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THE NON-PEPTIDYL LOW MOLECULAR WEIGHT RADICAL SCAVENGER IAC PROTECTS HUMAN PANCREATIC ISLETS FROM LIPOTOXICITY

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Key words: human pancreatic islet, oxidative stress, antioxidants, FFA, lipotoxicity.

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Abstract

Background. Chronic exposure to high free fatty acids (FFA) can lead to irreversible damage of beta-cell accounting for impaired insulin secretion. Multiple mechanisms concur in generating the damage, but activation of oxidative stress may contribute to the final toxic effect. To better understand the phenomenon of lipotoxicity in human beta-cells, we evaluated the effects of 24-h pre-culture with 1.0 mmol/l FFA on the function, survival and mRNA expression of several enzymes involved in the generation and scavenging of reactive oxygen species (ROS). **Material and Methods.** Human islets, prepared by collagenase digestion and density gradient purification from 9 pancreases of multiorgan donors were incubated for 24-hrs in the presence 1.0 mmol/l long-chain mixture (oleate:palmitate, 2:1) FFA, with or without 100 μ mol/l IAC, a non-peptidyl low molecular weight radical scavenger. At the end of incubation period, insulin secretion was measured by static incubation, and mRNA expression of insulin, Cu/Zn-SOD, Mn-SOD, Catalase, Glutathione peroxidase (GSH-px) and HO-1 by quantitative Real-Time RT-PCR. Nitrotyrosine levels were determined by an ELISA technique. **Results.** As compared to control incubation (Ctrl, no FFA), exposure to FFA was associated with impaired insulin release and reduced insulin mRNA expression. The presence of IAC in the incubation medium increased insulin release significantly and prevented changes in mRNA expression. Exposure to FFA was associated with oxidative stress as indicated by a significant accumulation of nitrotyrosine and IAC restrained such an increase. mRNA expression of Cu/Zn-SOD, Mn-SOD, Catalase, GSH-Px, and HO-1 were all modified after FFA exposure. These changes were partially prevented in the presence of IAC. **Conclusions.** In human islets 24-hr exposure to high FFA causes oxidative stress associated with changes of several enzymes involved in ROS scavenging. These effects were prevented by the use of an antioxidant molecule.

Introduction

Under physiological conditions, free fatty acids (FFAs) sustain basal insulin secretion in the fasted state and potentiate hormone release in response to glucose (Randle, 1998). In contrast, prolonged exposure to increased FFA concentrations, as to occurs in diabetes and obesity, has detrimental effects on pancreatic beta-cells (Lupi et al., 2002), a phenomenon termed lipotoxicity. Together with other investigators, we have previously reported that prolonged exposure of pancreatic human islets to FFAs causes a dose-dependent inhibition of glucose-stimulated insulin release (Lupi et al., 2002). The toxic effect is exerted through activation of multiple mechanisms including accumulation of malonyl CoA and long-chain fatty acyl CoA (Maestre et al., 2003), increased fatty acid oxidation and esterification (Segall et al., 1999), accumulation of ceramide (Maestre et al., 2003) and fatty-acid induced apoptosis (Lupi et al., 2002).

Both hyperglycemia and FFAs can enhance formation of reactive oxygen species (ROS), causing cell protein and DNA damage. ROS generation by FFAs has been demonstrated in rodent pancreatic islets (Carlsson et al, 1999) and beta-cell lines (Wang et al., 2004; Oprescu et al., 2007). Moreover, animal studies have shown that oxidative stress can decrease beta-cell secretory function both in vitro and in vivo (Tang et al., 2007). The Beta-cell appears more vulnerable to oxidative stress due to very low activities of antioxidant enzymes, as indicated by low gene expression of superoxide dismutase, catalase, and glutathione peroxidase (Lenzen et al., 1996).

