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Research Article

Rosiglitazone counteracts palmitate-induced β-cell dysfunction by suppression of MAP kinase, inducible nitric oxide synthase and caspase 3 activities

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Abstract. Chronic exposure of pancreatic islets to elevated levels of palmitate leads to β-cell dysfunction. We examined possible involvement of mitogen-activated protein kinases (MAPKs) and caspase-3 in palmitate-induced β-cell dysfunction and tested the influence of the anti-diabetic drug rosiglitazone (ROZ). Palmitate amplified glucose-stimulated augmentation of intracellular free calcium ($[Ca^{2+}]_i$) and insulin secretion in incubated islets. ROZ suppressed this amplification, whereas it modestly augmented glucose-induced increase in these events. ROZ suppressed short-term palmitate-induced phosphorylation of pro-apoptotic MAPKs, *i.e.*, SAPK/JNK and p38. Long-term islet culturing with palmitate induced inducible nitric oxide synthase (iNOS) and activated SAPK/JNK-p38. ROZ counteracted these effects. Both palmitate and cytokines activated caspase-3 in MIN6c4-cells and isolated islets. ROZ suppressed palmitate- but not cytokine-induced caspase-3 activation. Finally, after palmitate culturing, ROZ reversed the inhibitory effect on glucose-stimulated insulin release. We suggest that ROZ counteracts palmitate-induced deleterious effects on β-cell function *via* suppression of iNOS, pro-apoptotic MAPKs and caspase-3 activities, as evidenced by restoration of glucose-stimulated insulin release.

Keywords. Pancreatic islets, free fatty acids, insulin secretion, β-cell dysfunction.

Introduction

Pancreatic β-cells respond to free fatty acids (FFA) by integration of distinct and partially conflicting signals. Medium to long-chain FFA such as palmitate and oleate are known to have pleiotropic effects on the β-cell function [1, 2]. In contrast to the acute stimulatory action of palmitate on insulin release [2], long-term exposure of the endocrine pancreas to palmitate results in an adverse effect on β-cell function and survival [3, 4]. Previous studies have clearly demonstrated that an increase in intracellular generation of nitric oxide (NO) in the β-cell contributes to the development of several pathophysiological conditions [5–10]. It has been reported that inducible NO synthase (iNOS) in pancreatic islets is preferentially induced by macrophage-derived inflammatory agents such as various cytokines (IL-1β, TNF-α and IFN-γ). These agents are known to play a potential role in β-

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cell dysfunction and apoptosis in type 1 diabetes [9, 11–13]. Notably, we have presented evidence that also a noninflammatory and rather a metabolic induction of iNOS in the β-cell itself by elevated levels of glucose and/or FFA might be operating in otherwise healthy animals [4, 7, 8, 14–16]. Thus, we have found that an exaggerated NO production derived from islet iNOS is induced by the elevation of plasma FFA during long-term infusion of intralipids into healthy rats, and that this overproduction of NO might be implicated in the pathogenesis of β-cell dysfunction [4, 7, 8, 16]. These observations thus speak in favor of the idea that some signal(s) or metabolite(s) derived from FFA action could be involved in a noninflammatory induction of islet iNOS. It has previously been reported that hyperlipidemic patients display a markedly impaired insulin response to glucose and that hyperlipidemia is a prominent characteristic of type 2 diabetes [17]. However, the mechanisms by which FFA induce β-cell dysfunction and injury are not fully understood.

Recent studies have shown that two members of the mitogen-activated protein kinase (MAPK) superfamily, SAPK/JNK and p38, play an important role in cell dysfunction and apoptosis, whereas another member, p42/44, has an opposite effect and promotes cell proliferation, differentiation, and survival induced by a variety of cytokines or growth stimuli [18, 19]. Moreover, it has also been shown that various proinflammatory cytokines and many toxic compounds and chemotherapeutic agents might stimulate the activities of SAPK/JNK or p38, leading to cell death. In these processes caspase-3, a member of aspartate-specific cysteine proteases, is a well-known downstream executor [19–21]. The caspase families of enzymes are present within almost all cells as inactive forms and require a proteolytic process for activation [22] and thus their activation should be tightly controlled within the cell to avoid serious and deleterious consequences. Moreover, during recent years, a tight correlation between the activities of different caspases and β-cell dysfunction upon the presence of cytokines has been established [20, 21]. To our knowledge, the effects of palmitate on different MAPKs and their putative role in FFA-induced β-cell dysfunction have not been studied previously. Since it has been shown that PPARγ agonists such as the thiazolidinedione rosiglitazone (ROZ) might prevent the lipotoxic effect of FFA on the β-cells [23], we decided also to explore in more detail the influence of this drug on palmitate-induced β-cell signaling. In some initial experiments we studied short-term interactions between palmitate and ROZ by measuring intracellular Ca^{2+} ($[Ca^{2+}]_i$) and the activities of the MAPKs. We then examined long-time interactions between palmitate *versus* ROZ on the activities of MAPKs, iNOS and caspase-3 after 24-h culturing of islets or MIN6c4 cells. Finally, we tested the outcome of glucose- or glucose + palmitate-stimulated insulin release following the different culture conditions.

Materials and methods

Female mice of the NMRI strain (B & K, Sollentuna, Sweden) weighing 28–32 g were used throughout the experiments. They were given a standard pellet diet (B & K) and tap water *ad libitum*. All animals were housed in metabolic cages with constant temperature (22 °C) and 12-h light/dark cycles. The local animal welfare committee, Lund, Sweden, approved the experimental protocols.

Drugs and chemicals

Collagenase (CLS 4) was from Sigma (St. Louis, MO, USA). Fatty acid-free bovine serum albumin (BSA) was from Boehringer (Mannheim, Germany). The insulin radioimmunoassay kit was from Diagnostika (Falkenberg, Sweden). ROZ was kindly provided by GlaxoSmithKline (UK). All other chemicals were from Merck (Darmstadt, Germany) or Sigma. Palmitate was dissolved in ethanol (95 %). Before the experiments the stock solution was dissolved in the appropriate culture or incubation medium to achieve the desired concentrations of palmitate. According to the stepwise equilibrium method [24], the real concentration of palmitate in the presence of 1 % of fatty acid-free albumin is thus less than the given value in the figures. Due to a high binding capacity of BSA against FFA, the highest concentration of palmitate (1.0 mmol/l) gives rise to an approximate concentration of free palmitate of about 100 μmol/l [25].

Isolation of pancreatic islets

Preparation of mouse pancreatic islets was performed by retrograde injection of a collagenase solution *via* the bile-pancreatic duct [26]. Islets were then isolated and handpicked under a stereomicroscope at room temperature. The isolated islets were then subjected to different experimental procedures (see below).

