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Adiponectin counteracts cytokine- and fatty acid-induced apoptosis in the pancreatic beta-cell line INS-1

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Abstract

Aims/hypothesis. Pancreatic beta-cell apoptosis is a common feature of Type 1 and Type 2 diabetes and leptin exerts an anti-apoptotic function in these cells. The beta-cell line INS-1 was used to test the hypothesis that the adipocyte hormone adiponectin might mediate an anti-apoptotic effect comparable to leptin.
Methods. Apoptosis was induced by culturing cells with a cytokine combination (interleukin-1β/interferon-γ) or palmitic acid in absence or presence of leptin or the globular domain of adiponectin (gAcrp30), respectively.
Results. INS-1 cells had a prominent sensitivity towards cytokine- and fatty acid-induced apoptosis, resulting in about three- and six-fold increases in caspase 3 activation and DNA fragmentation, respectively. gAcrp30 strongly (50–60%) inhibited palmitic acid-induced apoptosis, with a weaker effect against cytokine-induced apoptosis (35%). The same result was observed for leptin with both adipokines being non-additive. Reduction of apoptosis by an inhibitor of IκB-kinase (IKK) indicated the involvement of the nuclear factor (NF)-κB pathway in both cytokine- and fatty acid-induced apoptosis, however, leptin and gAcrp30 were unable to block NF-κB activation. Cytokine- and fatty-acid-induced suppression of glucose/forskolin-stimulated insulin secretion was completely prevented through the action of gAcrp30, whereas leptin was only effective against lipotoxicity-mediated beta-cell dysfunction.
Conclusion/interpretation. Our data show that gAcrp30 partially rescues beta cells from cytokine- and fatty-acid-induced apoptosis and completely restores autoimmune- and lipotoxicity-induced dysfunction of insulin-producing cells. We suggest that gAcrp30 exerts its anti-apoptotic function without modulating NF-κB activation. This novel beta cell protective function of gAcrp30 might serve to counteract autoimmune- and lipotoxicity-induced beta-cell destruction. [Diabetologia (2004) 47:249–258]

Keywords INS-1 cells · Apoptosis · Adiponectin · Leptin · Cytokines · Lipotoxicity · NF-kappa B

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Abbreviations: Acrp30, adipocyte complement-related protein of 30 kDa · gAcrp30, globular domain of Acrp30 · IL-1β, interleukin-1β · IFN-γ, interferon-γ · IKK, IκB-kinase · NF-κB, nuclear factor-κB · TNF-α, tumor necrosis factor-α · annexin V-FITC, annexin V-fluorescein isothiocyanate · FCS, fetal calf serum · PBS, phosphate-buffered saline · PIB, phosphatase inhibitor buffer · BSA, bovine serum albumin · KRB, Krebs-Ringer bicarbonate

Type 1 diabetes is characterized by progressive destruction of pancreatic beta cells after lymphoid infiltration of the islet resulting in insulin deficiency and hyperglycaemia [1]. A number of pathological stimuli involved in Type 1 diabetes have been reported to induce beta-cell apoptosis including proinflammatory cytokines like interleukin-1β (lL-1β), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), nitric oxide, reactive oxygen species and Fas ligand [2, 3]. In the case of Type 2 diabetes, which is associated with peripheral insulin resistance and beta-cell dysfunction, recent evidence suggests a reduced beta-cell mass as a contributor to the pathogenesis of this chronic disease [4]. Moreover, fatty acid-induced apoptosis of pancreatic beta cells has been regarded as a critical determinant in switching from obesity and insulin resistance to Type 2 diabetes [5, 6].

Although the molecular mechanisms by which pathological stimuli implicated in the pathogenesis of Type 1 and Type 2 diabetes elicit beta-cell apoptosis have not yet been established, there is increasing evidence favouring convergence in signalling pathways towards common effectors of beta-cell apoptosis. Recent observations indicate that the intracellular signals involved in the beta-cell apoptotic cascade induced by immunological, inflammatory and metabolic stimuli include increases in intracellular calcium, cytochrom c, ceramide, free oxygen and nitric oxide (NO) radicals by activation of protein kinases and transcription factors such as AP-1 and nuclear factor (NF)-κB, which regulate various metabolic enzymes and genes involved in cell cycle progression and apoptosis [2]. One central signalling pathway that has been extensively investigated involves the cytotoxic effects of proinflammatory cytokines on pancreatic beta cells mediated through activation of the transcription factor NF-κB [7]. NF-κB activation represents a crucial event in cytokine-regulation of expression of several chemokines and cytokines [8], Fas expression [9], iNOS and COX-2 expression [10, 11], which are all regarded as essential factors for causing dysfunction and destruction of insulin-producing cells thus leading to the development of insulin-dependent diabetes. Importantly, a ceramide-dependent NF-κB-mediated up-regulation of iNOS expression was recently shown in response to palmitic acid, indicating a potential involvement of this transcription factor also in the regulation of beta-cell lipotoxicity and pathogenesis of Type 2 diabetes [6].

Recent work has identified a novel function of leptin consisting of the prevention of islet lipotoxicity by increasing β-oxidative metabolism of fatty acids and reducing lipogenesis [5, 6]. This protective action of leptin against beta-cell lipoapoptosis was accompanied by a reduction in ceramide synthesis and iNOS mRNA expression as well as prevention of fatty-acid-induced suppression of Bcl-2 expression thus inhibiting apoptotic death [4, 5, 6, 12]. Further, the effects of leptin on fatty acid metabolism (in muscle) are mediated through activation of 5'-AMP-activated protein kinase (AMPK) and subsequent down-regulation of acetyl coenzyme A carboxylase (ACC) [13, 14].