We have recently shown the efficacy of the new broad target cell penetrant antioxidant bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyloxy)decandioate di-hydrochloride (IAC) molecule on pancreatic human islets (Lupi et al., 2007). Twenty-four hours exposure of type 2 diabetic islets to IAC reduces oxidative stress and improves glucose-stimulated insulin secretion (Lupi et al., 2007). Because presently little information is available as for the direct action of FFA on oxidative stress on human pancreatic islets, in the present report we describe the effects of 24h exposure to FFA on islets prepared from non diabetes subjects, and the protective role of the antioxidant molecule IAC.

Materials and Methods

Islet preparation

For this study eight pancreases from non-diabetic multiorgan donors (age, 66 ± 13 years; gender, 5 males/4 females; body mass index, 25.7 ± 4.2 kg/m²) were used upon approval of the local Ethic Committee. Islets were prepared by collagenase digestion and density gradient purification, as previously reported (Lupi et al., 2002; Lenzen et al., 1996; Lupi et al., 2007). Three days after isolation, aliquots of approximately 500 hand-picked islets were free-floating cultured at 37 °C and 5% of CO₂ for 24h with or without 1.0 mmol/l long-chain fatty-acid mixture (oleate:palmitate, 2:1, 6% human albumin). Incubations were then repeated with or without 10 or 100 µmol/l bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidiny) decanedioate (IAC) (kindly provided by Medestea, Torino, Italy), added each 48h. The final pH of all media was buffered at pH 7.4.

Determination of oxidative stress

Oxidative stress in isolated islets was determined by measuring nitrotyrosine concentrations by an ELISA method as previously demonstrated (Lenzen et al., 1996). In 4 experiments we took advantage of IAC to measure oxidative stress also from nitroxide analysis by electron paramagnetic resonance (EPR) (Valgimigli et al., 2000; Valgimigli et al., 2001). This approach uses IAC as a probe since, after having accomplished its antioxidant role, IAC is transformed in the corresponding nitroxide that can be quantified by EPR. Therefore, the method allows simultaneous assessment of ROS production (by EPR) and use of IAC as an antioxidant agent (Lenzen et al., 1996; Valgimigli et al., 2000; Valgimigli et al., 2001).

Gene expression studies

Messenger RNA expression of Cu/Zn-SOD, Mn-SOD, catalase, glutathione peroxidase (GSH-px), HO-1, Bcl2, BAX, insulin, GLUT-2 and glucokinase (GK) were measured by quantitative Real-Time Reverse Transcription reaction (Real-Time RT-PCR) (Lenzen et al., 1996; Lupi et al., 2007). Total RNA was extracted from the islets by using the RNeasy Protect Mini Kit (QIAGEN) and quantified by absorbance at A₂₆₀/A₂₈₀ (ratio>1.65) nm in a Perkin-Elmer spectrophotometer. Its integrity was assessed after electrophoresis in 1.0% agarose gels by ethidium bromide staining. The primers and

probes sequences were obtained from PE Applied Biosystems (Pre-Developed TaqMan Assay Reagents Control Kits). The quantity of each cDNA sample was normalized to the housekeeping gene for cyclophilin A (n=8).

Assessment of Islets cell viability

A simultaneous double-staining procedure using fluoresceine diacetate (FDA) and propidium iodide (PI) for determination of cell viability in islet cell suspension was performed adapting the method of Jones KH et al. (Jones and Senft, 1985) to human pancreatic islets. Viable cells express bright green fluorescence, while non viable cells turn bright red. FDA (Inalco S.p.A., Milano - Italy) was prepared dissolving 1 mg powder in 1 ml of acetone and PI (Inalco S.p.A., Milano - Italy) was prepared dissolving 2 mg of powder in 1 ml of PBS. Briefly, aliquots of about 30 islets were washed twelve times in KREBS ringer bicarbonate and incubated for 30 min free-floating at 37 °C and 5% of CO₂ in 1 ml of M199 culture medium containing 4 µl of FDA and 2 µl of PI. At the end of the incubation period, islets were gently centrifuged, washed two times in PBS and re- suspended in 200 µl of MilliQ water. FDA and PI were immediately analyzed on fluorescence microplate reader with excitation respectively at 492 and 536-nm and emission respectively at 518 and 617-nm (n=8).