Fluorescent measurements of $[Ca^{2+}]_i$ concentrations

As previously described [27], $[Ca^{2+}]_i$ in the intact islets was measured using a dual-wavelength microfluorimetry with fura-2 as indicator dye. Briefly, after an overnight culture period, the islets were loaded with 3 μmol/l fura-2 (30 min) and then transferred to a 15 °C experimental chamber and were kept in place by a heat-polished glass pipette. The thermostatically controlled chamber was continuously perfused with a

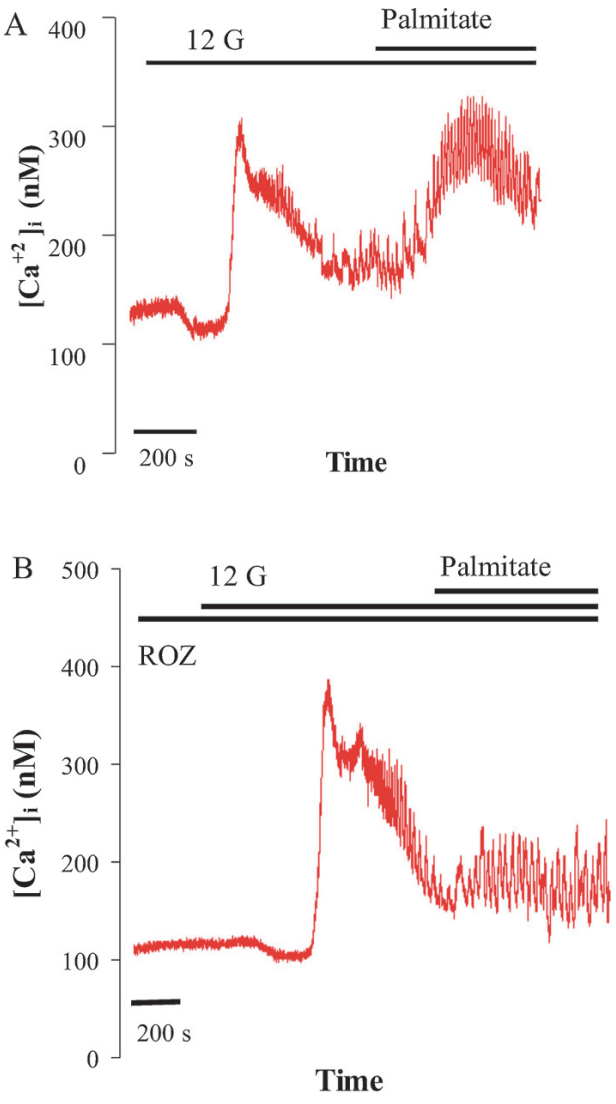

Figure 1. (*A*) Representative graph of glucose- and palmitate-stimulated increase in $[Ca^{2+}]_i$ in isolated intact islets. When glucose concentration was changed from 1 to 12 mmol/l a slight initial dip, followed by a prompt and rapid increase, in $[Ca^{2+}]_i$ was observed, which declined to a basal level a little higher than that of 1 mmol/l glucose. Addition of 1 mmol/l palmitate brought about a marked increase in $[Ca^{2+}]_i$ that, although gradually declining, was maintained at higher levels than with glucose alone. (*B*) The effects of glucose and palmitate on $[Ca^{2+}]_i$ when 1 µmol/l rosiglitazone (ROZ) was present during the experiments is shown. ROZ modestly amplified glucose-stimulated increase in $[Ca^{2+}]_i$, whereas it markedly suppressed the palmitate-stimulated amplification of $[Ca^{2+}]_i$.

buffer solution containing: 140 mmol/l NaCl, 3.6 mmol/l KCl, 2 mmol/l $NaHCO_3$, 0.5 mmol/l NaH_2PO_4, 0.5 mmol/l $MgSO_4$, 5 mmol/l HEPES, 2.6 mmol/l $CaCl_2$ and the test substances, *e.g.*, glucose and/or palmitate as indicated (pH was adjusted to 7.4).

Fluorescence signals were recorded using a microfluorimeter system (D104, PTI, Monmouth Junction, NJ, USA) with an emission wavelength of 510 nm at alternate 350/380 nm excitation wavelength and calibrated into $[Ca^{2+}]_i$ values using the equation previously described [27]. The fluorescence (F) ratio F_{350}/F_{380} was determined at a final ratio frequency of 10 Hz. $[Ca^{2+}]_i$ was then estimated using the equation given in [27] and a K_d of 224 nM. The maximum ratio (R_{max}) was achieved using 60 µmol/l ionomycin. Background subtraction was performed after quenching the fluorescence signal with 1 mmol/l $MnCl_2$. To work up the raw data in the detected signals we used the IGOR program.

Measurement of SAPK/JNK, p38 and p42/44 activities

Isolated islets (250 per vial) were incubated for 30 min in the presence of different test agents. After incubation, the islets were thoroughly washed and collected in ice-cold SDS buffer (200 µl) and stored at –20 °C for subsequent MAPK phosphorylation analysis. SAPK/JNK and p38 activation as well as p42/44 was determined using a phosphorylated SAPK/JNK, p38 and p42/44 assay kit including Western blots (Cell Signaling Technology, Inc, TX, USA) according to the manufacturer's instructions. Results were expressed as relative increase over GADPH in each tested group. In a parallel and similar experiment the islets were cultured for 24 h in the presence of the same agents prior to assay of SAPK/JNK, p38 and p42/44.

Measurement of iNOS activity

Isolated islets were either incubated for 2 h or cultured for 24 h. Thereafter, the islets (250 per vial) were thoroughly washed and collected in ice-cold DTT buffer (200 µl) and stored at –20 °C for subsequent analysis of iNOS activity. In brief, after sonication on ice, the buffer solution containing the islet homogenate was supplemented to contain 2.0 mmol/l NADPH and 0.2 mmol/l L-arginine in a total volume of 450 µl. The homogenate was then incubated at 37 °C under constant air bubbling (1.0 ml/min) for 3 h. Aliquots of the incubated medium (200 µl) were mixed with an equal volume of *o*-phthaldialdehyde reagent solution in a glass vial and then passed through an 1-ml Amprep CBA cation-exchange column for high-performance liquid chromatography (HPLC) analysis. The amount of L-citrulline formed (NO and L-citrulline are produced in equimolar concentrations) was then measured in a Hitachi F1000 fluorescence spectrophotometer (Merck) as previously described [15].

Protein

Protein was determined according to Bradford [28].