Recently, a further adipokine called adiponectin or adipocyte complement-related protein of 30 kDa (Acrp30), which is decreased in obesity and Type 2 diabetes, has been described to exert its beneficial functions on glucose and lipid metabolism as well as insulin sensitivity by activating AMPK in skeletal muscle and liver [15]. Interestingly, adiponectin has been shown to exert putative anti-atherogenic properties by suppressing TNF-α-induced NF-κB activation through a cAMP-dependent pathway, thus inhibiting inflammatory-induced gene transcription [16, 17]. These observations suggest that adiponectin could have therapeutic applications in diseases caused by excessive inflammatory responses.

The aim of this study was to test the hypothesis that adiponectin might act as an endogenous modulator of beta-cell responses to proinflammatory stimuli and increased fatty acids. Our data identify the C-terminal globular domain of adiponectin as a critical negative regulator of cytokine- and fatty-acid-induced apoptosis of pancreatic beta cells. We show that gAcrp30 mediates its beta-cell anti-apoptotic action without modulating NF-κB activation. Our data also provide evidence that gAcrp30 completely reverses cytokine- and lipotoxicity-induced dysfunction of insulin-producing cells. This novel beta cell protective action of gAcrp30 might serve to counteract diabetes-related beta-cell destruction by maintaining both function and survival of insulin-producing cells.

Materials and methods

Materials. Rat IL-1β was purchased from Biozol (Eching, Germany) and the recombinant rat IFN-γ was obtained from Calbiochem (Schwalbach, Germany). Palmitic acid was supplied by Sigma (Deisenhofen, Germany). Leptin and a highly specific IKK-inhibitor I229 were provided by Aventis (Frankfurt, Germany). I229 has submicromolar activity on the isolated IKK complex and is highly specific on IKK. Its general structure is described in PCT/EP00/05340. The recombinant C-terminal globular domain of adiponectin (gAcrp30) was a product from Tebu (Offenbach, Germany). Bovine serum albumin (BSA, Fraction V, fatty-acid free) was obtained from Boehringer Mannheim (Mannheim, Germany). Annexin V-FITC and the caspase 3 assay kit was provided by BD PharMingen (Heidelberg, Germany). Propidium iodide (PI) for discrimination of necrotic from apoptotic cells was product of MoBiTec (Göttingen, Germany). The ultrasensitive rat insulin ELISA kit was supplied by Mercodia (Uppsala, Sweden). The cell death detection ELISA kit was obtained from Roche Diagnostics (Mannheim, Germany). The nuclear extraction kit and TransAM NF-κB p65 chemiluminescence transcription factor assay kit were products from Active Motif (Rixensart, Belgium). Foetal calf serum (FCS), RPMI 1640 medium and penicillin/streptomycin were provided by Gibco (Eggenstein, Germany). The clonal glucose sensitive rat insulinoma cell line INS-1 was kindly provided by Dr. J. Seissler (Düsseldorf, Germany). All other chemicals were of the highest grade commercially available.

Cell culture. The rat insulinoma cell line INS-1 (passages 90–110) was grown in monolayer culture in RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum (FCS), 500 U/ml penicillin, 50 µg/ml streptomycin, 2 mmol/l glutamine and 50 µmol/l 2-mercaptoethanol in 75 cm^2 flasks in an atmosphere of 5% CO_2 at 37°C. Subconfluent cells were maintained in continuous passages by trypsinization of cultures 7 days after plating. The medium was changed every 72 h. Cell number was determined after cell dissociation with trypsin/EDTA at 37°C. Morphological and functional integrity of the cells was confirmed by electron microscopy and immunostaining as well as measuring glucose-stimulated insulin secretion (data not shown). INS-1 cells were routinely seeded at 3.5×10^5 cells/well of a 12-well plate for cell death detection experiments and insulin secretion studies and used on day 4 at 60–70% confluence.

Preparation of palmitic acid/BSA complex solution. A 1 mmol/l palmitic acid stock solution was prepared in 95% ethanol by incubating at 37°C. In parallel, 2% fatty acid-free BSA was solubilized in RPMI medium at 37°C. The palmitic acid/BSA complex solution was then prepared by incubating the components in a molar ratio of 3:1 at 37°C in a shaking water bath for 1 h. After sterile filtration it was used directly for INS-1 stimulation experiments. Control cultures were carried out in the presence of BSA/ethanol in RPMI without palmitic acid.

Determination of DNA fragmentation. Subconfluent INS-1 cells were incubated for 2 h in RPMI 1640 without FCS supplemented with 0.5% BSA. After this serum-free period the medium was removed and replaced with fresh RPMI 1640 containing 5% FCS and 0.5% BSA. Cells were then incubated with the cytokine combination IL-1β at 4 ng/ml and IFN-γ at 10 U/ml or palmitic acid coupled to 2% BSA at 250 µmol/l in the absence or presence of a specific IKK inhibitor (I229) (10 µmol/l) or leptin (200 ng/ml) and gAcrp30 (10 nmol/l) for 24 h at 37°C. After removing this medium, the cells were washed twice with ice cold PBS and lysed. The induced apoptosis was measured by the specific determination of mono- and oligonucleosomes in the cytoplasmatic fraction of cell lysates using a cell death detection ELISA kit. The assay is based on a quantitative sandwich-enzyme-immunoassay-principle using mouse monoclonal anti-histone and anti-DNA peroxidase antibodies. The relative degree of apoptotic cell death was photometrically determined by measuring the peroxidase activity of the immunocomplexes at 405 nm.