Insulin secretion study

Insulin secretion studies were performed by the batch incubation method, as previously detailed (Lupi et al., 2002; Lenzen et al., 1996; Lupi et al., 2007). Following a 45 min pre-incubation period at 3.3 mmol/l glucose, islets were kept at 37 °C for 45 min in Krebs-Ringer bicarbonate solution (KRB), 0.5% albumin, pH 7.4, containing 3.3 mmol/l glucose. At the end of this period, medium was completely removed and replaced with KRB containing either 3.3 or 16.7 mmol/l glucose. After additional 45 min incubation, media were removed. Samples (500 µl) from the different media were stored at -20 °C for determination of insulin concentrations by immunoradiometric assay technique (IRMA) (n=8).

Statistical analysis

Results are expressed as means $\pm$ SD. Comparison between two groups were performed by the two-tailed Student's t-test, whereas comparisons among multiple groups were done by ANOVA, followed by the Bonferroni correction to test differences between specific conditions.

Results

Oxidative stress evaluation

Incubation of human pancreatic islets in the presence of 1 mmol/l FFA was associated with increased of oxidative stress, as indicated by the EPR technique ($p < 0.001$) (Figure 1), and by accumulation of intracellular nitrotyrosine ($p < 0.05$) (Figure 2). Oxidative stress was paralleled by significantly reduction of Cu/Zn-SOD mRNA expression and by significant increase of catalase, GSH-px and HO-1 mRNA expression (Table 1).

As shown in Figure 2, 10 $\mu\text{mol/l}$ IAC was sufficient to reduce nitrotyrosine concentration by 32% ($p < 0.05$), while 100 $\mu\text{mol/l}$ IAC caused a 44% of reduction of the oxidative stress marker ($p < 0.05$). These changes were mirrored by consensual response in the mRNA expression of oxidant and antioxidant enzymes, as demonstrated in Table 1.

Islet function study

Oxidative stress induced by FFA was accompanied by marked changes in islet function, as indicated by impaired glucose-stimulated insulin release (Figure 3), and in a significant reduction of insulin, GLUT-2, and GK mRNA expression (Table 2). As shown in Figure 3 and Table 2, the adding of 10 and 100 $\mu\text{mol/l}$ IAC to culture media significantly increased the glucose-stimulated insulin secretion and gene expression.

Islets cell viability

The presence of FFA in culture medium resulted in significant reduction of cell viability (FFA: $21.99 \pm 17.18\%$. Ctrl: $77.42 \pm 15.53\%$), with 3.4 fold increase in the ratio between pro-apoptotic BAX/anti-apoptotic BCL2 gene expression (Table 3). Incubation of human islets with IAC preserved cell viability (IAC 10 $\mu\text{mol/l}$, $69.2 \pm 10.45\%$; IAC 100 $\mu\text{mol/l}$, $79.1 \pm 1.62\%$. both $p < 0.05$) and significantly reduced the BAX/BCL2 ratio (IAC 10 $\mu\text{mol/l}$, 0.91 ± 0.08 ; IAC 100 $\mu\text{mol/l}$, 0.57 ± 0.04 . both $p < 0.05$) (Table 3).

Conclusions

Lipotoxicity is a well known phenomenon accounting for impairment of beta-cell function (Ayvaz et al., 2002). It is believed to contribute to progressive decline of insulin secretion occurring in type 2 diabetic patients who commonly present chronic elevation of free-fatty acids (Poitout and Robertson, 2002). A large literature has illustrated the potential mechanisms responsible for toxic effect of FFA on the beta-cell (Poitout and Robertson, 2002; McGarry and Dobbins, 1999). Among these, oxidative stress may play a role. Our study, performed with human pancreatic islets support this view, because of direct evidence that exposure of islets to 1.0 mmol/l FFA is associated with 1. accumulation of nitrotyrosine, 2. consensual changes in the mRNA expression of oxidative stress-related enzymes, 3. impaired glucose-stimulated insulin release, and 4. lower cell viability along with BAX/BCI2 ratio. Finally, use of the novel antioxidant bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl) decanedioate (IAC) resulted in an almost complete protection from lipotoxicity.