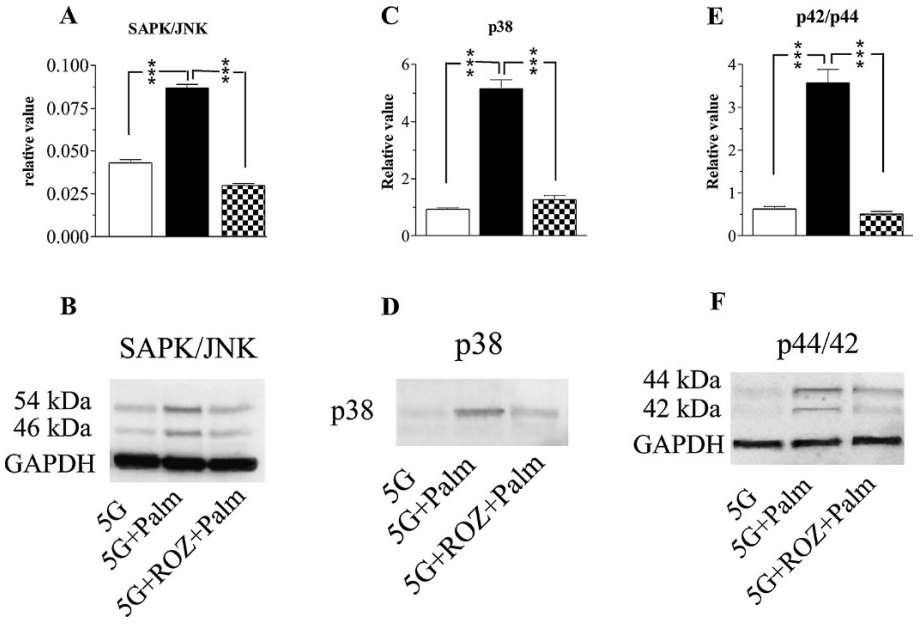

Figure 2. Suppressive effect of ROZ on short-term palmitate-induced activation of SAPK/JNK (*A*), p38 (C) and p42/44 (*E*). Pancreatic islets were preincubated with 5 mmol/l glucose (5G) alone or in combination with 1 μmol/l ROZ for 30 min, after which the medium was changed to an incubation medium containing either 5G, 5G + 1 mmol/l palmitate, or 5G + ROZ + palmitate and incubated for 30 min. After incubation, the islets were subjected to determination of SAPK/JNK, p38 and p42/44. The means ± SEM for five experiments in each group are shown. Asterisks (*) denote probability level of random difference; *** $p < 0.001$. Data for Western blots (*B*, *D* and *F*) are representative for five independent experiments in each group.

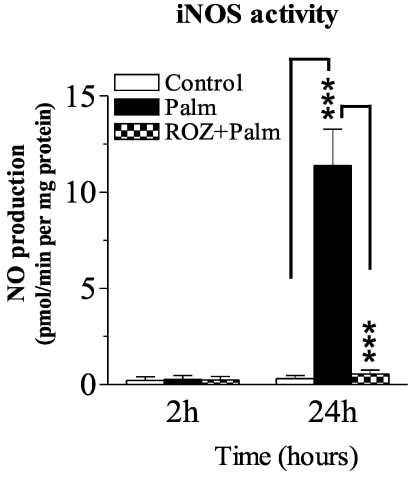

Figure 3. Inducible NO synthase (iNOS) activity measured as L-citrulline formation (pmol/min per mg protein) in isolated islets incubated for 2 h or cultured for 24 h at 5 mmol/l glucose (5G), 5G + 1 mmol/l palmitate, or 5G + 1 μmol/l ROZ + palmitate. The means ± SEM for five experiments in each group are shown. Asterisks (*) denote probability level of random difference; *** $p < 0.001$

Measurement of caspase-3 activity

The activity of caspase-3 was determined by monitoring the cleavage of a specific fluorogenic caspase-3 substrate Ac-DEVD-AMC (ac-Asp-Glu-Val-Asp-AMC; Upstate cell signaling solutions, NY, USA). The MIN6c4 cells were grown in monolayer culture with 10 % normal calf serum to 95 % confluence. The cells were thereafter washed with PBS prior to 24-h culture in culture medium containing either a basal glucose concentration of 5 mmol/l (5G), or 5G + palmitate (1 mmol/l) in the absence or presence of 1 μmol/l ROZ. After culturing, the cells were washed with PBS and then lysed with lysis buffer. Thereafter, the homogenates were allowed to react with the fluorogenic caspase-3 substrate in a 96-well plate (3 μl to each well) in a reaction buffer containing 20 mmol/l HEPES, 10 % glycerol and 2 mmol/l DTT. The mixtures were maintained at 37 °C for 60 min (darkness) and subsequently analyzed in a fluorometer (FLUO Star) equipped with excitation wavelength of 390 nm and emission wavelength of 460 nm. The results were correlated to the protein concentration of each well measured according to Bradford [28]. The effect of palmitate on the caspase-3 activity was compared to the effect induced by three cytokines (30 ng/ml IL-1β, 150 ng/ml TNF-α and 150 ng/ml IFN-γ). The same procedure was also performed with isolated islets.

Insulin secretion from MIN6c4 cells and isolated islets

Experiments with MIN6c4 cells. MIN6c4 cells were cultured in 48-well plates and, after reaching confluency, they were cultured for 24 h in the presence of different test agents as indicated in the figure legends. Thereafter the cells were washed and preincubated for 30 min at 37 °C in Krebs-Ringer bicarbonate buffer (pH 7.4), supplemented with 10 mmol/l HEPES, 1.0 % BSA and 1.0 mmol/l glucose. After preincubation the buffer was changed and the MIN6c4 cells were incubated at 1 or 12 mmol/l glucose ± 1 mmol/l palmitate for 60 min at 37 °C, to determine insulin secretion. Special care was taken to ensure that no cells were aspirated along with the collection of medium.

Experiments with isolated islets. Isolated islets were either used immediately after isolation and preincu-

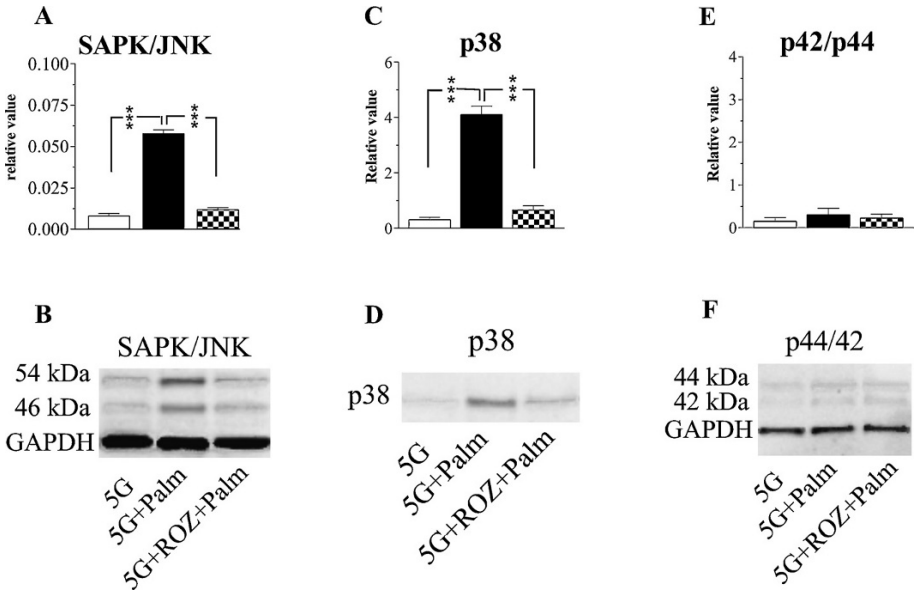

Figure 4. Suppressive effect of ROZ on palmitate-induced activation of SAPK/JNK (*A*), and p38 (*C*). p44/42 (*E*) was not affected by 24-h culturing with palmitate. Pancreatic islets were cultured for 24 h at 5 mmol/l glucose (5G), 5G + 1 mmol/l palmitate and 5G + 1 µmol/l ROZ + palmitate. Subsequently, the islets were thoroughly washed and subjected to determination of SAPK/JNK, p38 and p42/44. The means ± SEM for six experiments in each group are shown. Asterisks (*) denote probability level of random difference; *** $p<0.001$. Data for Western (*B*, *D* and *F*) blots are representative for six independent experiments in each group.