Caspase 3 assay. INS-1 cells were plated in 12-well-dishes (3.5×10^5 cells/well) and cultured in RPMI containing 10% FCS for 3 days. After reaching subconfluence the medium was removed and replaced with serum free RPMI supplemented with 0.5% BSA for 2 h, followed by an additional 16 h incubation in RPMI containing 5% FCS, 0.5% BSA and cytokines or palmitic acid at the indicated concentrations. The effect of the IKK-inhibitor, leptin and adiponectin was analysed in parallel incubations in the absence or presence of the cytokines and fatty acid, respectively. The DEVD-cleaving activity of the caspase 3 class of cystein proteases was determined in cell lysates using Ac-DEVD-AMC as fluorogenic substrate with the Caspase 3 assay kit (BD Biosciences) according to the manufacturer's protocol. The ability of cell lysates to cleave the specific caspase 3 substrate was quantified by ultraviolet (UV) spectrofluorometry using an excitation wavelength of 390 nm and an emission wavelength of 460 nm with a microplate reader. The corresponding peptide aldehyde Ac-DEVD-CHO was used to inhibit the caspase 3 activity and thus to ensure the specificity of the reaction (data not shown).

Annexin V/PI fluorescence staining and confocal microscopy. Following a 2-h pre-incubation under serum-free conditions the cells were kept for 24 h in RPMI containing 5% FCS, 0.5% BSA and the cytokine combination IL-1β/IFN-γ or palmitic acid at the indicated concentrations. After washing twice with cold PBS a double staining of cells with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) was carried out according to the recommended protocol and the cells were then embedded in vector fluorescent mounting medium. Images were produced by a confocal laser scanning microscope (Zeiss Axiovert fluorescence microscope) with a Zeiss Neofluar ×40/1.3 objective lens connected to a confocal scanner and a workstation.

Annexin V/PI double staining enables the differentiation of apoptotic cells from necrotic cells. The former were defined as PI negative, indicating an intact plasma membrane and annexin positive indicating the loss of membrane asymmetry.

Insulin secretion studies. INS-1 cells were cultured for 3 days in RPMI medium containing 11.1 mmol/l glucose and 10% FCS followed by a pre-incubation in serum-free medium for 2 h. Incubation was then continued in RPMI supplemented with 11.1 mmol/l glucose, 5% FCS and 4 ng/ml IL-1β plus 10 U/ml IFN-γ or 250 µmol/l palmitic acid in the absence or presence of 10 nmol/l adiponectin or 200 ng/ml leptin for 24 h at 37°C. The cells were then washed with Krebs-Ringer bicarbonate (KRB) solution, pH 7.4, containing 0.5% BSA and 0.5 mmol/l glucose and kept for 30 min in the same buffer. At the end of this challenge, the buffer was removed and replaced with fresh KRB supplemented with 15 mmol/l glucose in presence of 10 µmol/l forskolin for 30 min at 37°C. Insulin concentration in the various media was then determined by a commercial rat insulin enzyme immunoassay kit.

Determination of NF-κB activation. Subconfluent INS-1 cells were kept under serum-free conditions for 2 h and subsequently pre-incubated in the presence of 10 µmol/l IKK-inhibitor, 200 ng/ml leptin or 10 nmol/l gAcrp30 for 30 min followed by an additional stimulation with cytokines or palmitic acid at the indicated concentrations for 30 min. In control experiments the cells were incubated with 2.5 nmol/l TNF-α, a potent NF-κB activator, in absence or presence of the IKK-inhibitor. After removing this medium, the cells were washed with ice cold PBS/PIB and nuclear extracts were prepared using a hypotonic buffer with the nuclear extract kit (Active Motif) according to the manufacturer's protocol. The active NF-κB contained in cell extracts was then measured by its DNA-binding activity on immobilized oligonucleotides containing a specific consensus site using a NF-κB p65 transcription factor ELISA kit (Active Motif). This assay is based on a quantitative sandwich-enzyme-immunoassay-principle using a specific primary antibody that recognizes an epitope on the p65-subunit of activated and DNA-bound NF-κB and a peroxidase-conjugated secondary antibody. The relative degree of NF-κB activation was determined by measuring the peroxidase activity of the immunocomplexes by the chemiluminescence method and quantified using the LumiImager software.

Statistical analysis. Statistical analysis was carried out by using either Student's *t*-test or analysis of variance test (ANOVA) for comparison of more than two variables. All statistical analyses were done using Prism software (Graphpad, San Diego, Calif., USA). A p value of less than 0.05 was considered to be statistically significant. Corresponding significance levels are indicated in the figures.

Results

Characterization of induced beta-cell apoptosis by confocal microscopy. Exposure of INS-1 beta cells to the cytokine combination IL-1β (4 ng/ml)/IFN-1γ (10 U/ml) or palmitic acid (250 µmol/l) for 24 h and subsequent simultaneous treatment with annexin V-FITC and propidium iodide (PI), in order to discriminate apoptotic from necrotic cells, resulted in a marked increase in the number of apoptotic cells (green), as assessed by confocal microscopy (Fig. 1). The majority of treated cells had intact plasma mem-

