Afterwards, the cells were incubated for 10 min with Nile red (0.1 μg/mL), a selective fluorescence stain for intracellular neutral lipid droplets (Healy et al., 2003). Fluorescence was determined using the FL1 channel (green fluorescence—530/570 nm). Cells stained with Nile red fluorescence were evaluated as described above and the images were captured using an Axiovert 100M microscope, in combination with the Axiovision Program (Zeiss, Oberkochen, Germany).

2.7. Determination of fatty acid composition by HPLC

Total lipids were extracted from RINm5F cells as previously described (Folch et al., 1957). The lipids were saponified using 2 mL of an alkaline methanol solution (1 mol per mL NaOH in 90% methanol) at 37 °C for 2 h in a shaking water bath. Afterwards, the alkaline solution was acidified to pH 3 with HCl solution (1 mol/mL). Fatty acids were then extracted three times with 2 mL hexane. After the extraction procedure and saponification (Beyer and Jensen, 1989; Hamilton, 1992; Nishiyama-Naruke et al., 1998), the fatty acids were derivatized with 4-bromomethyl-7 coumarin (Abushufa et al., 1994) and the analysis performed in a liquid chromatographer (Shimadzu model LC-10A, Shimadzu, Kyoto, Japan). The samples were eluted using a C8 column (25 cm × 4.6 i.d., 5 μm of particles) with pre-column C8 (2.5 cm × 4.6 i.d., 5 μm of particles), 1 mL per minute of acetonitrile/water (77:23 by vol) flow and fluorescence detector (325 nm excitation and 395 emission) (Nishiyama-Naruke et al., 1998). The fatty acids were obtained from Sigma. Margaric (C17:0) acid was used to calculate recovery. The capacity factor (K'), elution sequence, linearity, recovery, precision, interference, and limit of detection were determined. The minimum limit of quantification of the fatty acids ranged from 1 to 10 ng. One curve of calibration for each standard, determining coefficients of correlation and regression, was obtained.

2.8. Statistical analysis

Results are expressed as means ± SEM. Statistical analyses were carried out using one-way ANOVA followed by Dunnett's test for multiple comparisons using the Prism analysis program (Graphpad, San Diego, CA, USA). The toxic values for 50% of cells were calculated directly from the graphics.

3. Results

The fatty acid composition of RINm5F cells was determined under control conditions. The toxicity of palmitic (PA), stearic (SA), oleic (OA), linoleic (LA), γ-linolenic (LNA), arachidonic (AA) and eicosapentaenoic (EPA) acids on this cell line was assessed after 24 h incubation by flow cytometry.

3.1. Determination of fatty acid composition by HPLC

The more abundant fatty acids present in RINm5F cells were the saturated palmitic (112 ± 7 ng/10^6 cells; 19%) and stearic (119 ± 15 ng/10^6 cells; 21%) acids. The value for oleic acid was lower (41 ± 3 ng/10^6 cells; 7%) and the polyunsaturated fatty acids represented 20% in total: linoleic (43 ± 6 ng/10^6 cells; 6%), γ-linolenic (16 ± 1 ng/10^6 cells; 3%), arachidonic (11 ± 0.5 ng/10^6 cells; 2%), eicosapentaenoic (28 ± 3 ng/10^6 cells; 5%) and docosahexaenoic (23 ± 3 ng/10^6 cells; 4%).

3.2. Cell membrane integrity

The polyunsaturated LNA and LA caused significant loss of RINm5F cell membrane integrity already at 0.1 mM and a nearly total loss at higher concentrations (Fig. 1B). The saturated FA SA, PA and the monounsaturated OA induced only slight changes in plasma membrane integrity at high concentrations (0.3 and 0.4 mM) (Fig. 1). AA and EPA, like the saturated FA, were only weakly toxic. The sequence of membrane toxicity was LNA = LA ≫ SA = PA = OA > AA = EPA.

