Apolipoprotein CIII Reduces Proinflammatory Cytokine-Induced Apoptosis in Rat Pancreatic Islets via the Akt Prosurvival Pathway

Joachim Størling, Lisa Juntti-Berggren, Gunilla Olivecrona, Michala C. Prause, Per-Olof Berggren, and Thomas Mandrup-Poulsen

Hagedorn Research Institute (J.S., M.C.P., T.M.-P.), 2820 Gentofte, Denmark; Rolf Luft Center for Diabetes Research (L.J.-B., P.-O.B., T.M.-P.), Karolinska Institutet, SE-171 77 Stockholm, Sweden; Department of Medical Biosciences (G.O.), Physiological Chemistry, Umeå University, 901 87 Umeå, Sweden; Center for Medical Research Methodology (T.M.-P.), Institute of Biomedical Science, University of Copenhagen, DK-2200 Copenhagen, Denmark; and Glostrup Research Institute (J.S.), Glostrup University Hospital, DK-2600 Glostrup, Denmark

Apolipoprotein CIII (ApoCIII) is mainly synthesized in the liver and is important for triglyceride metabolism. The plasma concentration of ApoCIII is elevated in patients with type 1 diabetes (T1D), and *in vitro* ApoCIII causes apoptosis in pancreatic β-cells in the absence of inflammatory stress. Here, we investigated the effects of ApoCIII on function, signaling, and viability in intact rat pancreatic islets exposed to proinflammatory cytokines to model the intraislet inflammatory milieu in T1D. In contrast to earlier observations in mouse β-cells, exposure of rat islets to ApoCIII alone (50 μg/ml) did not cause apoptosis. In the presence of the islet-cytotoxic cytokines IL-1β + interferon-γ, ApoCIII reduced cytokine-mediated islet cell death and impairment of β-cell function. ApoCIII had no effects on mitogen-activated protein kinases (c-Jun N-terminal kinase, p38, and ERK) and had no impact on IL-1β-induced c-Jun N-terminal kinase activation. However, ApoCIII augmented cytokine-mediated nitric oxide (NO) production and inducible NO synthase expression. Further, ApoCIII caused degradation of the nuclear factor κB-inhibitor inhibitor of κB and stimulated Ser473-phosphorylation of the survival serine-threonine kinase Akt. Inhibition of the Akt signaling pathway by the phosphatidylinositol 3 kinase inhibitor LY294002 counteracted the antiapoptotic effect of ApoCIII on cytokine-induced apoptosis. We conclude that ApoCIII in the presence of T1D-relevant proinflammatory cytokines reduces rat pancreatic islet cell apoptosis via Akt. **(*Endocrinology* 152: 3040–3048, 2011)**

Type 1 diabetes (T1D) is characterized by lack of insulin production due to an immune-mediated destruction of the insulin-producing pancreatic β-cells in the islets of Langerhans. In the process of β-cell destruction, proinflammatory cytokines, such as IL-1β and interferon (IFN)γ, have been implicated as effector molecules (1).

The plasma concentration of apolipoprotein CIII (ApoCIII) is elevated in T1D (2–5). ApoCIII resides on the surface of triglyceride-rich lipoproteins and high-density lipoproteins and is important in triglyceride metabolism (6). ApoCIII is mainly expressed in the liver, and overexpression of ApoCIII in mice causes hypertriglyceridemia due to an increase in the number of very low-density lipoprotein particles (7). In contrast, disruption of the ApoCIII gene results in hypotriglyceridemia (8). The effect of ApoCIII on plasma triglyceride levels is mediated by at least two different mechanisms: 1) inhibition of lipoprotein lipase activity, and 2) interference with the binding of lipoprotein particles to endothelial proteoglycans and specific lipoprotein receptors (9–12). *In vitro* ApoCIII also stimulates monocyte adhesion to vascular endothelial cells (13). Several reports have shown that *in vitro*, ApoCIII

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2010-1422 Received December 10, 2010. Accepted May 27, 2011.
First Published Online June 21, 2011

Abbreviations: Akt, Serine-threonine kinase; ApoCIII, apolipoprotein CIII; IκB, inhibitor of κB; IFN, interferon; IKK, IκB kinase; iNOS, inducible NO synthase; JNK, c-Jun N-terminal kinase; NFκB, nuclear factor κB; NO, nitric oxide; PI3K, phosphatidylinositol 3 kinase; T1D, type 1 diabetes; TLR, Toll-like receptor.

alone mimic the effects exerted by ApoCIII-containing lipoprotein particles (13–15).

ApoCIII induces apoptosis in the rat insulin-secreting cell lines RINm5F (2) and INS-1E (16) and in primary mouse β-cells not exposed to inflammatory stress (17). Moreover, haplotypes in the ApoCIII gene are associated with T1D (18). These findings collectively suggest that ApoCIII plasma levels and effects on β-cells may be of relevance to β-cell failure and destruction in T1D. However, the effects of ApoCIII on islets and β-cells in a T1D-relevant inflammatory environment, *i.e.* in the presence of proinflammatory cytokines, are unknown. Likewise, the molecular mechanisms explaining ApoCIII actions in β-cells are unclear.

In the current study, we investigated the functional effects and signaling mechanisms of ApoCIII alone or in the presence of proinflammatory cytokines in isolated intact rat islets. We find that in an inflammatory context, ApoCIII reduces islet cell apoptosis, which is associated with S473-phosphorylation of the serine-threonine kinase Akt. We speculate that the differential action of ApoCIII on β-cells in the presence or absence of inflammatory stress depends on a priming effect of ApoCIII on β-cell defense pathways that counteract cytokine toxicity.

Materials and Methods

Recombinant mouse IL-1β was obtained from BD Pharmingen (San Diego, CA), and recombinant rat IFNγ was from R&D Systems (Minneapolis, MN). ApoCIII (isoform 1) was purified from human serum by adsorption to a lipid emulsion and delipidation, followed by chromatography of the lipid-associated proteins under denaturing conditions in guanidium chloride and urea, respectively, as described (19). ApoCIII was dissolved in H_2O containing 0.1% trifluoric acid to a 2.5 μg/μl stock and stored at −80 C until use. The concentration chosen was 50 μg/ml based on the actual serum levels of ApoCIII found in plasma of recent-onset T1D subjects and similar to concentrations used on β-cell lines and ob/ob mouse β-cells (2). All reagents for SDS-PAGE were from Invitrogen (Carlsbad, CA). Antibody against inhibitor of κB (IκB) was obtained from Active Motif (Carlsbad, CA). Antibodies to Ser473-phosphorylated Akt, total Akt, Thr183/Tyr185-phosphorylated c-Jun N-terminal kinase (JNK)1/2, total JNK1/2, Thr202/Tyr204-phosphorylated ERK1/2, total ERK1/2, Thr180/Tyr182-phosphorylated p38, and total p38 were obtained from Cell Signaling (Beverly, MA). The phosphatidylinositol 3 kinase (PI3K) inhibitor LY294002, IκB kinase (IKK) inhibitor X, and the L-type Ca^{2+} channel blocker nimodipine were from Calbiochem (San Diego, CA) and were dissolved in dimethylsulfoxide.