bated (30 min) followed by incubation (30 min) or cultured for 24 h in the presence of different test agents as indicated in the figure legends. Thereafter, the cultured islets were washed and preincubated for 30 min at 37 °C in Krebs-Ringer bicarbonate buffer (as above) and 1.0 mmol/l glucose. After preincubation, the buffer was changed and the islets were incubated at 1 or 12 mmol/l glucose ± 1 mmol/l palmitate for 60 min at 37 °C. Each incubation vial contained 12 islets in 1.0 ml buffer solution and was gassed with 95 % O_2/5 % CO_2 to obtain constant pH and oxygenation. All incubations were performed in an incubation box at 30 cycles/min. An aliquot of the medium was removed immediately after incubation and frozen for the subsequent assay of insulin. The concentration of palmitate was chosen from ancillary dose-response studies in our laboratory showing a maximal effect at 1 mmol/l of this FFA, enabling us to safely detect significant changes of the measured parameters. A similar dose-response curve for another FFA, linoleic acid, was reported previously [2].

Statistics

The results are expressed as means ± SEM for the indicated number of observations or illustrated by an observation representative of a result obtained from different experiments. Probability levels of random differences were determined by analysis of variance followed by Tukey-Kramers' multiple comparisons test.

Results

Effect of ROZ on the increase in $[Ca^{2+}]_i$ and insulin secretion stimulated by glucose and palmitate

First, we wished to study the short-term influence of ROZ on palmitate-stimulated $[Ca^{2+}]_i$ and insulin release from isolated islets. As shown in Figure 1, when the islet was exposed to 12 mmol/l glucose, the prominent increase in $[Ca^{2+}]_i$ (both the initial peak and the following plateau of oscillations) was amplified when ROZ was present in the perfusion buffer. Quantification of repeated experiments showed that the Δ average $[Ca^{2+}]_i$, based on the calculation of Δ average time, was 112 ± 10 nmol/l for glucose alone *versus* 155 ± 15 nmol/l ($p<0.05$; $n=6$) when ROZ was included. As illustrated in Figure 1A, addition of 1 mmol/l palmitate to an islet already challenged with 12 mmol/l glucose resulted in a sustained increase in $[Ca^{2+}]_i$. Quantification showed that the Δ average $[Ca^{2+}]_i$, was 110 ± 10 nmol/l for glucose alone *versus* 185 ± 19 nmol/l ($p<0.001$; $n=6$) when palmitate was included. This effect of palmitate was markedly suppressed by ROZ, which was also confirmed by quantification of Δ average time calculations (194 ± 20 nmol/l for palmitate alone *versus* 116 ± 15 nmol/l ($p<0.01$; $n=6$) when ROZ was present (Fig. 1A and B). The action of ROZ to suppress the palmitate-stimulated elevation of $[Ca^{2+}]_i$ had its counterpart in a marked suppression of palmitate-induced insulin release. Glucose-stimulated insulin release, however, was amplified by ROZ. Thus, short-time insulin release (after 30-min incubation) at 12 mmol/l glucose was 0.36 ± 0.03 ng/islet in the absence and 0.51 ± 0.02 ng/islet in the presence of ROZ ($p<0.05$; $n=8$). In the presence of 12 mmol/l glucose,

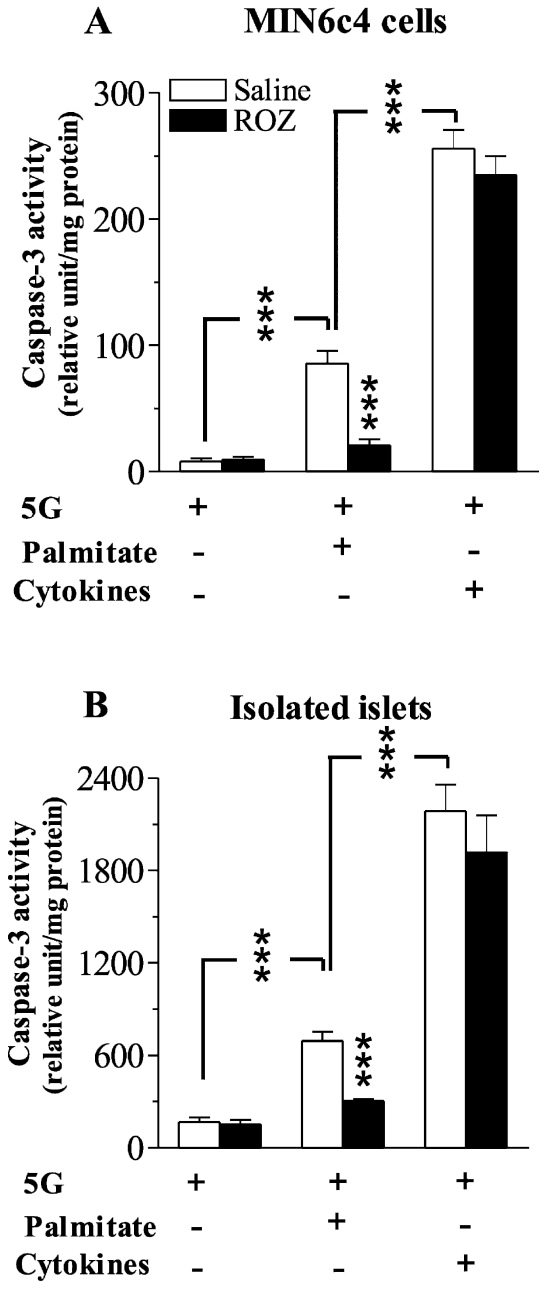

Figure 5. Caspase-3 activity in MIN6c4 cells (*A*) and isolated islets (*B*) cultured for 24 h. Effect of 1 mmol/l palmitate in absence or presence of 1 μmol/l ROZ at basal glucose concentration (5 mmol/l). For comparison, the effect of a triple-cytokine treatment (30 ng/ml IL-1β, 150 ng/ml TNF-α and 150 ng/ml IFN-γ), on caspase-3 activity is also shown. Caspase-3 activity in the cell or islet lysate was determined using caspase-3 assay kit. After the culture period the cells and the islets (175 islets/tube) were washed and lysed with lysis buffer on ice, and centrifuged at 2000 *g* at room temperature for 5 min. Supernatants were transferred to 96-well plates, and caspase-3 substrate (Ac-DEVD-AMC) and assay buffer were added. The plates were incubated at 37 °C and measured at excitation wavelength of 390 nm and emission wavelength of 460 nm. The values show caspase-3 activity–control blank per mg protein for each sample. Data are presented as means ± SEM. ($n = 8$–12). ***$p < 0.001$.

palmitate-stimulated insulin release 0.77±0.04, was markedly suppressed by ROZ to 0.54±0.02 ($p < 0.001$; $n = 8$ in each group).