3.3. DNA fragmentation

All FA, with the exception of AA, caused significant DNA fragmentation at 0.1 mM concentration or at higher

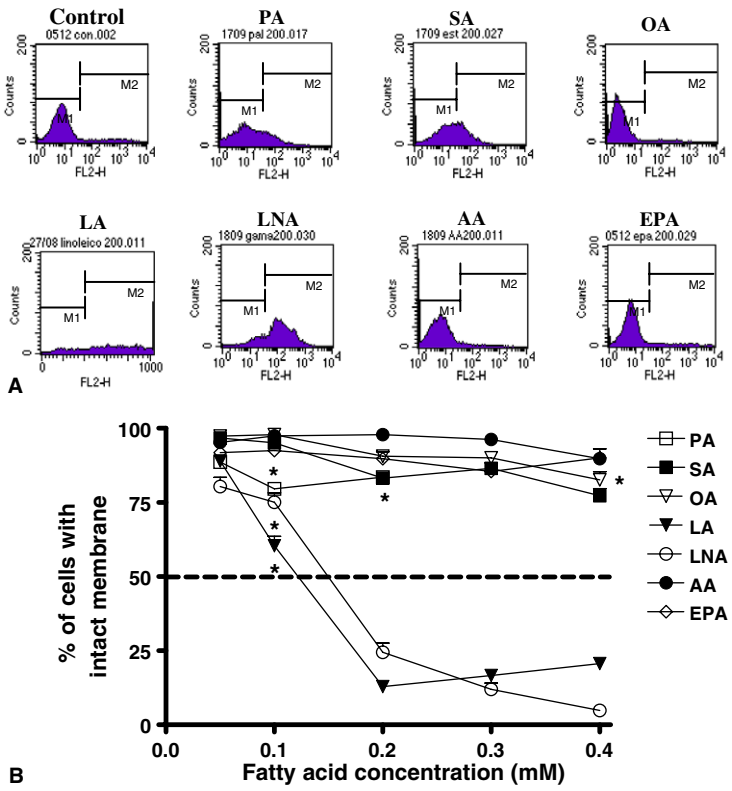

Fig. 1. Effect of fatty acids on membrane integrity in insulin-producing RINm5F cells. Cells were incubated for 24 h with fatty acids at various concentrations and analysed by flow cytometry. (A) Representative histograms of fluorescence of control and treated cells (0.2 mM fatty acids). The M1 area represents the low fluorescence of viable cells and M2 area the high fluorescence of cells with loss of membrane integrity cells. (B) The M1 values represent the means ± SEM of four different experiments carried out in triplicate. *$p < 0.05$ for the lowest concentrations of FA that caused a significant effect compared with untreated control cells (97%) (ANOVA plus Dunnett's test). The broken line indicates the estimated concentrations of FA at which 50% of the cells lost the plasma membrane integrity. The values are: 0.11 mM for LA and 0.15 mM for LNA. PA is insoluble in ethanol above 0.2 mM concentration. PA—palmitic acid, SA—stearic acid, LA—linoleic acid, LNA—γ-linolenic acid, AA—arachidonic acid, EPA—eicosapentaenoic acid.

concentrations (0.3–0.4 mM) in insulin-producing RINm5F cells (Fig. 2B). PA and LA proved to be the most toxic of the tested FA. EPA was the least toxic. The sequence of DNA toxicity was PA = LA > LNA = OA = SA > EPA ≫ AA.

3.4. Intracellular neutral lipids

FA differed also in their capacities to induce accumulation of neutral lipids (NLA) in insulin-producing RINm5F cells (Figs. 3 and 4). OA was the most effective in forming neutral lipid droplets, in a concentration-dependent manner, with maximal NLA observed at 0.2 mM concentration. Polyunsaturated FA with 18 carbons, LA and LNA, induced an intermediate lipid accumulation at low concentrations, and AA had an effect at high concentrations (0.3 and 0.4 mM) only. The saturated FA, PA and SA, like EPA, did not induce any significant lipid accumulation. The sequence of NLA accumulation was OA > LA = LNA > AA ≫ PA = SA = EPA.