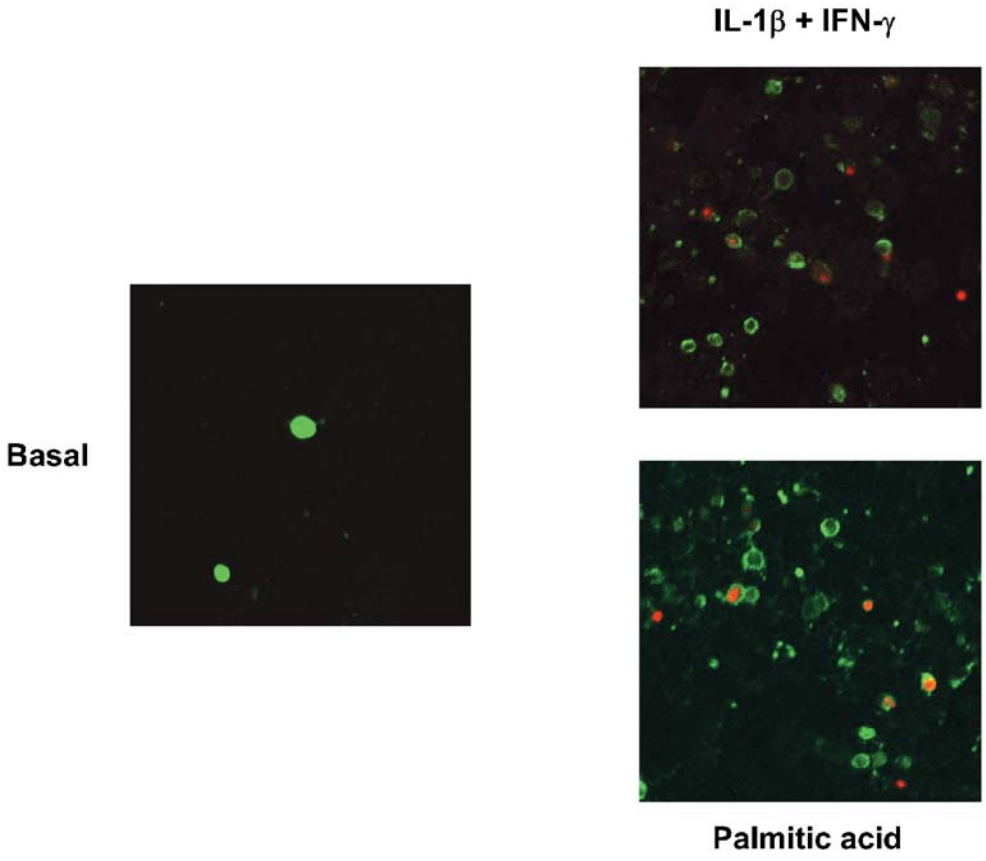

Fig. 1. Microscopic analysis of induced cell death in insulin-secreting beta cells in response to cytokines and fatty acids. INS-1 cells were incubated for 24 h with the indicated cytokine combination or palmitic acid (coupled to BSA in a molar ratio 3:1) and subsequently subjected to a double fluorescence staining with annexin V/PI and qualitative analysis by confocal microscopy as described. Images obtained from a representative experiment are shown. *Green* (annexin V): apoptotic cells; *red* (PI): necrotic cells

branes, impermeable to the DNA-binding dye PI (red) (Fig. 1). Therefore, both cytokine- and fatty acid-induced beta-cell death represent mainly apoptotic cell death accompanied by a negligible necrotic component under these experimental conditions. This is consistent with previous observations indicating apoptosis as the predominant form of both cytokine- [18, 19] and saturated fatty acid-induced beta-cell death [20] in rat and human islets.

Effect of leptin and gAcrp30 on cytokine- and fatty acid-induced apoptosis. Caspases are converted to active enzymes during apoptosis by proteolytic cleavage, subsequently leading to cleavage of various cellular key regulatory proteins thus triggering programmed cell death [21, 22]. We assessed apoptosis quantitatively by determining activation of caspase 3 as a typical biochemical change in the execution phase of programmed cell death. As shown in Fig. 2, a prominent increase in caspase 3 activity (three–four-fold) was observed after treating the beta-cells with the cytokine combination IL-1β/IFN-γ or palmitic acid, respectively, in agreement with data reported in the literature [11, 23]. Exposure of INS-1 cells to leptin or gAcrp30 did not modify caspase 3 activity under control conditions (not shown in the Figure). However, a significant reduction of cytokine-induced caspase 3 activation was obtained in the presence of leptin or gAcrp30 (35–45%) (Fig. 2a). The prominent increase of caspase 3 activation in response to palmitic acid was strongly suppressed by both leptin and gAcrp30 to an equal extent (about 70%) (Fig. 2B).

In order to further elucidate the role of leptin and gAcrp30 actions in the regulation of cytokine- and fatty acid-induced programmed cell death, we measured nucleosomal release as an early biochemical feature and quantitative marker of apoptosis [24]. Incubating INS-1 cells with the cytokine combination IL-1β/IFN-γ or palmitic acid resulted in a pronounced stimulation of DNA fragmentation (six–seven fold) (Fig. 3), whereas leptin and gAcrp30 showed no significant influence on nucleosomal release by untreated control cells (not shown in the Figure). Cytokine-induced DNA fragmentation was partially inhibited in the presence of leptin or gAcrp30, respectively (35%) (Fig. 3a). Leptin and gAcrp30 exerted a comparable strong inhibitory action on fatty acid-induced DNA fragmentation (50–60%). Leptin and gAcrp30 showed no synergistic effects on cytokine- or fatty acid-in-