Effect of palmitate on islet MAPK activities in the absence and presence of ROZ

We next investigated whether palmitate in short-time experiments (30 min) could influence the phosphorylation of SAPK/JNK, p38 and p42/p44 in isolated islets and to what extent this effect could be modulated by ROZ. Addition of 1 mmol/l palmitate to the incubation media (at a basal glucose concentration of 5 mmol/l) resulted in an increased activation of SAPK/JNK (Fig. 2A), p38 (Fig. 2C) and p42/p44 (Fig. 2E) in the islets. This effect was markedly suppressed by 1 μmol/l ROZ as also illustrated by Western blots (Fig. 2B, D and F).

Influence of ROZ on palmitate-induced islet iNOS activity

As seen in Figure 3, no iNOS activity could be detected during a 2-h incubation of islets with 1 mmol/l palmitate. However, when islets were cultured for 24 h with 1 mmol/l palmitate, a strong upregulation of iNOS activity was detected (Fig. 3). The palmitate-induced iNOS activity was markedly suppressed by ROZ (1 μmol/l) (Fig. 3).

Long-term interactions of palmitate and ROZ on islet MAPK activities

The long-term (24 h) effect of palmitate in the absence or presence of ROZ on the phosphorylation of SAPK/JNK, p38 and p42/p44 in the isolated islets was also investigated. The increased activities of SAPK/JNK (Fig. 4A) and p38 (Fig. 4C) were still evident after long-term culturing with 1 mmol/l palmitate, and also strongly suppressed by 1 μmol/l ROZ. The stimulatory effect of palmitate on p42/p44 (Fig. 4D) was, however, not observed after long-term culturing of islets with palmitate (Fig. 4). Associated Western blots are illustrated in Figure 4B, D and F.

Long-term effects of palmitate *versus* cytokines on caspase-3 activity in MIN6c4 cells and isolated islets in the absence or presence of ROZ

We used MIN6c4 insulinoma cells and isolated islets cultured for 24 h to study the effect of palmitate in the absence and presence of ROZ on caspase-3 activity. The ability of palmitate to stimulate caspase-3 activity was also compared to that of a mixture of cytokines (IL-1β, TNF-α and IFN-γ), which is known to induce caspase-3 activation [29]. As shown in Figure 5A and B, palmitate was capable of inducing a marked increase in caspase-3 activity in both MIN6c4 cells (Fig. 5A) and isolated islets (Fig. 5B), although this

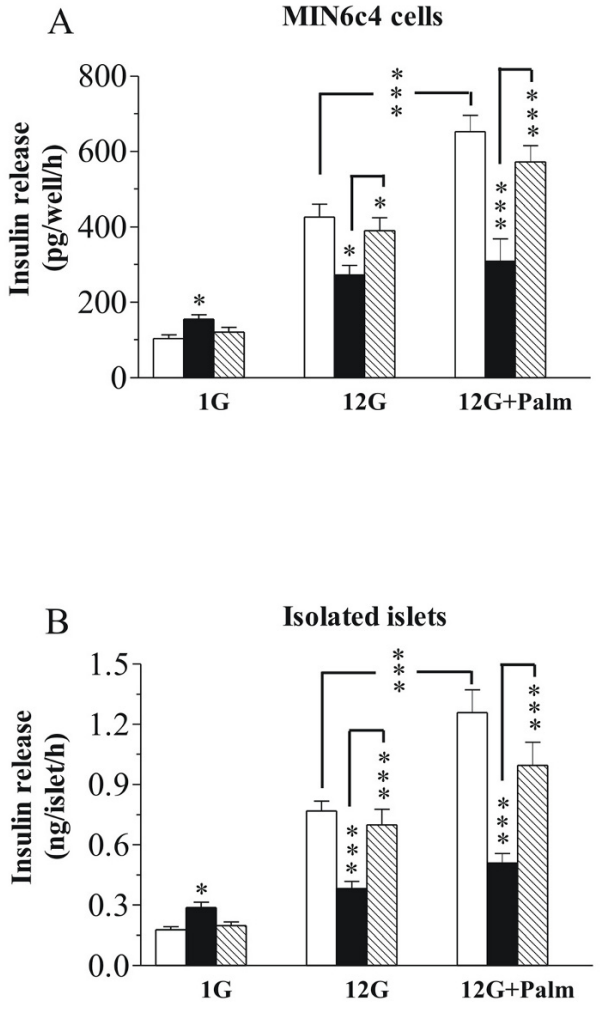

Figure 6. Effects of glucose (12 mmol/l) and palmitate (1 mmol/l) on insulin secretion from MIN6c4 cells (*A*) or isolated islets (*B*) that have been exposed to 1 mmol/l palmitate (black columns) or palmitate + 1 μmol/l ROZ (hatched columns) at 5 mmol/l glucose for 24 h. Basal controls (5 mmol/l glucose) are denoted by open columns. After culture, MIN6c4 cells and islets were washed and preincubated at 1 mmol/l glucose for 30 min followed by incubation for 60 min at 1 mmol/l glucose (1G) or 12 mmol/l glucose (12G) or glucose + palmitate (1 mmol/l). The values are means ± SEM for ten observations in each group for MIN6c4 cells (*A*) and eight observations in each group for isolated islets (*B*). Asterisks (*) denote probability level of random difference *versus* controls; * $p < 0.01$, *** $p < 0.001$.

effect was less pronounced compared to that of the cytokine mixture (Fig. 5). The effect of palmitate was markedly suppressed by 1 μmol/l ROZ, while the increase induced by the cytokines was unaffected. ROZ by itself had no appreciable effect on caspase-3 activity at basal glucose (5 mmol/l).

Effects of long-term culturing of MIN6c4 cells or isolated islets with palmitate with or without ROZ on insulin release challenging by glucose or glucose and palmitate

The insulin secretory response of MIN6c4 cells or isolated islets to glucose and palmitate after a culturing period for 24 h at 5 mmol/l glucose (5G), 5G + 1 mmol/l palmitate or 5G + 1 μmol/l ROZ + palmitate was studied. Figure 6A illustrates that insulin release was slightly increased at 1 mmol/l glucose when MIN6c4 cells had been cultured with palmitate but not when ROZ was present. The stimulatory action of glucose or glucose + palmitate on insulin release was markedly attenuated after MIN6c4 had been cultured with palmitate alone for 24 h but not when ROZ was present during the culturing period (Fig. 6A). The insulin secretory pattern of isolated mouse pancreatic islets to glucose or glucose + palmitate was similar, although more pronounced compared with that observed for MIN6c4 cells. As seen in Fig. 6B, the stimulatory action of glucose or glucose + palmitate on insulin release was markedly attenuated when islets had been cultured with palmitate for 24 h. This deleterious effect by palmitate was greatly reversed when ROZ was present during the culture period (Fig. 6B).