4. Discussion

In the present study, the toxicity of different FA to insulin-producing RINm5F cells has been assessed by measurement of both increase in DNA fragmentation and loss of plasma membrane integrity. DNA fragmentation was induced by all tested FA (palmitic, linoleic, γ-linolenic, oleic, stearic end eicosapentaenoic), except by arachidonic. Loss of membrane integrity was mainly caused by the polyunsaturated linoleic and γ-linolenic acids. In general, the concentrations of the FA that induced DNA fragmentation were lower than those leading to loss of plasma membrane integrity. This is suggestive that FA caused death of insulin-producing RINm5F cells mainly by apoptosis. OA caused much less DNA fragmentation in insulin-producing RINm5F cells than PA, confirming observations by others in primary beta cells and insulin-producing cell lines (Cnop et al., 2001; Maedler et al., 2001). It is noteworthy that OA represents only 7% of the total FA in contrast to PA that represents 19%. The effect of OA on DNA fragmentation was observed in 50% of cells at 0.2 mM; this is in the range of physiological plasma concentrations (0.07–0.39 mM, Tietz, 1994). With respect to loss of plasma membrane integrity, the two FA did not cause a significant effect.

Addition of OA to culture medium leads to NLA being well tolerated by CHO (Chinese hamster ovary) cells, whereas an excess of PA is poorly incorporated into

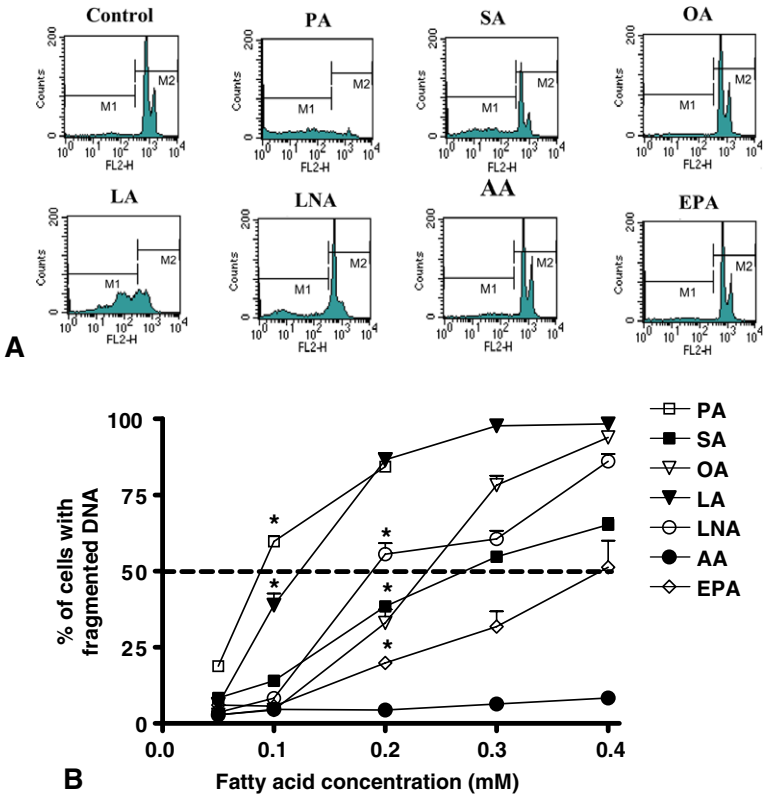

Fig. 2. Effect of fatty acids on DNA fragmentation in insulin-producing RINm5F cells. Cells were incubated for 24 h with fatty acids at various concentrations and analysed by flow cytometry. (A) Representative histograms of fluorescence of control and treated cells (0.2 mM fatty acids). The M1 area represents the low fluorescence of DNA fragments and M2 area the high fluorescence of intact DNA. (B) The M1 values represent the means ± SEM of four different experiments carried out in triplicate. *$p < 0.05$ for the lowest concentrations of FA that caused a significant effect compared to untreated cells (2%) (ANOVA plus Dunnett's test). The broken line indicates the estimated concentrations of FA at which 50% of the cells underwent DNA fragmentation. The values are: 0.08 mM for PA, 0.12 mM for LA, 0.19 mM for LNA, 0.24 mM for AO, 0.27 mM for SA and 0.4 mM for EPA. PA is insoluble in ethanol above 0.2 mM concentration. PA—palmitic acid, SA—stearic acid, LA—linoleic acid, LNA—γ-linolenic acid, AA—arachidonic acid, EPA—eicosapentaenoic acid.