Islet isolation and preculture

Pancreata from approximately 5-d-old outbred Wistar rats (Taconic, Hudson, NY) were collagenase digested and islets isolated by Percoll gradient purification (GE Healthcare, Princeton, NJ). Islets were precultured for approximately 5–7 d in complete RPMI 1640 medium (11 mM glucose) containing 10% newborn calf serum and 100 U/ml penicillin and 100 μg/ml streptomycin (all from Invitrogen) at 37 C in a humidified atmosphere of 5% CO_2. On the day of experimentation, islets were washed in fresh culture medium, handpicked, and placed in appropriate dishes (Nunc, Auburn, AL) in RPMI 1640 medium supplemented with 10% fetal calf serum and penicillin + streptomycin. A similar volume of H_2O with 0.1% trifluoric acid or dimethylsulfoxide was added to all conditions as control for the solvents used to dissolve the ApoCIII and the pharmacological inhibitors. In all experiments with ApoCIII and cytokines, islets were first preincubated overnight with ApoCIII alone before coexposure to cytokines and ApoCIII for various time periods.

Measurement of islet apoptosis

Islet apoptotic cell death was determined by Cell Death Detection assay (Roche, Indianapolis, IN) according to the manufacturer's protocol. Briefly, 10 or 25 islets in duplicates were exposed to the desired experimental conditions for the specified time periods. In all experiments with cytokines, islets were preexposed to 50 μg/ml ApoCIII overnight before addition of cytokines. In addition, solvents used for ApoCIII, LY294002 or nimodipine were added to all conditions in relevant experiments. Islets were then transferred to 1.5 ml Eppendorf tubes and mildly centrifuged to pellet the islets. Culture medium was removed and islets lysed in 200 μl of lysis buffer. Lysates were then centrifuged at 200 × *g* to pellet the nuclei, and 20 μl of the supernatant were loaded on a streptavidin-coated multiwell dish. After 2 h of incubation with biotin-conjugated antihistone antibody and peroxidase-conjugated anti-DNA antibody, 2,2'-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt substrate was added and absorbance measured at 405 and 490 nm. After correction for background absorbance, apoptosis rates were calculated as specified by the manufacturer and presented either as fold induction compared with untreated control islets or as percentage of islets exposed to cytokines.

Insulin measurement

Accumulated insulin release to the culture medium from 25 islets in duplicates was measured by competitive ELISA as described previously (20).

Western blotting

For preparation of whole islet lysates, 100–300 islets per condition were used. Islets were lysed for 30 min on ice in lysis buffer containing 20 mM Tris-acetate (pH 7.0), 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1% vol/vol Triton X-100, 5 mM sodium pyrophosphate, 10 mM sodium glycerophosphate, 1 mM benzamidine, 1 mM dithiothreitol, 1 mM Na_3VO_4, and 4 μg/ml leupeptin. Detergent-insoluble material was pelleted by centrifugation at 15,000 × *g* for 5 min at 4 C. After protein concentration measurements of the lysates by the Bradford method, SDS-PAGE was performed, and care was taken to load exact equal protein amounts of the lysates. After transfer to nitrocellulose membranes, membranes were probed with desired primary antibodies overnight. Membranes were then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h. Chemiluminescence was captured digitally using a Fuji LAS3000 (Fujifilm, Tokyo, Japan). Quantification of band intensities was done using Multi Gauge software (Fujifilm). Control experiments where the nitrocellu-

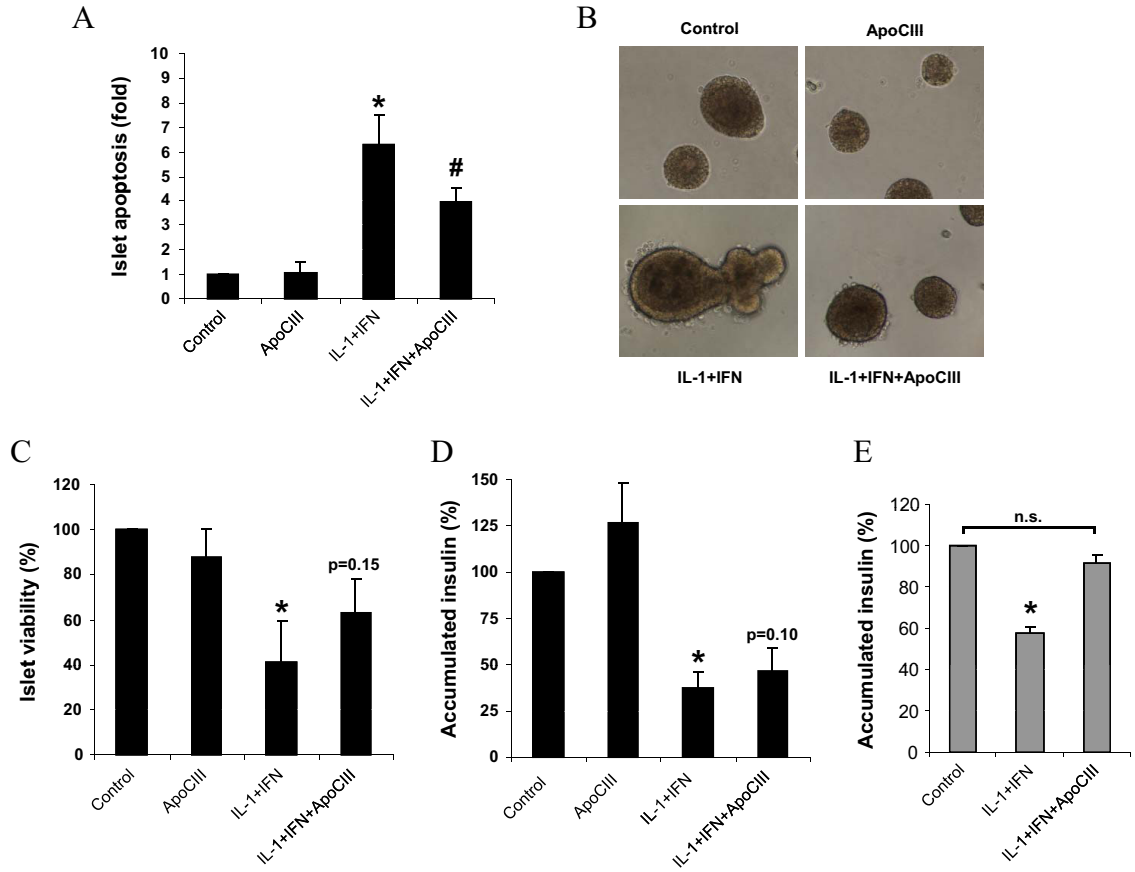

FIG. 1. ApoCIII reduces islet apoptosis in response to cytokines. A, Islets were preexposed to 50 μg/ml ApoCIII overnight before exposure to IL-1β (150 pg/ml) + IFNγ (5 ng/ml) for 24 h. After islet lysis, apoptotic cell death was determined by measurements of histone-DNA complexes present in the cytoplasmic fraction of cells. Data are means ± SE of n = 5. B, Representative images of islets taken after treatment as in A. C, Islets were preexposed to 50 μg/ml ApoCIII overnight before exposure to IL-1β (150 pg/ml) + IFNγ (5 ng/ml) for 2 d. Mitochondrial activity was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data are means ± SE of n = 3–5 for each condition. D, Accumulated insulin in the culture medium from the experiment in C was measured. E, Islets were preexposed to 50 μg/ml ApoCIII overnight before exposure to IL-1β (150 pg/ml) + IFNγ (5 ng/ml) for 24 h. Accumulated insulin in the culture medium was measured. Data are means ± SE of n = 3. In all panels, *, $P \leq 0.05$ *vs.* control; # $P = 0.02$ *vs.* IL-1β + IFNγ. n.s., Non-significant.

lose membranes were stained by the protein dye Ponceau were performed to assure equal protein loading.