Discussion

Glucose tolerance tests performed in hyperlipidemic subjects often reveal a reduced insulin secretory response to glucose [30, 31]. Chronic elevation of plasma FFA has long been recognized as a major risk factor for β-cell dysfunction and the subsequent development of type 2 diabetes. Although β-cell dysfunction has been observed in pancreatic islets cultured with FFA in *in vivo* animal models of hyperlipidemia and in clinical observations of subjects with diabetes-prone obesity [1, 4, 7, 8, 16, 31], there is still controversy about the relevance of different target molecules in mediating lipotoxic effects of FFA in the β-cell that lead to the pathogenesis of type 2 diabetes. It has been claimed that lipotoxicity only in combination with glucotoxicity has a deleterious effect on islet hormone secretion [17, 32]. However, growing evidence including our own observations [4, 7, 8, 16] strongly suggests that lipotoxicity *per se* may contribute to β-cell dysfunction by several mechanisms. We have reported [4, 7, 8, 16] that hyperlipidemia induced experimentally by infusion of intralipid to rats for 8 days resulted in a markedly enhanced NO generation through a pronounced induction of iNOS expression and activity in the islets. Our present *in vitro* data confirm our previous *in vivo*

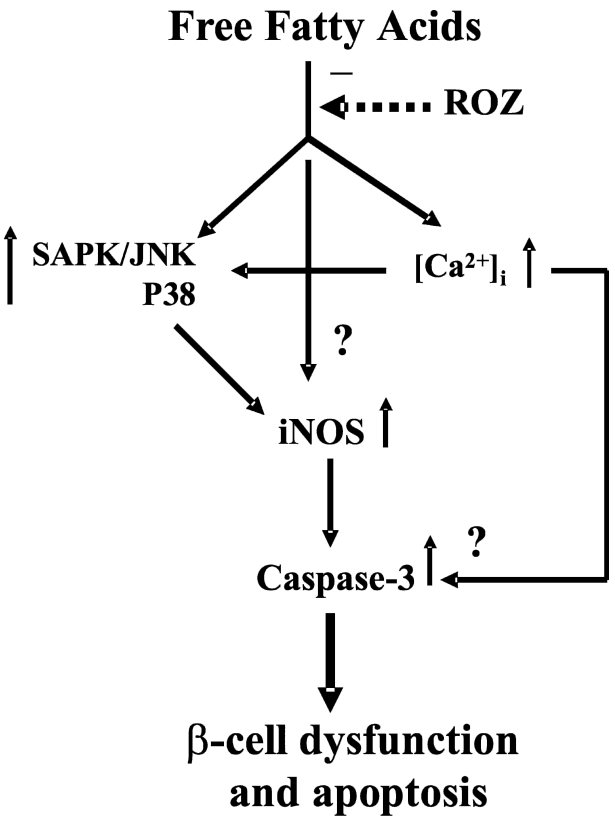

Figure 7. Proposed scheme suggesting an early step for the interaction of ROZ with the free fatty acid signaling pathways in the pancreatic β-cell leading to prevention of β-cell dysfunction/ apoptosis.

reports, and demonstrate that the ability of palmitate to induce iNOS activity is time dependent and cannot be observed during a short-term exposure of the islets to palmitate. Importantly, in the present study, we have shown that the thiazolidinedione ROZ markedly suppressed palmitate-induced iNOS activity in the islets, thus reducing a pathological level of NO generation. This is also of importance for the production of other related reactive molecules such as peroxynitrite ($ONOO^-$) that might adversely modulate β-cell function [13]. High levels of NO have been reported to induce apoptotic cell death by increasing the activities of caspases, *i.e.* caspase-9, caspase-8 and the most important member of the caspase family, caspase-3, in several cell types including pancreatic islets, macrophages, thymocytes, certain neurons and tumor cells [22]. The pro-apoptotic mechanisms of NO alone or in combination with $ONOO^-$ also include protein and DNA oxidation [13], lipid peroxidation [17, 33], protein S-nitrosylation [34] and endoplasmic reticulum stress with subsequent caspase activation [35]. Hence, NO might directly induce β-cell dysfunction through caspase activation [21, 35].

The importance of $[Ca^{2+}]_i$ as a signal molecule necessary for a normal insulin response to glucose or FFA is becoming increasingly clear [25, 36]. In this context it should be mentioned that a pulsatile rather than a sustained elevation of $[Ca^{2+}]_i$ plays a critical role for a proper insulin secretion by the β-cell [37]. Our present measurement of $[Ca^{2+}]_i$ showed that the glucose-stimulated increase of the $[Ca^{2+}]_i$ levels was markedly augmented after addition of palmitate. Interestingly, ROZ had a suppressive action on palmitate-induced $[Ca^{2+}]_i$ and also on palmitate-stimulated insulin release. Conversely, in the presence of glucose, ROZ induced an increase in $[Ca^{2+}]_i$ as well as in insulin release. These data strongly suggest that the interaction of ROZ with β-cell signaling generated by palmitate is distinct from that of glucose. A positive modulation of glucose-stimulated insulin release by ROZ has previously been reported from experiments in the perfused rat pancreas [38]. In contrast, long-term stimulation of the β-cell accompanied by a sustained increase in $[Ca^{2+}]_i$ might have adverse effects on the β-cell function. Hence a substantial body of evidence shows that an exaggerated increase in $[Ca^{2+}]_i$ is involved in the generation of other signal molecules important for the activation of a variety of target proteins, including phosphorylation of the different MAPKs [39]. Many stress- and inflammation-related kinases such as SAPK/JNK, p38, p42/44 and PKCs have been shown to mediate both inflammation and/or FFA-induced insulin resistance in hepatocytes, adipocytes and muscle tissues [40, 41]. The present investigation has identified SAPK/JNK and p38 as possible players not in short-term but in long-term palmitate-induced β-cell dysfunction. Our results show that a prolonged exposure of the β-cell to palmitate, which is reminiscent of the situation seen in obesity and obese diabetes, can induce β-cell dysfunction by activating SAPK/JNK and p38, while p42/44, which is growth-promoting, is unaffected. Hence our findings are consistent with the notion that the chronic presence of saturated FFA such as palmitate is important for fat-induced β-cell dysfunction. In addition, our results demonstrated for the first time that the pro-apoptotic MAPKs signaling pathway is suppressed by ROZ in islet tissue both after short-time and long-time incubation. Previous studies by other investigators have demonstrated that SAPK/JNK, p38 and p42/44, three members of the MAPK superfamily, are activated by cytokines [42]. However, activation of p42/44 (anti-apoptotic) and SAPK/JNK, p38 (pro-apoptotic) exerts opposite effects on cytokine-induced β-cell dysfunction. We found here that palmitate activated all three MAPKs in short-term experiments, while only SAPK/JNK and p38 were increased in long-term experiments. Importantly, however,

ROZ suppressed SAPK/JNK and p38 in both types of experiments, suggesting a marked protective capacity of this thiazolidinedione. Although the detailed mechanisms responsible for the regulation of SAPK/JNK, p38 and p42/44 activation in short-term palmitate-stimulated islets are not known, one can speculate that high levels of $[Ca^{2+}]_i$ alone or in combination with other palmitate-derived signals may exert a stimulatory effect on the phosphorylation of p42/44, SAPK/JNK and p38.