triglycerides provoking apoptosis of these cells (Listenberger et al., 2003). Similar results were obtained in experiments carried out with rat pancreatic beta cells (Cnop et al., 2001; Maedler et al., 2001). In our study, PA and SA, saturated fatty acids, showed a potent toxic effect and both did not cause detectable triglyceride accumulation, in spite of its high abundance in the FA composition in whole cells, about 20% each. LA and LNA induced a marked loss of cell membrane integrity and DNA fragmentation and caused very low lipid accumulation. Therefore, the toxicity of PA, SA, LA and LNA to insulin-producing RINm5F cells is related to low lipid accumulation. These findings are in line with those obtained by Cnop et al. (2001) for rat beta cells. These authors also observed an inverse correlation between lipotoxicity and FA incorporation into triglycerides (Cnop et al., 2001). However, the cellular mechanisms that determine whether excess lipid accumulation is well tolerated or is cytotoxic remain largely unknown. In spite of the information above, evidence is presented herein that FA toxicity and NLA is not strictly inversely correlated and does not apply to all FA in the studied cell line. AA and EPA together comprise 7% of the total FA present in RINm5F cells. Neither AA nor EPA induced significant neutral lipid accumulation (NLA). However, these FA did

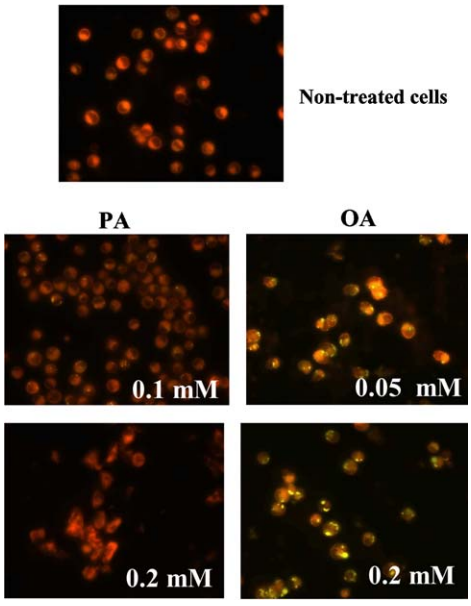

Fig. 3. Neutral lipid accumulation in cells treated with palmitic or oleic acids. Cells were treated as described in Section 2 and analysed by fluorescence microscopy using filters 530/570 mm. The triglyceride accumulation was observed as green light spots inside the cells. PA—palmitic acid, OA—oleic acid.