Statistical analyses

Statistical significance of differences between groups was determined by paired one- or two-tailed *t* test where appropriate.

Results

ApoCIII reduces rat islet cell apoptosis in the presence of proinflammatory cytokines

To examine whether ApoCIII affects islet function and viability in the presence or absence of proinflammatory cytokines, we preincubated intact rat islets overnight with ApoCIII (50 μg/ml) and then exposed them to a combination of IL-1β (150 pg/ml) and IFNγ (5 ng/ml) in the continued presence of ApoCIII for 24 h. The concentration of 50 μg/ml ApoCIII is in the range of what we have used previously for *in vitro* experiments and is based on the actual serum levels of ApoCIII found in type 1 diabetics (2). As opposed to what has been observed in insulin-secreting cell lines and purified mouse β-cells, exposure of intact healthy rat islets to ApoCIII alone did not cause apoptotic cell death (Fig. 1A). As expected, cytokines significantly increased islet apoptosis. When coincubated with cytokines, ApoCIII reduced the cytotoxic effect of cytokines by approximately 50%, indicating an antiapoptotic effect of ApoCIII in the presence of cytokines. Consistent with this, islets exposed to cytokines alone had markedly disrupted morphology and tended to aggregate, a phenomenon that was partly prevented by ApoCIII (Fig. 1B). We also measured islet mitochondrial activity by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and did not observe any effect of exposure of islets to ApoCIII for 3 d (Fig. 1C). Cytokines reduced mitochondrial activity to 41% of control islets, whereas in the presence of ApoCIII, cytokines reduced mitochondrial activity to 63% of control islets (Fig. 1C). Finally, we measured whether insulin release to the culture medium was affected by ApoCIII in the presence or absence of cytokines. Islets

exposed for 3 d to ApoCIII alone released a similar amount of insulin compared with untreated control islets (Fig. 1D). Cytokines caused a pronounced suppression of islet insulin release (to 38% of control islets), which was slightly reversed (to 47%) by coincubation with ApoCIII. Reducing the exposure time to 24 h resulted in complete protection against cytokine-induced suppression of insulin release by ApoCIII (Fig. 1E). We also performed an experiment on isolated islets from adult mice (NMRI) to exclude that the protective effect of ApoCIII on cytokine-stimulated apoptosis was specific for rat islets. We found that exposure of mouse islets to IL-1β (150 pg/ml) plus IFNγ (5 ng/ml) for 24 h caused a 2.29-fold increase in apoptosis, which was reduced to 1.49-fold when islets were coincubated with cytokines and ApoCIII (n = 1). Collectively, these findings suggest that ApoCIII affords protection against cytokine-induced islet cell death and impairment of β-cell function.

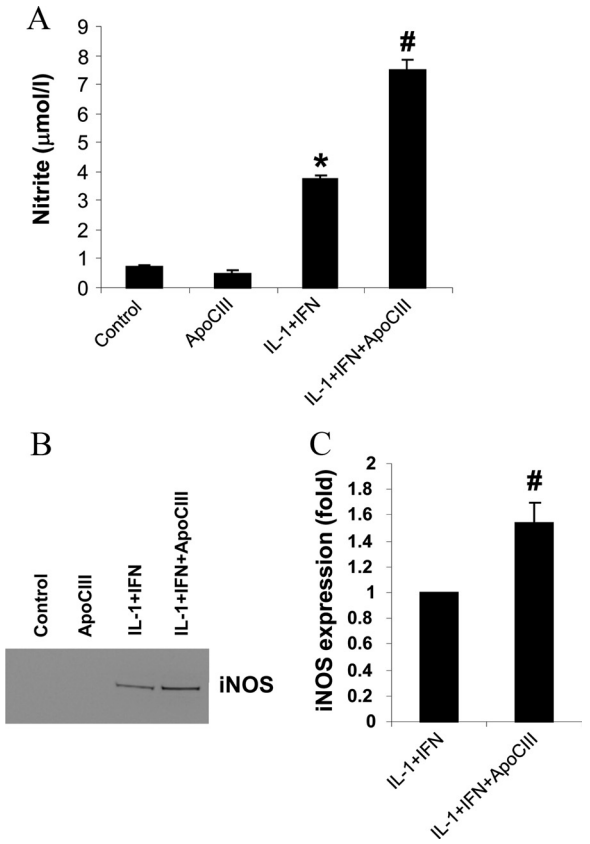

FIG. 2. ApoCIII increases cytokine-induced islet NO production and iNOS expression. A, Islets were preexposed to 50 μg/ml ApoCIII overnight before exposure to IL-1β (150 pg/ml) + IFNγ (5 ng/ml) for 2 d. NO production was determined as accumulated nitrite in the culture medium. Data are means ± SE of n = 3. B, Islets were preexposed to 50 μg/ml ApoCIII overnight before exposure to IL-1β (150 pg/ml) + IFNγ (5 ng/ml) for 24 h. Islet lysates were subjected to immunoblot analysis using an anti-iNOS antibody. Immunoblot shown is representative of seven separate experiments. C, Quantitative analysis of iNOS expression. Data are means ± SE of n = 7. In A and C, *, $P < 0.005$ *vs.* control; and #, $P < 0.005$ *vs.* IL-1 + IFN.

Cytokine-induced nitric oxide (NO) production is augmented by ApoCIII

Because the cytotoxic effects of cytokines on rodent islet cells are at least partly mediated via generation of NO (21), a possible mechanism behind ApoCIII-mediated antiapoptotic effects could be via inhibition of NO production. To address this, we determined whether ApoCIII modulates islet production of NO after exposure to cytokines. Surprisingly, we found that IL-1β + IFNγ-induced islet NO production as determined by accumulated nitrite in the culture medium was augmented by ApoCIII coincubation (Fig. 2A). Consistently, cytokine-induced expression of inducible NO synthase (iNOS) enzyme as determined by immunoblotting was increased 54% by ApoCIII (Fig. 2, B and C). These results suggest that ApoCIII increases cytokine-induced iNOS expression and NO production in rat islets.

Because we have previously reported that blocking Ca^{2+} influx via L-type Ca^{2+} channels can prevent effects of ApoCIII (2), we next examined whether L-type Ca^{2+} influx mediates the effects of ApoCIII in rat islets. In accordance with our previous findings on insulin-secreting cells (22), the L-type Ca^{2+} channel blocker nimodipine (10 μM) reduced cytokine-induced islet apoptosis (Fig. 3). However, in combination with ApoCIII, nimodipine did not further modulate the protective effect of ApoCIII, suggesting that L-type channel Ca^{2+} influx is not required for the antiapoptotic effect of ApoCIII.