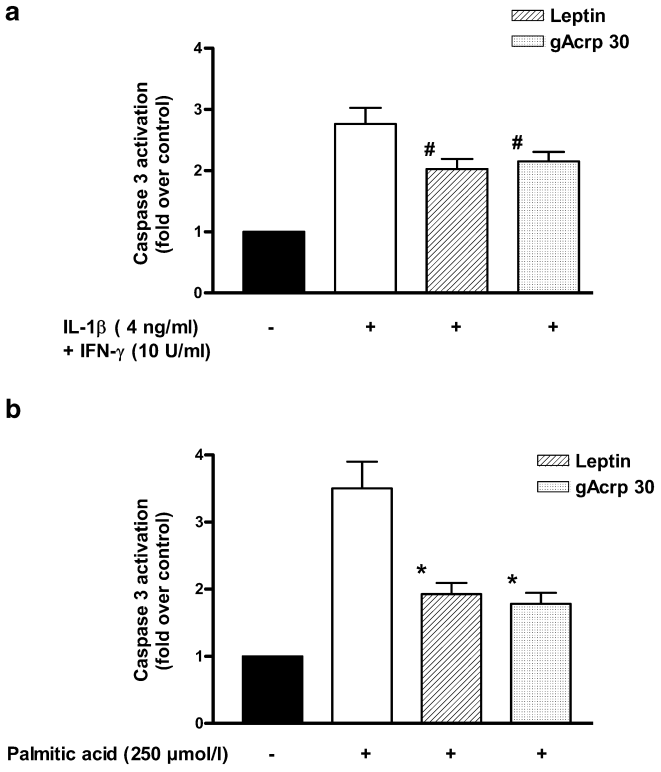

Fig. 2a, b. Caspase 3 activation in response to cytokines and fatty acids and modulation by leptin and gAcrp30. INS-1 cells were exposed for 16 h to cytokines (**a**) or fatty acids/BSA (3:1) (**b**) and leptin at 200 ng/ml or gAcrp30 at 10 nmol/l and processed for determination of caspase 3. Results are expressed relative to untreated control cells and are mean values ± SEM of four to five separate experiments. #Significantly different from the cytokine-stimulated value, $p<0.05$; *Significantly different from the palmitic acid-stimulated value, $p<0.001$

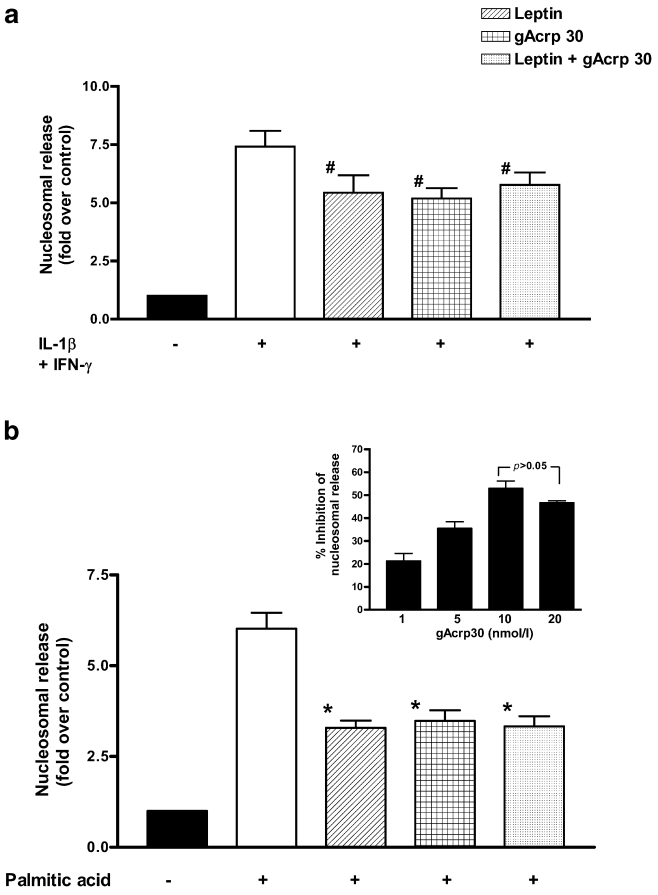

Fig. 3a, b. Nucleosomal release in response to cytokines and fatty acids and modulation by leptin and gAcrp30. INS-1 cells were exposed to cytokines (**a**) or fatty acids/BSA (3:1) (**b**) and adipokines for 24 h. Apoptosis was assessed by determination of oligonucleosomal accumulation in the cytosolic extracts using a cell death detection ELISA kit. Nucleosomal release is expressed relative to untreated control cells. Data are mean values ± SEM of five to six separate experiments. #Significantly different from the cytokine-stimulated value, $p<0.01$; *Significantly different from the palmitic acid-stimulated value, $p<0.001$. *Inset*: INS-1 cells were incubated with fatty acids and increasing concentrations of gAcrp30 for 24 h. Inhibition of nucleosomal release was then determined as outlined in **b**. Data are mean values ± SEM (n=3–4)

duced nucleosomal release (Fig. 3a, b), suggesting a common signalling mechanism for the anti-apoptotic action of these fat-derived hormones.

We then determined the dose-dependent anti-apoptotic effect of gAcrp30 by incubating INS-1 cells with 250 µmol/l palmitic acid in the presence of 1–20 nmol/l gAcrp30 for 24 h and subsequently measuring DNA fragmentation. At all concentrations a significant suppression of fatty acid-induced nucleosomal release was observed reaching maximal inhibition at 10 nmol/l (Fig. 3b inset). To our knowledge, the physiological plasma concentrations of gAcrp30 remain unknown so far, thus making a correlation to the concentrations used in the present study not feasible. Recently, the concentration-dependent activation of fatty acid oxidation by gAcrp30 in muscle cells was reported reaching a maximum at around 5 nmol/l [25], which is in good agreement with our results.

Effect of leptin and gAcrp30 on cytokine- and fatty acid-induced NF-κB activation. The proapoptotic role of the NF-κB pathway in response to proinflammatory cytokines is well established in pancreatic beta cells [7, 8, 9, 10, 11]. Recent work also indicates a direct involvement of this transcription factor in the regulation of beta-cell lipotoxicity [6]. We determined the role of NF-κB activation in cytokine- and fatty acid-induced apoptosis in the pancreatic beta-cell line INS-1 by inhibiting this pathway with the highly specific IKK-inhibitor I229. The cytokine- and palmitic acid-induced apoptosis measured at the level of caspase 3 activation and nucleosomal release was strongly reduced by the presence of the IKK-inhibitor (60–70%), thus confirming the proapoptotic role of the NF-κB pathway in the insulin-producing cell line INS-1 (Table 1).