These results support previous observations indicating oxidative stress as an effector of lipotoxicity. In agreement with some previous data of ours (Lupi et al., 2002), prolonged exposure to FFA caused cytostatic and pro-apoptotic effect. In that experience we reported that the increased amount of dead cell following FFA exposure was accounted for by increased apoptosis. Therefore, even if not directly tested here, we are confident that activation of apoptotic process is the main reason for poor cell survival. In support of this interpretation is the marked changes in the ratio between mRNA expression of pro-apoptotic protein BAX and the anti-apoptotic one, BCI2. FFA-induced apoptosis has been shown to be mediated by ceramide accumulation in beta-cell (Summers, 2006) but, rather than being an alternative mechanism, oxidative stress can be accelerated by ceramide as suggested by Shimabukuro et al. (Shimabukuro et al., 1998).

Previous work (Piro et al., 2002) showed that nicotinamide could prevent FFA-induced apoptosis but no direct assessment of oxidative stress was performed in that study. Interestingly, we have previously reported an anti lipotoxic effect of PPAR-gamma agonists (Lupi et al., 2004). Although not directly observed in that study, glitazones possess antioxidant properties (May and Qu, 2000). At present we provide direct assessment for that, by determination of nitrotyrosine and EPR measurement, showing that FFA can indeed result in ROS generation. Similar results have been obtained in rat islets incubated in the presence of palmitate (Lameloise et al., 2001). However, in these animals,

the effect of FFA-induced oxidative stress has been questioned since no effect on glucose-stimulated insulin secretion was observed upon 72h exposure of rat islets to FFA, ceramides, or ROS formation (Moore et al., 2004). Whether such a resistance may be explained by slight uncoupling electron transport observed under similar condition (Joseph et al., 2004) remains unclear.

In spite of apparent relationship between FFA and ROS generation, the mechanism by which oxidative stress is generated remains partially understood. In insulin secreting cells, FFA can inhibit respiratory chain (Koshkin et al., 2003), while ceramide has been shown to affect its efficiency (Schönfeld and Wojtczak, 2007). Moreover, FFA can activate NADPH-oxidase as indicated by increased expression of p47^{phox} (Morgan et al., 2007). Whatever the mechanism is, the effect of IAC was evident and marked. When, added to the culture medium together with FFA only minor activation of oxidative stress occurred as indicated by non significant changes of nitrotyrosine concentration and prevention of both cytostatic and pro-apoptotic effects. Gene expression and activity of the main antioxidant enzymes are much lower in pancreatic islets than in other tissues (Tiedge et al., 1998; Lenzen et al., 1996). We, therefore, have directly assessed responses of mRNA expression of Cu/Zn-SOD, catalase, Mn-SOD, GSH-px and HO-1. In response to FFA exposure, SODs mRNA expression decreased while catalase, GSH-px and HO-1 increased. All these changes were, at least partially, reverted by IAC, again emphasizing a direct relationship between FFA availability, formation of oxidative stress, and cytostatic and pro-apoptotic effects.

Our results may also have some practical implications. Type 2 diabetic patients, particularly those with abdominal obesity, in addition to hyperglycemia, are characterized by increased FFA levels (Golay et al., 1990). Therefore, the use of antidiabetic agents that may reduce plasma FFA levels while exerting an antioxidant effect may have some potential in preserving beta-cell function.

In conclusion, reducing oxidative stress in islet cell exposed to FFA seems to be an effective method to improve beta-cell function and survival, suggesting that the antioxidant treatment may protect human islet beta-cell against injuries of lipotoxicity.

Legend to Figures

Figure 1. Increased oxidative stress in FFA islets compared to Ctrl islets, evaluated by EPR radical-probe technique (* $p < 0.001$)

Figure 2. Nitrotyrosine level in Ctrl compared to FFA islets, with or without addition of IAC. The marker of oxidative stress level is significantly higher in FFA respect to Ctrl islets. IAC was able to significantly reduce the nitrotyrosine concentration respect to FFA alone (*= $p < 0.05$ vs Ctrl by Bonferroni t-test correction; #= $p < 0.05$ vs FFA alone by Bonferroni t-test correction).