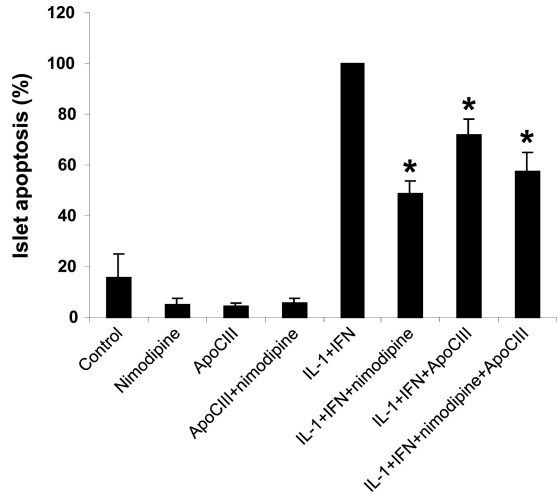

FIG. 3. Calcium channel inhibition does not modulate the protective effect of ApoCIII. Islets were preexposed to 50 μg/ml ApoCIII in the presence or absence of 10 μM of the Ca^{2+} channel blocker nimodipine overnight before exposure to IL-1β (150 pg/ml) + IFNγ (5 ng/ml) for 24 h. After islet lysis, apoptotic cell death was determined by measurements of histone-DNA complexes present in the cytoplasmic fraction of cells. Apoptosis rate in islets treated with cytokines alone was set to 100%. Data are means ± SE of n = 3. *, $P < 0.05$ *vs.* IL-1β + IFNγ.

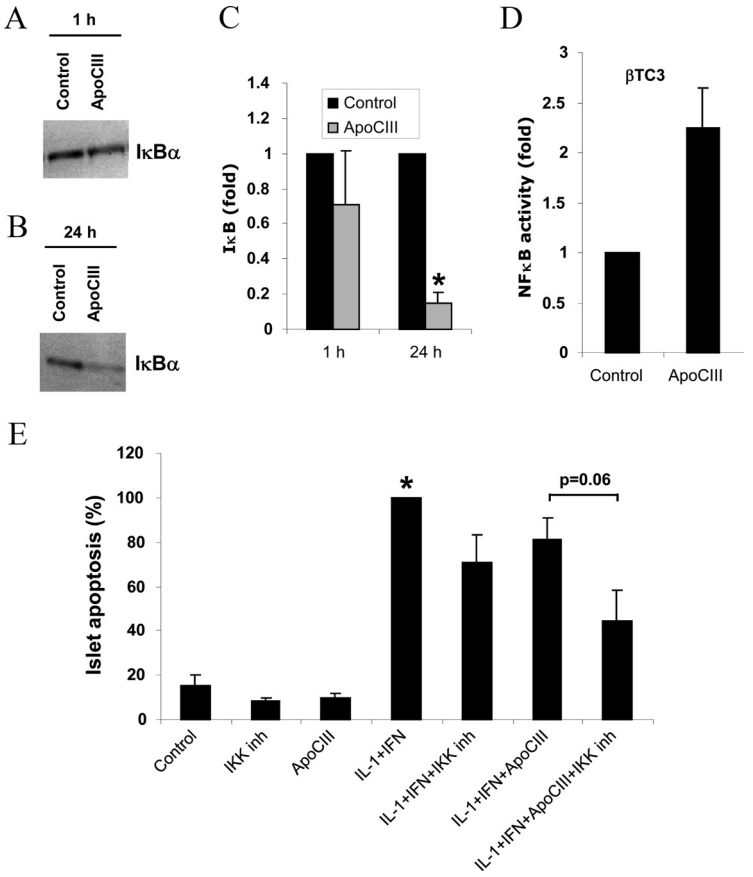

FIG. 4. ApoCIII stimulates IκB degradation in islets. Islets were exposed to 50 μg/ml ApoCIII for 1 h (A) or 24 h (B). Islet lysates were subjected to immunoblot analysis using an anti-IκB antibody. Immunoblots shown are representative of three separate experiments. C, Quantitative analysis of IκB degradation. Data are means ± SE of n = 3. *, $P < 0.01$ *vs.* control. D, βTC3 cells were transfected with a NFκB-driven luciferase reporter gene construct. Cells were left untreated or exposed to IL-1β (150 pg/ml) for 4 h. Luciferase activity was determined in lysates, and data are mean of two experiments. E, Islets were preexposed to 50 μg/ml ApoCIII in the presence or absence of 10 μM IKK inhibitor X overnight before exposure to IL-1β (150 pg/ml) + IFNγ (5 ng/ml) for 24 h. After islet lysis, apoptotic cell death was determined by measurements of histone-DNA complexes present in the cytoplasmic fraction of cells. Apoptosis rate in islets treated with cytokines alone was set to 100%. Data are means ± SE of n = 4. *, $P < 0.05$ *vs.* control.

ApoCIII activates nuclear factor κB (NFκB)

To examine other potential mechanisms underlying the antiapoptotic effect of ApoCIII, we investigated the possible effects of ApoCIII on the NFκB and MAPK signaling pathways. Both of these pathways have been established as crucial signaling mediators in response to different stimuli, including cytokines, glucose, and hormones in pancreatic β-cells. There was no significant effect of ApoCIII on the degradation of the NFκB inhibitor IκB in islets exposed to ApoCIII for 1 h (Fig. 4, A and C). However, a 24-h exposure to ApoCIII caused a pronounced degradation of IκB (Fig. 4, B and C), indicative of NFκB activation. We also examined the ability of ApoCIII to stimulate NFκB activity in insulin-secreting βTC3 cells by reporter gene assay using an NFκB-driven luciferase construct. It was observed that ApoCIII stimulated approximately a 2-fold up-regulation in NFκB activity (Fig. 4D). To investigate the contribution of NFκB to the protective effect of ApoCIII on cytokine-induced islet apoptosis, we blocked NFκB signaling using a small molecule inhibitor of the IκB kinase (IKK). We observed a strong tendency for synergistic protection against cytokine-induced apoptosis by IKK inhibition and ApoCIII (Fig. 4E).

ApoCIII does not affect IL-1β-induced JNK activation

Because a previous study suggested that ApoCIII stimulates activation the p38 MAPK in insulin-secreting INS-1E cells (16), we sought to investigate whether ApoCIII can activate MAPK in rat islets. Thus, islets were exposed to ApoCIII for 24 h, and then the contents of phosphorylated, activated forms of ERK1/2, p38, and JNK1/2 were determined in islet extracts. In contrast to the clear effect of a 1-h exposure to IL-1β, which was used as positive control, there were no effects of ApoCIII on either of the MAPK (Fig. 5, A and B). Thus, at least within the same time and dose window where ApoCIII caused IκB degradation, ApoCIII did not stimulate phosphorylation of MAPK in islets.