In order to further characterize the role of the NF-κB pathway in the anti-apoptotic action of leptin and

Table 1. Effect of IKK-inhibitor I229 on cytokine- and fatty acid-induced apoptosis in INS-1 cells

Treatment	I299	Caspase 3 activation (fold over control)	Nucleosomal release (fold over control)
IL-1β + IFN-γ	–	2.80±0.26	7.40±0.67
	+	1.55±0.14[a]	3.58±0.36[a]
Palmitic acid	–	3.50±0.4	6.00±0.44
	+	1.66±0.12[b]	3.46±0.32[b]

Determination of cytokine- and palmitic acid-induced caspase 3 activation and nucleosomal release was performed in absence or presence of the IKK-inhibitor I299 at 10 μmol/l. Results are expressed relative to untreated control cells and are mean values ± SEM of four to five separate experiments. [a] Significantly different from the cytokine-stimulated value, $p<0.001$; [b] Significantly different from the palmitic acid-stimulated value, $p<0.001$

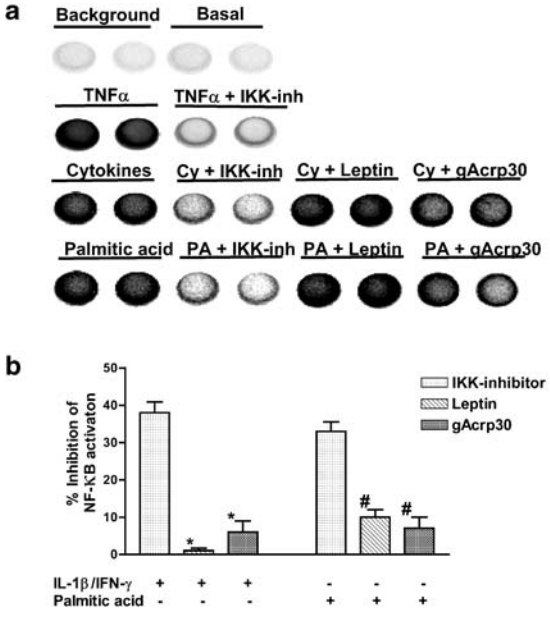

Fig. 4a, b. NF-κB activation in response to cytokines and fatty acids and modulation by leptin and gAcrp30. INS-1 cells were exposed to the IKK-inhibitor I229 and adipokines for 30 min and subsequently stimulated with cytokines or palmitic acid/BSA (3:1) for 30 min. In control experiments TNF-α was used as potent NF-κB activator. Nuclear extracts were prepared and processed for determination of NF-κB activation by measuring its DNA-binding capacity using a chemiluminescence-based ELISA kit. (**a**) LumiImager signals obtained from duplicate cell culture wells of a representative experiment are shown. (**b**) Signals were quantified using the LumiImager software. Results are expressed as a percentage inhibition of cytokine- and palmitic acid-induced NF-κB activation and are mean values ± SEM of three separate experiments. *No significant inhibition of the cytokine-stimulated NF-κB activation and not significantly different from each other with $p>0.05$; #No significant inhibition of the palmitic acid-stimulated NF-κB activation and not significantly different from each other, $p>0.05$

gAcrp30 we assessed the direct effect of the adipokines on cytokine- and fatty acid-induced NF-κB activation. Exposure of INS-1 cells to the cytokine combination IL-1β/IFN-γ or palmitic acid resulted in a pronounced NF-κB activation (15–20 fold over basal) comparable to the TNF-α control (Fig. 4a). Inhibition of IKK induced a strong suppression of this inflammatory response exerting a comparable effect against the different stimuli tested (Fig. 4a, b). However, neither leptin nor gAcrp30 could reverse cytokine- and palmitic acid-induced NF-κB activation (Fig. 4a, b), indicating that these adipokines counteract the cytokine- and fatty acid-induced beta-cell death without modulating NF-κB activation.

Effect of cytokines, palmitic acid, gAcrp30 and leptin on insulin secretion. It is well established that proinflammatory cytokines play a critical role in the development of beta-cell dysfunction by reducing insulin production and secretion, thereby contributing to the pathogenesis of Type 1 diabetes [26, 27]. There is increasing evidence that acute exposure of pancreatic beta cells to high concentrations of fatty acids results in an increase of insulin release, whereas chronic exposure induces desensitization and suppression of secretion [28, 29], possibly contributing to the progressive beta-cell dysfunction in Type 2 diabetes.

In this study we determined the ability of INS-1 cells to secrete insulin upon stimulation with 15 mmol/l gucose in presence of 10 μmol/l forskolin, an activator of adenylyl cyclase known to stimulate the cAMP/protein kinase A (PKA) system and thereby potentiating the effect of glucose on insulin exocytosis [30]. In order to elucidate the long term effects of cytokines and fatty acids on glucose/forskolin-induced insulin secretion and the influence of leptin and gAcrp30 on this process, INS-1 cells were exposed to the cytokine combination IL-1β/IFN-γ or palmitic acid in absence or presence of these compounds at the indicated concentrations for 24 h before determination of insulin secretion.

