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Apolipoprotein CIII links islet insulin resistance to β-cell failure in diabetes

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Conflict of interest: POB is co-founder and CEO of Biocrine, a biotech company and MHA, IL, BL, MK and EI are serving as consultants for Biocrine. MJG and RMC are employees of Isis Pharmaceuticals, Inc.
Abstract

Insulin resistance and β-cell failure are the major defects in type-2 diabetes mellitus (T2DM). However, the molecular mechanisms linking these two defects remain unknown. Elevated levels of apolipoprotein CIII (apoCIII) are associated, not only with insulin resistance, but also with cardiovascular disorders and inflammation. We now demonstrate that local apoCIII production is connected to pancreatic islet insulin resistance and β-cell failure. An increase in islet apoCIII causes deranged regulation of β-cell cytoplasmic free Ca$^{2+}$-concentration ([Ca$^{2+}$]_i), increased mitochondrial metabolism, apoptosis and promotion of a local inflammatory milieu. Decreasing apoCIII in vivo results in improved glucose tolerance and pancreatic apoCIII knock-out islets transplanted into diabetic mice, with high systemic levels of the apolipoprotein, demonstrate a normal [Ca$^{2+}$]_i response pattern and no hallmarks of inflammation. Hence, under conditions of islet insulin resistance locally produced apoCIII is an important diabetogenic factor responsible for β-cell incapacitation and may constitute a novel target for the treatment of T2DM.

Significance statement

Insulin resistance and β-cell failure are the major defects in type-2 diabetes mellitus (T2DM). We now demonstrate that an increase in apolipoprotein CIII (apoCIII) within the islets of Langerhans, as a consequence of local insulin resistance, causes deranged regulation of β-cell cytoplasmic free Ca$^{2+}$-concentration ([Ca$^{2+}$]_i), increased mitochondrial metabolism, apoptosis and promotion of an intra-islet inflammatory milieu. Decreasing apoCIII in vivo in animals with insulin resistance results in improved glucose tolerance and pancreatic apoCIII knock-out islets transplanted into diabetic mice, with high systemic levels of the apolipoprotein, demonstrate a normal [Ca$^{2+}$]_i response pattern and no hallmarks of inflammation. Hence,
under conditions of islet insulin resistance locally produced apoCIII is an important
diabetogenic factor responsible for β-cell incapacitation and may constitute a novel target for
the treatment of T2DM.

Glucose homeostasis in mammals is tightly controlled by a balance between peripheral tissue
insulin sensitivity with corresponding glucose uptake, and regulated insulin secretion by
pancreatic β-cells. If this balance is disturbed diabetes develops. The progression of type-2
diabetes mellitus (T2DM) occurs in phases. The initial phase is characterized by peripheral
tissue insulin resistance with a β-cell compensatory response of increasing mass and insulin
secretion. With prolonged hyperinsulinemia however, β-cells start to fail, causing defects in
insulin secretion and eventually increased β-cell apoptosis (1, 2). Although being crucial for
the development of novel diabetes therapies, the direct link between insulin resistance and β-
cell failure in T2DM is still not known.

Apolipoprotein CIII (apoCIII) resides on apoB lipoproteins and high density lipoproteins and
regulates their metabolism by inhibiting lipoprotein lipase (3, 4). The expression of apoCIII
is increased by insulin deficiency and insulin resistance (5, 6) and recently it has been
shown that hyperglycemia induces apoCIII transcription (7). On top of high levels of
circulating triglycerides, mice overexpressing apoCIII are more susceptible to high-fat diet
induced diabetes (8). In humans, elevated levels of circulating apoCIII are associated with a
number of adverse effects including cardiovascular problems, inflammation and insulin
resistance (9, 10). Conversely, humans with a life-long reduction of the apolipoprotein, due to
mutations in the apoCIII gene, benefit from a favorable pattern of lipoproteins, increased
insulin sensitivity and longevity, lower incidence of hypertension and protection against cardiovascular diseases (11, 12).

We now show that apoCIII serves as a link between insulin resistance and β-cell failure in T2DM. The mechanistic explanation is that specific insulin resistance within the pancreatic islet leads to local expression of apoCIII, resulting in an autocrine negative feedback loop for β-cell function and survival.