Because ApoCIII appeared not to have effects on MAPK alone, we next examined whether overnight pretreatment with ApoCIII modulates IL-1β-induced MAPK activation. We focused on JNK, because this MAPK is most important for cytokine-induced β-cell death. By both immunoblotting of phosphorylated JNK1/2 and by *in vitro* kinase assay on islet extracts using c-Jun as substrate, we found no evidence for effects of ApoCIII on JNK activation by IL-1β (Fig. 6, A–C). This finding suggest that the antiapoptotic effect of ApoCIII is not associated with modulation of cytokine-induced activation of JNK.

ApoCIII stimulates phosphorylation of Akt

Protein kinase B/Akt is activated in a PI3K-dependent manner and involved in cell survival and growth (23). We have found previously that cytokines inhibit the activity of Akt in pancreatic β-cells, thereby leading to apoptosis, an effect antagonized by IGF-I (24). We investigated whether ApoCIII had an impact on Akt activity as determined by Ser473-phosphorylation. It was observed that ApoCIII caused an increase in S473-phosphorylated Akt (Fig. 7, A and B), suggesting activation of the PI3K/Akt pathway. To

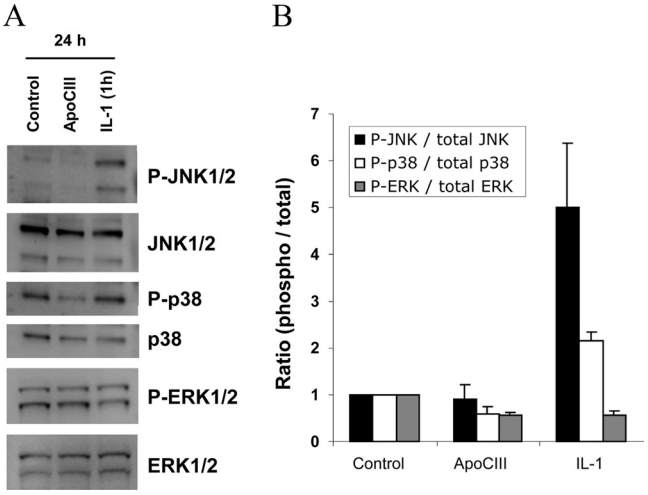

FIG. 5. ApoCIII does not stimulate phosphorylation of MAPK in islets. A, Lysates from islets exposed to 50 μg/ml ApoCIII for 24 h or to 150 pg/ml IL-1β for 1 h (positive control stimulus) were subjected to immunoblot analysis using antibodies to phosphorylated (P) or total forms of JNK, p38, and ERK. Immunoblots shown are representative of four separate experiments. B, Quantitative analyses of phosphorylated and total JNK, p38, and ERK. Data are means ± SE of n = 4.

get insight into the mechanisms underlying ApoCIII-stimulated Akt phosphorylation, we examined the involvement of NFκB. We found that an inhibitor of IKK resulted in a reduction in ApoCIII-induced Ser473-phosphorylation of Akt (n = 3) (data not shown). To examine the contribution of increased Akt signaling to the antiapoptotic effect of ApoCIII, we took advantage of LY294002, an inhibitor of PI3K that prevents phosphorylation and activation of Akt. As seen in Fig. 7C, the antiapoptotic effect of ApoCIII was counteracted by coincubation with 1 μM LY204002. These findings indicate that ApoCIII exerts its antiapoptotic effect, at least partly, via the PI3K/Akt signaling pathway.

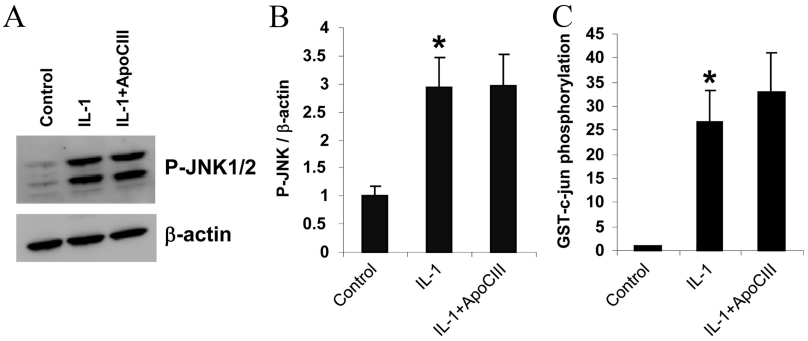

FIG. 6. ApoCIII does not modulate IL-1β-induced phosphorylation (P) of JNK. A, lysates from islets exposed to 50 μg/ml ApoCIII overnight and then to 150 pg/ml IL-1β for 1 h were subjected to immunoblot analysis using antibodies to phosphorylated or total forms of JNK1/2. Immunoblots shown are representative of three experiments. B, Quantitative analyses of phosphorylated and total JNK, p38, and ERK. Data are means ± SE of n = 3. C, islets were preexposed to 50 μg/ml ApoCIII overnight before exposure to 150 pg/ml IL-1β for 1 h. After islet lysis, JNK activity in crude lysates was measured by *in vitro* kinase assay using glutathione-S-transferase-c-Jun as substrate. Data are means ± SE of n = 4. *, $P < 0.05$ *vs.* control.

Discussion

The plasma concentration of ApoCIII is elevated in T1D and may potentially influence β-cell function and apoptosis. However, the functional effects of ApoCIII on pancreatic β-cells exposed to inflammatory stress have not been studied. Here, we examined effects of ApoCIII on intact rat pancreatic islets exposed to proinflammatory cytokines to mimic the intraislet inflammatory environment in T1D. Our findings provide evidence that in isolated rat islets exposed to inflammatory stress, ApoCIII reduces cytokine-mediated apoptosis, at least in part via the survival kinase Akt.

We found that exposure to ApoCIII (50 μg/ml) had no impact on rat islet apoptosis or chronic insulin release. These findings indicate that in contrast to previous findings obtained with the insulin-secreting cell lines RINm5F and INS-1E, and with primary single mouse β-cells, where ApoCIII induced cell death (2, 16, 17), ApoCIII does not have cytotoxic effects on intact whole rat islets. There might be several explanations for these different effects of ApoCIII. First, there could be species and age differences. In the present study, we used islets from neonatal Wistar rats, whereas we used islet cells from adult ob/ob mice in a previous study (17). Second, the use of single islet cells or tumorigenic cells *vs.* intact whole islets could make a difference as to the functional effects of ApoCIII and to the effectiveness whereby ApoCIII affects the β-cells. Third, pretreatment and exposure times to ApoCIII as well as source of ApoCIII and differences in culture conditions could potentially impact on the outcome of ApoCIII effects. Whether ApoCIII has antiapoptotic effects under inflammatory conditions in other cell types and tissues than islets is currently not known and should be examined.