Glucose stimulation resulted in a 2.5-fold increase of insulin release when compared to the basal glucose-free state (data not shown), whereas the addition of forskolin induced a marked potentiation (15-fold over basal control) of the glucose-responsiveness of INS-1 cells (Fig. 5), consistent with previous data reported in this clonal pancreatic cell line [31]. Cytokines and palmitic acid induced a marked (two–three fold) decrease of glucose/forskolin-stimulated insulin release

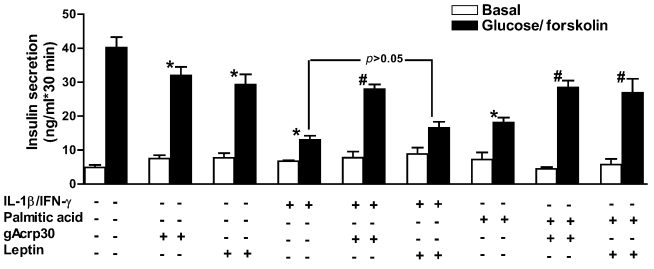

Fig. 5. Effect of cytokines, palmitic acid, leptin and gAcrp30 on insulin secretion in insulin-producing beta cells. INS-1 cells were subjected to a glucose-free incubation for 30 min and then stimulated with 15 mmol/l glucose in presence of 10 μmol/l forskolin for 30 min. The effects of IL-1β/IFN-γ, palmitic acid, and the adipokines leptin and gAcrp30 on basal and stimulated insulin secretion was assessed by incubating cells in the presence of the tested agents for 24 h prior to stimulating with glucose/forskolin. Insulin content in the supernatants was determined using a commercial rat insulin ELISA assay. Data are expressed in absolute amounts of insulin secreted into the culture medium during the incubation period of 30 min and are mean values ± SEM from four to seven separate experiments. *Significantly different from the glucose/forskolin-stimulated control value, $p<0.05$. #Not significantly different from their corresponding control values, $p>0.05$

(Fig. 5), in complete agreement with recently published data [23, 26, 27, 29, 32]. Neither IL-1β/IFN-γ nor palmitic acid affected basal insulin secretion under our experimental conditions (Fig. 5). Indeed, prolonged exposure to palmitic acid has been reported not to induce any significant changes in basal insulin release in rat pancreatic islets [20], whereas the role of cytokines in this process remains controversial [33, 34], probably depending on the exposure time, the cytokine combination and/or the cell model. Leptin and gAcrp30 did not affect basal insulin secretion, however both adipokines reduced glucose/forskolin-stimulated insulin release (Fig. 5). This is in good agreement with current evidence indicating that leptin inhibits insulin secretion induced by cellular cAMP in INS-1 cells without affecting the basal value [35, 36]. Importantly, both cytokine- and palmitic acid-induced suppression of glucose/forskolin-stimulated insulin release was completely prevented by the presence of gAcrp30 (Fig. 5). Leptin was able to fully reverse palmitic acid-mediated impairment of insulin secretion with no significant effect on cytokine-induced beta-cell dysfunction (Fig. 5).

Discussion

In this investigation we have identified a novel function of the C-terminal globular domain of adiponectin, gAcrp30, consisting of the inhibition of cytokine- and fatty acid-induced apoptosis and dysfunction of pancreatic beta cells. Adiponectin is structurally similar to complement factor C1q consisting of a N-terminal collagenous region and a trimeric C-terminal globular domain and undergoes oligomerization which probably exerts a regulatory function on its activity [37, 38]. Recent work describes potent physiological activities of this fat-derived hormone involving regulation of glucose and lipid metabolism [39]. Adiponectin serum concentrations are reduced in a variety of obese and insulin resistant states [40] and very recent data suggest that this adipokine could represent a link between intra-abdominal fat, insulin resistance and an atherogenic lipoprotein profile in humans [41]. Indeed, treatment with recombinant adiponectin has been shown to ameliorate insulin resistance in obese and lipoatrophic mice by increasing fatty acid oxidation in muscle [42]. Recent observations provide evidence that a proteolytic cleavage product of human adiponectin including the C-terminal domain (gAcrp30) circulates in human plasma at low abundance [43]. This globular gAcrp30 has been described to be more potent than full-length Acrp30 in increasing fatty acid oxidation in muscle and causing weight loss in mice [43]. Very recently, two receptors for adiponectin (AdipoR1 and R2) have been identified with tissue-specific expression and distinct ligand binding affinities despite the high structural homology [44]. According to this study, the AdipoR1 which is most abundantly expressed in skeletal muscle, represents a high-affinity receptor for globular adiponectin concomitantly exerting only a low binding affinity for the full-length protein, which might explain the reported stronger signalling potency of gAcrp30 in muscle. To our knowledge, we report here for the first time on a beta-cell protective action of gAcrp30 against cytokine- and fatty acid-induced apoptosis.

Proinflammatory cytokines are considered to play a crucial role in the autoimmune-mediated beta-cell apoptosis leading to Type 1 diabetes development [2], whereas glucotoxicity and lipotoxicity represent the principal contributors involved in beta-cell failure in Type 2 diabetes [45]. Moreover, fatty acid-induced beta-cell apoptosis has been associated with obesity-related Type 2 diabetes [5, 6]. Consistently, in our study both the cytokine combination IL-1β/IFN-γ and palmitic acid induced a prominent apoptosis in the rat pancreatic beta-cell line INS-1, as reflected by a strong increase in caspase 3 activation and DNA fragmentation, respectively. Leptin exerted a prominent inhibitory function against palmitic acid-induced apoptosis both at the caspase 3 and DNA fragmentation level. This is consistent with previous observations of the beta-cell protective function of leptin against lipoapoptosis through triglyceride depletion and increases in fatty acid oxidation [12]. In addition, prevention of fatty acid-induced Bcl-2 suppression was also found to be a critical step of leptin-mediated beta-cell anti-lipoapoptosis [5]. The present results clearly show a novel anti-apoptotic function of leptin against cytokine-induced beta-cell-programmed cell death,

consisting of a significant reduction of IL-1β/IFN-γ-induced apoptosis in rat pancreatic beta cells. A key finding of the present investigation consists of the observation that gAcrp30 partially rescues beta cells against both cytokine- and fatty acid-induced apoptosis. The dose-dependent suppression of palmitic acid-mediated beta-cell apoptosis by gAcrp30 reached a maximum at 10 nmol/l. This is in good correlation with the reported maximal activity of gAcrp30 towards fatty acid oxidation in muscle at a concentration of around 5 nmol/l [25]. Consistent with our findings it has recently been reported that overexpression of gAcrp30 could prevent diabetes in vivo by increasing not only insulin sensitivity but also plasma insulin levels, in contrast to the action of PPAR γ agonists, thus suggesting a direct protective effect of this adipokine on beta-cells [46]. We did not observe an additivity between the anti-apoptotic functions of gAcrp30 and leptin against cytokine- and fatty acid-induced apoptosis suggesting a common signalling pathway for these adipokines in pancreatic beta cells.