Figure 3. Stimulation Index (ratio between 16.7 mmol/l and 3.3 mmol/l glucose-stimulated insulin release) in Ctrl islets, FFA islets and FFA islets exposed or not to 10 and 100 $\mu\text{mol/l}$ IAC.

Table 1. Cu/Zn-SOD, Catalase, Mn-SOD, GSH-px HO-1 mRNA expression in Ctrl islets and FFA islets before and after exposure to 10 or 100 $\mu\text{mol/l}$ IAC.

	Cu/Zn-SOD	Catalase	Mn-SOD	GSH-px	HO-1
Ctrl	1	1	1	1	1
FFA 1.0 mmol/l	0.510 $\pm$ 0.134*	2.703 $\pm$ 0.971*	0.361 $\pm$ 0.107	1.841 $\pm$ 0.416*	2.242 $\pm$ 0.701*
FFA+IAC 10 $\mu\text{mol/l}$	0.619 $\pm$ 0.124*	1.590 $\pm$ 0.223	1.875 $\pm$ 0.592*	1.521 $\pm$ 0.286*	1.309 $\pm$ 0.073
FFA+IAC 100 $\mu\text{mol/l}$	0.623 $\pm$ 0.222*	1.264 $\pm$ 0.185	1.828 $\pm$ 0.941*	1.339 $\pm$ 0.068	1.159 $\pm$ 0.352

Measurements were made by real-time RT-PCR, and normalized for values in ctrl.

*= $p < 0.05$ vs Ctrl by Bonferroni t-test correction

Table 3. Pro-apoptotic BAX and anti-apoptotic Bcl2 mRNA expression in Ctrl islets and FFA islets before and after exposure to 10 or 100 $\mu\text{mol/l}$ IAC.

	BAX	Bcl2	BAX/Bcl2
Ctrl	1	1	1
FFA 1.0 mmol/l	1.55 $\pm$ 0.07*	0.45 $\pm$ 0.06*	3.44 $\pm$ 0.80*
FFA+IAC 10 $\mu\text{mol/l}$	1.0 $\pm$ 0.14 [#]	1.10 $\pm$ 0.28 [#]	0.91 $\pm$ 0.08 [#]
FFA+IAC 100 $\mu\text{mol/l}$	0.79 $\pm$ 0.01* [#]	1.39 $\pm$ 0.04* [#]	0.57 $\pm$ 0.04 [#]

Measurements were made by real-time RT-PCR, and normalized for values in ctrl.

*= $p < 0.05$ vs Ctrl by Bonferroni t-test correction

[#]= $p < 0.05$ vs FFA alone by Bonferroni t-test correction

Table 2. Insulin, GLUT-2 and Glucokinase (GK) mRNA expression in Ctrl islets and FFA islets before and after exposure to 10 or 100 $\mu\text{mol/l}$ IAC.

	Insulin	GLUT2	GK
Ctrl	1	1	1
FFA 1.0 mmol/l	0.47 $\pm$ 0.05*	0.40 $\pm$ 0.14*	0.56 $\pm$ 0.06*
FFA+IAC 10 $\mu\text{mol/l}$	1.0 $\pm$ 0.14 [#]	0.65 $\pm$ 0.06* [#]	1.40 $\pm$ 0.28* [#]
FFA+IAC 100 $\mu\text{mol/l}$	1.15 $\pm$ 0.07* [#]	0.95 $\pm$ 0.07 [#]	1.35 $\pm$ 0.07* [#]

Measurements were made by real-time RT-PCR, and normalized for values in ctrl.

*= $p < 0.05$ vs Ctrl by Bonferroni t-test correction

#= $p < 0.05$ vs FFA alone by Bonferroni t-test correction

Figure 1

Figure 2

Figure 3

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