Caution should be exerted when extrapolating *in vitro* findings to *in vivo* observations in T1D patients. Obviously, the inflammatory context in the present study is a simplified model of *in vivo* conditions during inflammation. For example, increased concentrations of ApoCIII *in vivo* may affect β-cell function at several additional levels than those examined in the present study. First, because overexpression of ApoCIII causes hypertriglyceridemia (7) and because it is well known that free fatty acids derived from triglycerides are known to impair β-cell function and cause β-cell apoptosis (25), one could imagine that increased levels of ApoCIII indirectly by causing higher levels of triglycerides has a negative effect on β-cell function and viability. In accordance with this, there is a correlation between higher ApoCIII serum levels and increased triglycerides in patients with

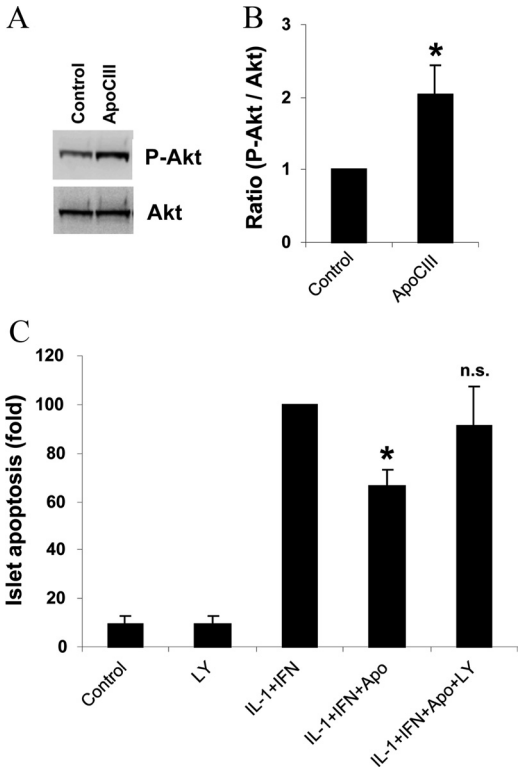

FIG. 7. ApoCIII stimulates phosphorylation (P) of Akt in islets. A, Islets were exposed to 50 μg/ml ApoCIII for 24 h. Islet lysates were subjected to immunoblot analysis using anti-P-Akt and anti-Akt antibodies. Immunoblots shown are representative of five separate experiments. B, Quantitative analysis of Akt phosphorylation. Data are means ± SE of n = 5. *, $P = 0.02$ *vs.* IL-1β + IFNγ. C, Islets were left untreated or preexposed to 1 μM LY294002 for 1 h and then exposed to 50 μg/ml ApoCIII overnight before exposure to IL-1β (150 pg/ml) + IFNγ (5 ng/ml) for 24 h. After islet lysis, apoptotic cell death was determined by measurements of histone-DNA complexes present in the cytoplasmic fraction of cells. Data are means ± SE of n = 4. *, $P = 0.02$ *vs.* IL-1β + IFNγ. n.s., Nonsignificant *vs.* IL-1β + IFNγ.

T1D (26). Second, human THP-1 monocytes are activated by ApoCIII (13), and in endothelial cells, ApoCIII causes up-regulation of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1, leading to increased monocyte adhesion to endothelial cells (14). If ApoCIII also up-regulates intercellular adhesion molecule-1 in β-cells is unclear, but if so, such an effect in combination with ApoCIII-mediated monocyte activation would led to increased islet immune infiltration, an effect that most likely would have detrimental effects on the β-cells in the presence of an intact immune system *in vivo*. Third, even though IL-1β + IFNγ is a highly relevant and widely used T1D *in vitro* stimulus, T1D pathogenesis *in vivo* is much more complicated, and many other immune mediators and mechanisms are involved in causing β-cell destruction. Thus, taking these reflections and our present findings into consideration, it seems plausible that the functional effects of ApoCIII on β-cells are highly dependent on the exact biological context, *i.e.* cosignals, the inflammatory state, and stimulus-secretion coupling activity. It will therefore be of high importance to examine the role and effects of ApoCIII *in vivo* in relevant animal models of T1D. For example, it should be tested whether injections of ApoCIII into NOD mice can prevent or revert diabetes. It would also be interesting to generate NOD mice transgenic for ApoCIII to see how this will affect diabetes development.

ApoCIII caused PI3K-dependent Ser473-phosphorylation of Akt in islets. Akt is known to be activated by various mitogenic and antiapoptotic stimuli in β-cells and to be deactivated by diverse apoptotic stimuli (23, 24, 27, 28). Opposite to our findings in islets, it has been reported that ApoCIII decreases insulin-induced phosphorylation of Akt in vascular endothelial cells (29). Hence, the activating effect of ApoCIII on Akt may be specific for islets/β-cells. Consistent with an antiapoptotic function of Akt in islets, the PI3K inhibitor LY294002 abrogated the antiapoptotic effect of ApoCIII, suggesting that Akt is required for the protective effect of ApoCIII. In addition to activation of Akt, ApoCIII also augmented cytokine-induced iNOS expression and NO production, which is normally associated with proapoptotic signaling and necrosis in β-cells. This may therefore seem a paradox, *i.e.* that ApoCIII both causes increased survival signaling and proapoptotic signaling, but we speculate that the activation of Akt survival signaling outweighs the apoptotic and necrotic contributions of NO. The relative contribution of activated proapoptotic *vs.* antiapoptotic pathways to

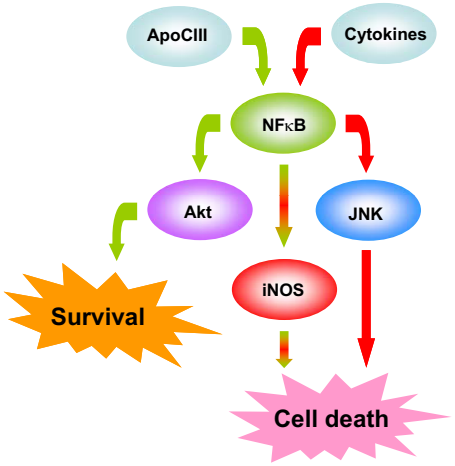

FIG. 8. Model for ApoCIII-mediated protection against cytokine-induced islet cell apoptosis. Both ApoCIII and cytokines activate NFκB. In the presence of ApoCIII alone, NFκB leads to activation of the Akt kinase, which signals survival and antiapoptosis. In the presence of both ApoCIII and cytokines, a more pronounced NFκB activation is induced compared with cytokines alone. Besides causing Akt activation, this increase in NFκB signaling causes a higher expression of iNOS and thus NO production compared with that stimulated by cytokines alone. Moreover, cytokines lead to proapoptotic signaling via JNK. Thus, in the presence of cytokines, ApoCIII signaling via Akt tips the balance toward survival despite of a higher production of NO. *Green arrows* indicate signaling induced by ApoCIII, whereas *red arrows* indicate signaling induced by cytokines. *Two-colored arrows* indicate signaling that are increased by both ApoCIII and cytokines.

ApoCIII effects on β-cells may vary between species and models, helping explain why ApoCIII has apoptotic effects in some systems and antiapoptotic effects in others. In Fig. 8, we suggest a model for how ApoCIII in the presence of proinflammatory cytokines may mediate its protective effects.