Overproduction of NO is known to induce apoptosis in beta cells [11, 47] with NF-κB activation representing a critical event in the enhanced expression of inducible nitric oxide synthase (iNOS), at least in response to proinflammatory cytokines [2]. Recent findings show that fatty acid-induced beta-cell apoptosis is mediated via de novo ceramide formation, which activates NF-κB-mediated up-regulation of iNOS expression at least in response to palmitic acid [6]. The prominent suppression of apoptosis by a highly specific IKK-inhibitor observed in the present study confirms the pivotal role of the NF-κB pathway in cytokine- and fatty acid-induced beta-cell death. A partial protection against cytokine-induced apoptosis was also obtained in human islets [48] and the pancreatic beta-cell line MIN6 [49] by blocking NF-κB activation. However, cytokine-induced NF-κB DNA-binding activity was not affected by gAcrp30 or leptin, indicating that the adipokines mediate their anti-apoptotic actions without modulating NF-κB activation. In contrast, full-length adiponectin has been shown to exert putative anti-atherogenic properties by suppressing TNF-α-induced IκB-α phosphorylation and subsequent NF-κB activation in endothelial cells, thus inhibiting inflammatory-induced gene transcription [16]. However, recent work provides evidence for an oligomerization state-dependent stimulation of NF-κB signalling by adiponectin in muscle cells showing that only hexameric and larger isoforms of Acrp30 stimulate this pathway, whereas trimeric Acrp30 or gAcrp30 do not affect the activation of this transcription factor [38], which correlates with our observation. Certainly, we cannot rule out a cross-talk between signalling pathways of gAcrp30 and NF-κB at a level downstream of the activation of this transcription factor.

In addition to the anti-apoptotic action of gAcrp30 we also provide evidence for a protective role of this adipokine against both cytokine- and fatty acid-induced functional impairment in beta cells. Thus, the IL-1β/IFN-γ- and palmitic acid-mediated strong suppression of glucose/forskolin-stimulated insulin secretion was completely prevented by the presence of gAcrp30. In contrast, leptin only ameliorated fatty acid-induced beta-cell dysfunction. In contrast, a recent study has reported that leptin could not ameliorate lipotoxicity, however at lower concentrations than used in this study [32]. It is worth noting that the physiological relevance of leptin action for insulin secretion remains controversial due to a number of conflicting reports in the literature. Nonetheless, the main body of evidence suggests that physiological leptin concentrations in normal rodents do not affect glucose-induced insulin secretion but inhibit the potentiation of this pathway involving cAMP or PLC/PKC activation [35, 36], which agrees with our observation. It is likely that inherent characteristics of insulin-producing cells, being of different origins, and/or methodological differences in concentration and incubation period could explain the observed discrepancy in the actions of leptin on insulin secretion.

The impairment of glucose/forskolin-induced insulin secretion caused by cytokines and fatty acids may underlie: (i) a reduction of the intact functional beta-cell number either by an increased apoptosis- or a reduced proliferation-rate; (ii) an inhibition of the insulin secretory pathway; (iii) a suppression of insulin biosynthesis. Previous data have shown a lipotoxicity-induced decrease in ^{3}H-thymidine incorporation and a down-regulation of IRS-1/2 gene expression in INS-1 cells [50]. Further, suppression of the acetyl-CoA carboxylase expression with a resulting exaggerated fatty acid oxidation has been implicated as the critical trigger of lipotoxicity-mediated beta-cell dysfunction [51]. Cytokine-induced suppression of beta-cell sensitivity to glucose-induced insulin exocytosis has been associated with an impairment of mitochondrial function via the generation of NO and decrease in the ATP/ADP ratio in rat islets [34]. Recent studies provide evidence for a decreased insulin content in response to long-term exposure of rat pancreatic islets to proinflammatory cytokines and fatty acids [20, 26]. However, fatty acid-induced perturbation of insulin secretion and biosynthesis has been found not to correlate with the intracellular loss of the hormone [52]. Additional work is needed to identify the precise molecular mechanisms underlying the beta-cell functional impairment by cytokines and fatty acids and the protective effects of gAcrp30 and leptin against these processes. At the present stage we do not know if the protective effects of gAcrp30 and leptin against cytokine- and fatty acid-induced beta-cell dysfunction and apoptosis observed in the clonal pancreatic beta-cell line INS-1 are also operative in primary pancreatic

beta cells, and this limitation of our study must be taken into account.

In conclusion, this study shows that the C-terminal globular domain of adiponectin partially rescues beta cells from both cytokine- and fatty acid-induced apoptosis and completely restores functional impairment under pathological conditions involved in Type 1 and Type 2 diabetes. Thus, gAcrp30 might counteract autoimmune- and lipotoxicity-induced beta-cell destruction by affecting both function and survival of beta cells. Our data suggest that the beta-cell protective function of gAcrp30 is mediated without affecting NF-κB activation. Treatment with gAcrp30 in the prediabetic phase or replenishment of gAcrp30 in obesity and insulin-resistant states could provide a novel strategy for both Type 1 and Type 2 diabetes therapy.

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