In previous studies reported by other investigators, almost a maximal SAPK/JNK, p38 and p42/44 activation was observed at ~30 min after incubation of either isolated islets or β-cell lines with different inflammatory agents, *i.e.*, IL-1 β, TNF-α and IFN-γ [29, 42]. The activities of SAPK/JNK and p38 remained elevated until appearance of apoptosis [39, 42, 43]. The time course of palmitate-stimulated activation of SAPK/JNK and p38 in our study might speak in favor of these signaling pathways being operated upstream of induction of iNOS expression in the islets since no iNOS activity could be detected in short-term incubation of islets with palmitate. In this context, it is interesting that we found that the increase in caspase-3 activity after 24-h culture of isolated islets and MIN6c4 cells in the presence of palmitate was markedly suppressed by ROZ, while ROZ had no effect on the increase induced by a mixture of cytokines (IL-1 β, TNF-α and IFN-γ). These results suggest that FFA-induced and cytokine-induced β-cell dysfunction are differentially regulated. This is in accordance with a recent report [44] suggesting that FFA and cytokines induce pancreatic β-cell dysfunction by different mechanisms. These authors also suggested that an apoptotic effect of FFA was independent of an increased iNOS-derived NO production, because they found only a marginal augmentation of iNOS mRNA. However, they did not measure the activity of the iNOS protein. Hence we propose that a post-translationally induced NO production is an important part of palmitate-induced β-cell dysfunction, although an additional NO-independent pathway cannot be excluded.

Finally, another important finding in the present study is that treatment with ROZ markedly improved β-cell function measured after a glucose challenge. Thus, a marked suppression of insulin release stimulated by glucose or glucose + palmitate in short-time incubations following 24-h culture with palmitate was greatly reversed when isolated islets or MIN6c4 cells were cultured with palmitate + ROZ. These experiments suggest that ROZ achieves its β-cell protection *via* suppression of an exaggerated and sustained increase in $[Ca^{2+}]_i$ induced by FFA and also by inhibition of MAPK activation as well as nitrosative stress. Moreover, data from a parallel investigation in our laboratory using G protein-coupled receptor 40 (GPR40) antisense treatment (unpublished) suggest that the protective effect of ROZ on FFA-induced β-cell dysfunction might be exerted at GPR40.

In summary, our present study demonstrates that palmitate-induced β-cell dysfunction is most likely achieved by increasing the induction of pro-apoptotic molecules, such as iNOS-derived NO, and pro-apoptotic signaling pathways, such as SAPK/JNK and p38 MAPKs, and finally increasing the activity of apoptotic enzymes such as caspase-3. Moreover, and most importantly our results clearly demonstrate that ROZ markedly suppressed these signals and restored β-cell function during its long-term presence together with a high level of palmitate. This suggests that ROZ has an essential and beneficial action on the pancreatic β-cell in addition to its anti-diabetic effects through its well-known cellular target PPARγ in peripheral tissues. A proposed scheme for the effects of FFA in inducing β-cell dysfunction as recorded in the present study is shown in Figure 7. Because ROZ counteracted all the deleterious parameters measured, we are inclined to suggest that ROZ elicits its effect through interaction with an early step in the FFA signaling pathway (Fig. 7).

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1 Nolan, C. J., Madiraju, M. S., Delghingaro-Augusto, V., Peyot, M. L. and Prentki, M. (2006) Fatty acid signaling in the beta-cell and insulin secretion. Diabetes 55 Suppl 2, S16–S23.
2 Salehi, A., Flodgren, E., Nilsson, N. E., Jimenez-Feltstrom, J., Miyazaki, J., Owman, C. and Olde, B. (2005) Free fatty acid receptor 1 (FFA(1)R/GPR40) and its involvement in fatty-acid-stimulated insulin secretion. Cell Tissue Res. 322, 207–215.
3 Qader, S. S., Salehi, A., Hakanson, R., Lundquist, I. and Ekelund, M. (2005) Long-term infusion of nutrients (total parenteral nutrition) suppresses circulating ghrelin in food-deprived rats. Regul. Pept. 131, 82–88.
4 Qader, S. S., Jimenez-Feltstrom, J., Ekelund, M., Lundquist, I. and Salehi, A. (2007) Expression of islet inducible nitric oxide synthase and inhibition of glucose-stimulated insulin release after long-term lipid infusion in the rat is counteracted by PACAP27. Am. J. Physiol. Endocrinol. Metab. 292, E1447–1455.
5 Mosen, H., Salehi, A., Henningsson, R. and Lundquist, I. (2006) Nitric oxide inhibits, and carbon monoxide activates, islet acid alpha-glucoside hydrolase activities in parallel with glucose-stimulated insulin secretion. J. Endocrinol. 190, 681–693.
6 Salehi, A., Carlberg, M., Henningson, R. and Lundquist, I. (1996) Islet constitutive nitric oxide synthase: Biochemical determination and regulatory function. Am. J. Physiol. 270, C1634–C1641.