We found that ApoCIII caused degradation of IκB in pancreatic islets. This observation is consistent with recent studies showing that ApoCIII stimulates IκB degradation in endothelial cells (14, 15). The role of NFκB in β-cells is debated. Several studies have reported that β-cell NFκB activation in response to IL-1β with or without IFNγ is proapoptotic, because blockade of NFκB reduces cell death (30–32). However, others have reported that NFκB is antiapoptotic in response to TNFα + IFNγ (33, 34), and NOD mice with β-cell-specific NFκB blockade have accelerated diabetes, further suggesting an antiapoptotic effect of NFκB (34). Thus, whether NFκB signaling in β-cells is pro- or antiapoptotic depends on the specific biological settings and stimuli. The downstream events of ApoCIII-induced NFκB activation in rat islets are not known. Our observation that ApoCIII potentiates cytokine-induced iNOS expression could indicate that ApoCIII-mediated NFκB activation contributes to iNOS expression under inflammatory conditions. However, we also have unpublished data that indicate that an inhibitor of the upstream IKK decreases ApoCIII-mediated Ser473-phosphorylation of Akt (our unpublished data). This may suggest that ApoCIII-induced NFκB activation leads to activation of Akt and thereby protection against cytokine-mediated β-cell death.

At present, it is not known how ApoCIII activates signaling in islets. In THP-1 monocytes, ApoCIII activates NFκB via Toll-like receptor (TLR)-2 (35). Thus, ApoCIII-induced activation of NFκB and monocytes could be blocked by an anti-TLR2 blocking antibody, and ApoCIII effects were blocked in peripheral blood monocytes from TLR2-deficient mice. Whether TLR2, which is also expressed in islets and β-cells (36–38), mediates the signaling and functional effects of ApoCIII in islets and whether TLR2 priming may desensitize β-cells to proapoptotic cytokine signaling awaits clarification.

Of note, a study reported that a haplotype block in the promoter region of the ApoCIII gene is associated with T1D (18). This haplotype block contains two promoter variants within a negative insulin-response element. Both promoter variants attenuate the repression of promoter activity exerted by insulin (18). Hence, seen in the light of our findings, there may be an interesting relationship between ApoCIII and insulin levels and β-cell function. Thus, insulin secretion from β-cells may lead to suppression of hepatic ApoCIII expression, which may in turn modulate the susceptibility of the β-cells to undergo apoptosis under proinflammatory conditions. It is likely that both genetic and environmental factors that cause modulation of the balance between ApoCIII and insulin levels are of importance for β-cell destruction in T1D.

In conclusion, we show that ApoCIII stimulates IκB degradation and phosphorylation of the survival kinase Akt in primary pancreatic rat islets. These effects are associated with antiapoptotic effects under proinflammatory settings resembling those of T1D. Hence, our findings, together with previous knowledge of ApoCIII effects on β-cells, suggest that ApoCIII may have dual functional effects, the outcome determined by the exact biological context. Further investigations are needed to fully clarify the role and effects of ApoCIII in pancreatic β-cells, especially in relationship to the development of T1D.

Acknowledgments

We thank the technical expertise and help of Fie Hillesø, Tine Drengsgaard, and Hanne Foght.

Address all correspondence and requests for reprints to: Joachim Størling, Ph.D., Hagedorn Research Institute, Niels Steensens Vej 1, 2820 Gentofte, Denmark. E-mail: jstq@hagedorn.dk.

This work was supported by funding from The Danish Research Council, The Swedish Research Council, The Family Erling-Persson Foundation, The Swedish Diabetes Foundation, Bert von Kantzow's Foundation, Barndiabetesfonden, and Novo Nordisk A/S.

Disclosure Summary: J.S., T.M.-P., G.O., and L.J.-B. have nothing to disclose. P.-O.B. is involved in a biotech company, Biocrine, which is developing apolipoprotein CIII as a novel target for the treatment of diabetes and its complications.

References

1. **Donath MY, Størling J, Berchtold LA, Billestrup N, Mandrup-Poulsen T** 2008 Cytokines and β-cell biology: from concept to clinical translation. Endocr Rev 29:334–350
2. **Juntti-Berggren L, Refai E, Appelskog I, Andersson M, Imreh G, Dekki N, Uhles S, Yu L, Griffiths WJ, Zaitsev S, Leibiger I, Yang SN, Olivecrona G, Jörnvall H, Berggren PO** 2004 Apolipoprotein CIII promotes Ca2+-dependent β cell death in type 1 diabetes. Proc Natl Acad Sci USA 101:10090–10094
3. **Reverter JL, Sentí M, Rubiés-Prat J, Lucas A, Salinas I, Pizarro E, Pedro-Botet J, SanmartÆ A** 1993 Lipoprotein composition in the insulin-deficient non-acidotic phase of type I diabetic patients and early evolution after the start of insulin therapy. Clin Chim Acta 223:113–120
4. **Blackett P, Sarale DC, Fesmire J, Harmon J, Weech P, Alaupovic P** 1988 Plasma apolipoprotein C-III levels in children with type I diabetes. South Med J 81:469–473
5. **Briones ER, Mao SJ, Palumbo PJ, O'Fallon WM, Chenoweth W, Kottke BA** 1984 Analysis of plasma lipids and apolipoproteins in insulin-dependent and noninsulin-dependent diabetics. Metabolism 33: 42–49
6. **Fredenrich A** 1998 Role of apolipoprotein CIII in triglyceride-rich lipoprotein metabolism. Diabetes Metab 24:490–495
7. **Ito Y, Azrolan N, O'Connell A, Walsh A, Breslow JL** 1990 Hyper-