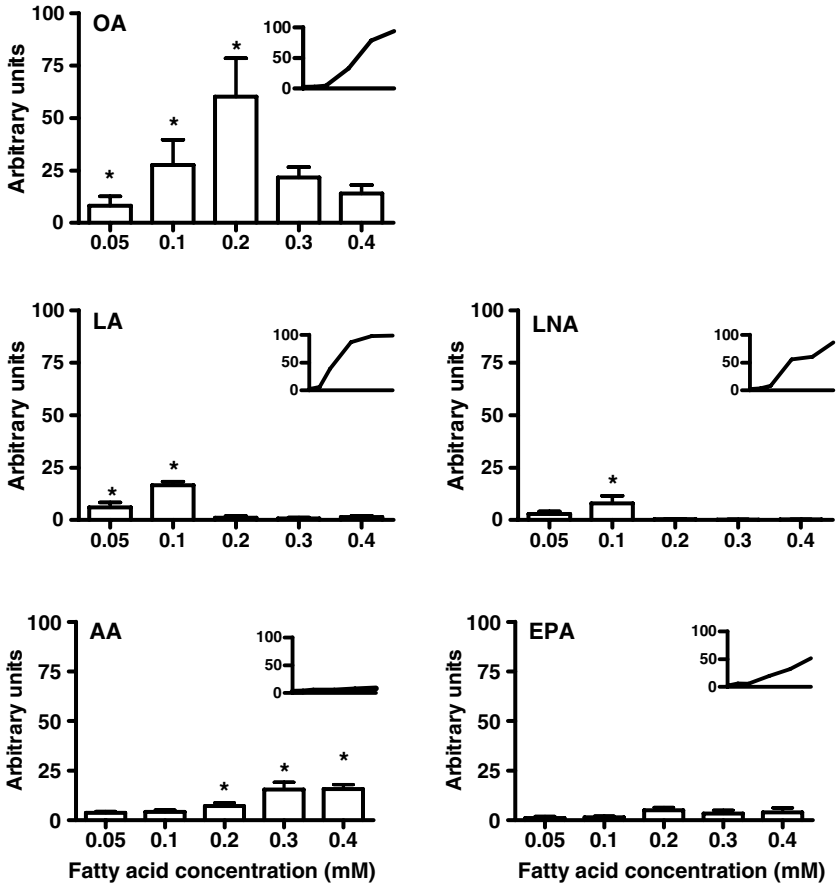

Fig. 4. Neutral lipid accumulation in cells treated with different fatty acids. Cells were treated with FA at various concentrations (0.05–0.4 mM) and analysed by flow cytometry. The bar graphics express arbitrary unities of neutral lipid accumulation as compared with non-treated cells, which were considered as 1. The values are presented as mean ± SEM of nine wells from three different experiments. $^{*}p < 0.05$ compared to untreated cells (ANOVA plus Dunnett's test). The graphics inserted on the top of the bar graphics show the toxicity curves (expressing the percentage of cells with fragmented DNA) of each fatty acid. OA—oleic acid, LA—linoleic acid, LNA—γ-linolenic acid, AA—arachidonic acid, EPA—eicosapentaenoic acid.

not disrupt the plasma membrane and AA did not cause an increase in the percentage of cells with fragmented DNA. Similarly, Healy et al. (2003) observed that polyunsaturated and monounsaturated fatty acids increased NLA but also caused apoptosis of differentiated neutrophil HL-60 cells (Healy et al., 2003). It is interesting to note that LA had a protective effect against the PA induction of membrane integrity loss in RINm5F cells (Beeharry et al., 2004). Monounsaturated FA also protected insulin-producing BRIN-BD11 cells against apoptosis induced by saturated FA (Welters et al., 2004).

In addition to be esterified into triglycerides, FA can also regulate the activity of metabolic pathways that lead to both neutral lipid synthesis and degradation. Unsaturated fatty acids serve as ligands for transcription factors such as the peroxisome proliferator-activated receptor γ (Kliewer et al., 1997), which is associated with the regulation of enzymes of triglyceride synthesis. The 24 h incubation with FA, as performed in our study, may allow this effect of the polyunsaturated FA to occur. It is possible that unsaturated FA activate signaling pathways that promote triglyceride storage or inhibit triglyceride hydrolysis. In an attempt to investigate this possibility, Listenberger et al. (2003) found that inhibitors of protein kinase C or phosphatidylinositol-3-kinase have no effect on unsaturated FA-induced triglyceride storage in CHO cells (Listenberger et al., 2003).

Increased triglyceride accumulation in CHO cells in response to the addition of OA to the incubation medium, also observed in the present study, may reflect a preference of some enzymes in the triglyceride synthetic pathway for unsaturated FA substrates (Listenberger et al., 2003) or increased stability of lipid droplets containing a higher percentage of certain unsaturated acyl chains. On the other hand, a recent study has shown that higher free fatty acid oxidation rates prevent accumulation of triglyceride in UCP-2 knockout mouse islets (Joseph et al., 2004). The authors postulated that UCP-2 and thus fatty acid oxidation activity plays a key role in the toxicity of FA in pancreatic beta cells. Although the toxicity of individual FA on insulin-producing RINm5F cells was clearly shown herein, the toxic effects of combinations of FA have not yet been investigated in detail.

Taken as whole, the toxic effect of the FA on insulin-producing RINm5F cells varied irrespective of the chain length and the degree of unsaturation. In these cells PA and

LA exhibited the highest toxicity, whereas AA was not toxic. There was no correlation between cytotoxicity and the abundance of the FA in the cells. In addition, the toxicity of most tested FA was inversely related to low NLA, except for AA and EPA. The results of this study contribute to the understanding of the role of FA in the impairment of pancreatic beta cell function that occurs in type 2 diabetes and obesity.

Acknowledgements

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