7 Salehi, A., Ekelund, M., Henningsson, R. and Lundquist, I. (2001) Total parenteral nutrition modulates hormone release by stimulating expression and activity of inducible nitric oxide synthase in rat pancreatic islets. Endocrine 16, 97–104.
8 Salehi, A., Ekelund, M. and Lundquist, I. (2003) Total parenteral nutrition-stimulated activity of inducible nitric oxide synthase in rat pancreatic islets is suppressed by glucagon-like peptide-1. Horm. Metab. Res. 35, 48–54.
9 Mandrup-Poulsen, T. (1996) The role of interleukin-1 in the pathogenesis of IDDM. Diabetologia 39, 1005–1029.
10 Eizirik, D. L., Delaney, C. A., Green, M. H., Cunningham, J. M., Thorpe, J. R., Pipeleers, D. G., Hellerstrom, C. and Green, I. C. (1996) Nitric oxide donors decrease the function and survival of human pancreatic islets. Mol. Cell. Endocrinol. 118, 71–83.
11 Corbett, J. A. and McDaniel, M. L. (1992) Does nitric oxide mediate autoimmune destruction of beta-cells? Possible therapeutic interventions in IDDM. Diabetes 41, 897–903.
12 Corbett, J. A., Wang, J. L., Hughes, J. H., Wolf, B. A., Sweetland, M. A., Lancaster, J. R. Jr. and McDaniel, M. L. (1992) Nitric oxide and cyclic GMP formation induced by interleukin 1 beta in islets of Langerhans. Evidence for an effector role of nitric oxide in islet dysfunction. Biochem. J. 287, 229–235.
13 Delaney, C. A. and Eizirik, D. L. (1996) Intracellular targets for nitric oxide toxicity to pancreatic beta-cells. Braz. J. Med. Biol. Res. 29, 569–579.
14 Jimenez-Feltstrom, J., Lundquist, I. and Salehi, A. (2005) Glucose stimulates the expression and activities of nitric oxide synthases in incubated rat islets: An effect counteracted by GLP-1 through the cyclic AMP/PKA pathway. Cell Tissue Res. 319, 221–230.
15 Henningsson, R., Salehi, A. and Lundquist, I. (2002) Role of nitric oxide synthase isoforms in glucose-stimulated insulin release. Am. J. Physiol. Cell Physiol. 283, C296–304.
16 Ekelund, M., Qader, S. S., Jimenez-Feltstrom, J. and Salehi, A. (2006) Selective induction of inducible nitric oxide synthase in pancreatic islet of rat after an intravenous glucose or intralipid challenge. Nutrition 22, 652–660.
17 Robertson, R. P., Harmon, J., Tran, P. O. and Poitout, V. (2004) Beta-cell glucose toxicity, lipotoxicity, and chronic oxidative stress in type 2 diabetes. Diabetes 53 Suppl 1, S119–S124.
18 Cai, Y., Martens, G. A., Hinke, S. A., Heimberg, H., Pipeleers, D. and Van de Casteele, M. (2007) Increased oxygen radical formation and mitochondrial dysfunction mediate beta cell apoptosis under conditions of AMP-activated protein kinase stimulation. Free Radic. Biol. Med. 42, 64–78.
19 Kyaw, M., Yoshizumi, M., Tsuchiya, K., Kirima, K. and Tamaki, T. (2001) Antioxidants inhibit JNK and p38 MAPK activation but not ERK 1/2 activation by angiotensin II in rat aortic smooth muscle cells. Hypertens. Res. 24, 251–261.
20 Liadis, N., Murakami, K., Eweida, M., Elford, A. R., Sheu, L., Gaisano, H. Y., Hakem, R., Ohashi, P. S. and Woo, M. (2005) Caspase-3-dependent beta-cell apoptosis in the initiation of autoimmune diabetes mellitus. Mol. Cell. Biol. 25, 3620–3629.
21 Montolio, M., Tellez, N., Biarnes, M., Soler, J. and Montanya, E. (2005) Short-term culture with the caspase inhibitor z-VAD.fmk reduces beta cell apoptosis in transplanted islets and improves the metabolic outcome of the graft. Cell Transplant. 14, 59–65.
22 Sadowski-Debbing, K., Coy, J. F., Mier, W., Hug, H. and Los, M. (2002) Caspases – Their role in apoptosis and other physiological processes as revealed by knock-out studies. Arch. Immunol. Ther. Exp. (Warsz) 50, 19–34.
23 Lupi, R., Del Guerra, S., Marselli, L., Bugliani, M., Boggi, U., Mosca, F., Marchetti, P. and Del Prato, S. (2004) Rosiglitazone prevents the impairment of human islet function induced by fatty acids: Evidence for a role of PPARgamma2 in the modulation of insulin secretion. Am. J. Physiol. Endocrinol. Metab. 286, E560–E567.
24 Spector, A. A., Fletcher, J. E. and Ashbrook, J. D. (1971) Analysis of long-chain free fatty acid binding to bovine serum albumin by determination of stepwise equilibrium constants. Biochemistry 10, 3229–3232.
25 Olofsson, C. S., Salehi, A., Holm, C. and Rorsman, P. (2004) Palmitate increases L-type Ca^{2+} currents and the size of the readily releasable granule pool in mouse pancreatic beta-cells. J. Physiol. 557, 935–948.
26 Gotoh, M., Maki, T., Kiyoizumi, T., Satomi, S. and Monaco, A. P. (1985) An improved method for isolation of mouse pancreatic islets. Transplantation 40, 437–438.
27 Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440–3450.
28 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
29 Laychock, S. G., Sessanna, S. M., Lin, M. H. and Mastrandrea, L. D. (2006) Sphingosine 1-phosphate affects cytokine-induced apoptosis in rat pancreatic islet beta-cells. Endocrinology 147, 4705–4712.
30 Robertson, R. P., Harmon, J., Tran, P. O., Tanaka, Y. and Takahashi, H. (2003) Glucose toxicity in beta-cells: Type 2 diabetes, good radicals gone bad, and the glutathione connection. Diabetes 52, 581–587.
31 Unger, R. H. (1995) Lipotoxicity in the pathogenesis of obesity-dependent NIDDM. Genetic and clinical implications. Diabetes 44, 863–870.
32 Poitout, V. and Robertson, R. P. (2008) Glucolipotoxicity: Fuel excess and {beta}-cell dysfunction. Endocr. Rev. 29, 351–366.
33 Yaney, G. C. and Corkey, B. E. (2003) Fatty acid metabolism and insulin secretion in pancreatic beta cells. Diabetologia 46, 1297–1312.
34 Jaffrey, S. R., Erdjument-Bromage, H., Ferris, C. D., Tempst, P. and Snyder, S. H. (2001) Protein S-nitrosylation: A physiological signal for neuronal nitric oxide. Nat. Cell Biol. 3, 193–197.
35 Araki, E., Oyadomari, S. and Mori, M. (2003) Endoplasmic reticulum stress and diabetes mellitus. Intern. Med. 42, 7–14.
36 Henquin, J. C., Ishiyama, N., Nenquin, M., Ravier, M. A. and Jonas, J. C. (2002) Signals and pools underlying biphasic insulin secretion. Diabetes 51 Suppl 1, S60–S67.
37 Salehi, A., Qader, S. S., Grapengiesser, E. and Hellman, B. (2005) Inhibition of purinoceptors amplifies glucose-stimulated insulin release with removal of its pulsatility. Diabetes 54, 2126–2131.
38 Yang, C., Chang, T. J., Chang, J. C., Liu, M. W., Tai, T. Y., Hsu, W. H. and Chuang, L. M. (2001) Rosiglitazone (BRL 49653) enhances insulin secretory response *via* phosphatidylinositol 3-kinase pathway. Diabetes 50, 2598–2602.
39 Khoo, S., Gibson, T. B., Arnette, D., Lawrence, M., January, B., McGlynn, K., Vanderbilt, C. A., Griffen, S. C., German, M. S. and Cobb, M. H. (2004) MAP kinases and their roles in pancreatic beta-cells. Cell Biochem. Biophys. 40, 191–200.
40 Mendelson, K. G., Contois, L. R., Tevosian, S. G., Davis, R. J. and Paulson, K. E. (1996) Independent regulation of JNK/p38 mitogen-activated protein kinases by metabolic oxidative stress in the liver. Proc. Natl. Acad. Sci. USA 93, 12908–12913.
41 Harper, S. J. and LoGrasso, P. (2001) Signalling for survival and death in neurones: The role of stress-activated kinases, JNK and p38. Cell. Signal. 13, 299–310.
42 Kim, W. H., Lee, J. W., Gao, B. and Jung, M. H. (2005) Synergistic activation of JNK/SAPK induced by TNF-alpha and IFN-gamma: Apoptosis of pancreatic beta-cells via the p53 and ROS pathway. Cell. Signal. 17, 1516–1532.
43 Kaneto, H., Nakatani, Y., Kawamori, D., Miyatsuka, T., Matsuoka, T. A., Matsuhisa, M. and Yamasaki, Y. (2005) Role of oxidative stress, endoplasmic reticulum stress, and c-Jun N-terminal kinase in pancreatic beta-cell dysfunction and insulin resistance. Int. J. Biochem. Cell Biol. 37, 1595–1608.
44 Kharroubi, I., Ladriere, L., Cardozo, A. K., Dogusan, Z., Cnop, M. and Eizirik, D. L. (2004) Free fatty acids and cytokines induce pancreatic beta-cell apoptosis by different mechanisms: Role of nuclear factor-kappaB and endoplasmic reticulum stress. Endocrinology 145, 5087–5096.