triglyceridemia as a result of human apo CIII gene expression in transgenic mice. Science 249:790–793
8. **Maeda N, Li H, Lee D, Oliver P, Quarfordt SH, Osada J** 1994 Targeted disruption of the apolipoprotein C-III gene in mice results in hypotriglyceridemia and protection from postprandial hypertriglyceridemia. J Biol Chem 269:23610–23616
9. **Narayanaswami V, Ryan RO** 2000 Molecular basis of exchangeable apolipoprotein function. Biochim Biophys Acta 1483:15–36
10. **Clavey V, Lestavel-Delattre S, Copin C, Bard JM, Fruchart JC** 1995 Modulation of lipoprotein B binding to the LDL receptor by exogenous lipids and apolipoproteins CI, CII, CIII, and E. Arterioscler Thromb Vasc Biol 15:963–971
11. **Jong MC, Hofker MH, Havekes LM** 1999 Role of ApoCs in lipoprotein metabolism: functional differences between ApoC1, ApoC2, and ApoC3. Arterioscler Thromb Vasc Biol 19:472–484
12. **Gangabadage CS, Zdunek J, Tessari M, Nilsson S, Olivecrona G, Wijmenga SS** 2008 Structure and dynamics of human apolipoprotein CIII. J Biol Chem 283:17416–17427
13. **Kawakami A, Aikawa M, Libby P, Alcaide P, Luscinskas FW, Sacks FM** 2006 Apolipoprotein CIII in apolipoprotein B lipoproteins enhances the adhesion of human monocytic cells to endothelial cells. Circulation 113:691–700
14. **Kawakami A, Aikawa M, Alcaide P, Luscinskas FW, Libby P, Sacks FM** 2006 Apolipoprotein CIII induces expression of vascular cell adhesion molecule-1 in vascular endothelial cells and increases adhesion of monocytic cells. Circulation 114:681–687
15. **Kawakami A, Aikawa M, Nitta N, Yoshida M, Libby P, Sacks FM** 2007 Apolipoprotein CIII-induced THP-1 cell adhesion to endothelial cells involves pertussis toxin-sensitive G protein- and protein kinase Cα-mediated nuclear factor-κB activation. Arterioscler Thromb Vasc Biol 27:219–225
16. **Sol EM, Sundsten T, Bergsten P** 2009 Role of MAPK in apolipoprotein CIII-induced apoptosis in INS-1E cells. Lipids Health Dis 8:3
17. **Refai E, Dekki N, Yang SN, Imreh G, Cabrera O, Yu L, Yang G, Norgren S, Rössner SM, Inverardi L, Ricordi C, Olivecrona G, Andersson M, Jörnvall H, Berggren PO, Juntti-Berggren L** 2005 Transthyretin constitutes a functional component in pancreatic β-cell stimulus-secretion coupling. Proc Natl Acad Sci USA 102:17020–17025
18. **Hokanson JE, Kinney GL, Cheng S, Erlich HA, Kretowski A, Rewers M** 2006 Susceptibility to type 1 diabetes is associated with ApoCIII gene haplotypes. Diabetes 55:834–838
19. **Bengtsson-Olivecrona G, Olivecrona T** 1991 Phospholipase activity of milk lipoprotein lipase. Methods Enzymol 197:345–356
20. **Nielsen K, Karlsen AE, Deckert M, Madsen OD, Serup P, Mandrup-Poulsen T, Nerup J** 1999 β-Cell maturation leads to in vitro sensitivity to cytotoxins. Diabetes 48:2324–2332
21. **Eizirik DL, Pavlovic D** 1997 Is there a role for nitric oxide in β-cell dysfunction and damage in IDDM? Diabetes Metab Rev 13:293–307
22. **Størling J, Zaitsev SV, Kapelioukh IL, Karlsen AE, Billestrup N, Berggren PO, Mandrup-Poulsen T** 2005 Calcium has a permissive role in interleukin-1β-induced c-jun N-terminal kinase activation in insulin-secreting cells. Endocrinology 146:3026–3036
23. **Gonzalez E, McGraw TE** 2009 The Akt kinases: isoform specificity in metabolism and cancer. Cell Cycle 8:2502–2508
24. **Størling J, Binzer J, Andersson AK, Züllig RA, Tonnesen M, Lehmann R, Spinas GA, Sandler S, Billestrup N, Mandrup-Poulsen T** 2005 Nitric oxide contributes to cytokine-induced apoptosis in pancreatic β cells via potentiation of JNK activity and inhibition of Akt. Diabetologia 48:2039–2050
25. **Haber EP, Procópio J, Carvalho CR, Carpinelli AR, Newsholme P, Curi R** 2006 New insights into fatty acid modulation of pancreatic β-cell function. Int Rev Cytol 248:1–41
26. **Klein RL, McHenry MB, Lok KH, Hunter SJ, Le NA, Jenkins AJ, Zheng D, Semler AJ, Brown WV, Lyons TJ, Garvey WT, RG** 2004 Apolipoprotein C-III protein concentrations and gene polymorphisms in type 1 diabetes: associations with lipoprotein subclasses. Metabolism 53:1296–1304
27. **Li L, El-Kholy W, Rhodes CJ, Brubaker PL** 2005 Glucagon-like peptide-1 protects β cells from cytokine-induced apoptosis and necrosis: role of protein kinase B. Diabetologia 48:1339–1349
28. **Natalicchio A, De Stefano F, Orlando MR, Melchiorre M, Leonardini A, Cignarelli A, Labarbuta R, Marchetti P, Perrini S, Laviola L, Giorgino F** 2010 Exendin-4 prevents c-Jun N-terminal protein kinase activation by tumor necrosis factor-α (TNFα) and inhibits TNFα-induced apoptosis in insulin-secreting cells. Endocrinology 151:2019–2029
29. **Kawakami A, Osaka M, Tani M, Azuma H, Sacks FM, Shimokado K, Yoshida M** 2008 Apolipoprotein CIII links hyperlipidemia with vascular endothelial cell dysfunction. Circulation 118:731–742
30. **Eldor R, Yeffet A, Baum K, Doviner V, Amar D, Ben-Neriah Y, Christofori G, Peled A, Carel JC, Boitard C, Klein T, Serup P, Eizirik DL, Melloul D** 2006 Conditional and specific NF-κB blockade protects pancreatic β cells from diabetogenic agents. Proc Natl Acad Sci USA 103:5072–5077
31. **Heimberg H, Heremans Y, Jobin C, Leemans R, Cardozo AK, Darville M, Eizirik DL** 2001 Inhibition of cytokine-induced NF-κB activation by adenovirus-mediated expression of a NF-κB super-repressor prevents β-cell apoptosis. Diabetes 50:2219–2224
32. **Giannoukakis N, Rudert WA, Trucco M, Robbins PD** 2000 Protection of human islets from the effects of interleukin-1β by adenoviral gene transfer of an Iκ B repressor. J Biol Chem 275:36509–36513
33. **Chang I, Kim S, Kim JY, Cho N, Kim YH, Kim HS, Lee MK, Kim KW, Lee MS** 2003 Nuclear factor κB protects pancreatic β-cells from tumor necrosis factor-α-mediated apoptosis. Diabetes 52:1169–1175
34. **Kim S, Millet I, Kim HS, Kim JY, Han MS, Lee MK, Kim KW, Sherwin RS, Karin M, Lee MS** 2007 NF-κ B prevents β cell death and autoimmune diabetes in NOD mice. Proc Natl Acad Sci USA 104:1913–1918
35. **Kawakami A, Osaka M, Aikawa M, Uematsu S, Akira S, Libby P, Shimokado K, Sacks FM, Yoshida M** 2008 Toll-like receptor 2 mediates apolipoprotein CIII-induced monocyte activation. Circ Res 103:1402–1409
36. **Kiely A, Robinson A, McClenaghan NH, Flatt PR, Newsholme P** 2009 Toll-like receptor agonist induced changes in clonal rat BRIN-BD11 β-cell insulin secretion and signal transduction. J Endocrinol 202:365–373
37. **Böni-Schnetzler M, Boller S, Debray S, Bouzakri K, Meier DT, Prazak R, Kerr-Conte J, Pattou F, Ehses JA, Schuit FC, Donath MY** 2009 Free fatty acids induce a proinflammatory response in islets via the abundantly expressed interleukin-1 receptor I. Endocrinology 150:5218–5229
38. **Ehses JA, Meier DT, Wueest S, Rytka J, Boller S, Wielinga PY, Schraenen A, Lemaire K, Debray S, Van Lommel L, Pospisilik JA, Tschopp O, Schultze SM, Malipiero U, Esterbauer H, Ellingsgaard H, Rütti S, Schuit FC, Lutz TA, Böni-Schnetzler M, Konrad D, Donath MY** 2010 Toll-like receptor 2-deficient mice are protected from insulin resistance and β cell dysfunction induced by a high-fat diet. Diabetologia 53:1795–1806