Results

Insulin signaling in ob/ob islets. The ob/ob mouse is known to progressively develop insulin resistance and a transient hyperglycemia (13). Using this mouse model at the age of 4-12 weeks, we found that blood glucose and body weight increased progressively (Fig. 1A and B) and that non-fasting insulin levels remained unchanged higher than in control animals (Fig. 1C). We determined the mRNA expression levels of gck, irs1, irs2, vamp2, snap25 and rab27a, genes that have been demonstrated to be controlled by insulin receptor (IR) activated phosphatidylinositol 3-kinase (PI3K) activity (14, 15). All genes were downregulated in 12 weeks old ob/ob islets (Fig. 1D). This is consistent with previous findings where a significantly reduced PI3K activity was found in islets from ob/ob mice at 12 weeks of age compared with islets from age-matched ob/lean littermates (16, 17).

Progressive change in FoxO1-regulated gene expression in ob/ob islets over time. One of the ways in which insulin induces changes in gene expression is through the phosphorylation cascade of Akt and its downstream target forkhead transcription factor FoxO1. Insulin-mediated activation of Akt leads to phosphorylation of FoxO1 with its nuclear exclusion and loss of transcriptional activity. This signaling cascade has been shown to be crucial in maintaining β-cell function and proliferation during either insulin resistance or insult/injury
We determined levels of phospho-Akt and phospho-FoxO1 in ob/ob islets. There was a significant reduction in phospho-Akt (Ser473) and phospho-FoxO1 (Ser256) in islets from 12-weeks old ob/ob mice compared to islets from 4-weeks old ob/ob mice (Fig. 1E). Total Akt protein levels within these islets were not significantly different across time. Total FoxO1 protein levels in the islet could not be successfully measured because of poor signal from immunoblots, reflecting low expression of FoxO1. To circumvent this, we determined mRNA levels of FoxO1 and found no significant difference in all the age groups studied (Fig. 1E).

The levels of nuclear FoxO1 was higher in islets from 12-weeks old ob/ob mice compared to islets from ob/lean. This was shown both in vivo, using the anterior chamber of the eye (ACE) transplantation technique (21), (Fig.1F) and in vitro (Fig.1G and H). Hence, the simultaneous reduction of phospho-FoxO1 and increase in nuclear FoxO1 further corroborates the above described changes in ob/ob islet gene expression, since irs1, irs2, vamp2 and rab27a have been suggested to be repressed by FoxO1(14).

Insulin receptor-mediated repression of FoxO1 is pivotal in maintaining the balance between carbohydrate and fat metabolism in hepatocytes (3). In insulin-resistant hepatocytes, reduced FoxO1 phosphorylation, and hence its activation as a transcription factor, promotes the expression of apoCIII, a modulator of circulating triglycerides, glucose-6-phosphatase, a key gluconeogenic enzyme, and mttp1, a microsomal triglyceride transfer protein that is involved in lipoprotein assembly (22). To explore if a similar mechanism occurs in ob/ob islets with declining levels of phospho-FoxO1, we determined the mRNA expression levels of the FoxO1 target genes apoCIII, g6pc2 (islet specific glucose-6-phosphatase) and mttp1. There was a significant increase in ob/ob islet mRNA expression of all three genes across time (Fig. 2A and S1A and B). Together, these data suggest that insulin resistance at the islet level leads to a change in the expression of genes that are positively or negatively regulated by FoxO1. Of these, apoCIII expression stands out as a novel islet factor (Fig. 2A).
finding since this apolipoprotein is known to be involved in both β-cell dysfunction (23) and type-1 diabetes mellitus (24, 25).

**ApoCIII is increased in islets and serum in diabetes and systemically decreasing it in vivo improves the diabetic phenotype.** As there was a three-fold increase in apoCIII mRNA levels in islets from 12-weeks old ob/ob mice, without any difference in liver and intestine apoCIII expression (Fig. 2A, B and C), we decided to determine the systemic levels of apoCIII. The immunoblots showed increased apoCIII levels in serum of ob/ob mice (Fig. S2A and B). Given that liver is the main source of apoCIII, the higher levels of circulating apoCIII cannot be explained by the enhanced islet release of the apolipoprotein, but rather reflects reduced cellular uptake of triglyceride-rich (VLDL) remnants, similar to what has been previously reported (26).

To explore the effects of decreasing apoCIII in vivo, ob/ob mice were treated with antisense oligonucleotides specific for apoCIII or an inactive control. These antisense oligonucleotides decrease apoCIII by more than 50% (Fig. 2D). The lowering of apoCIII led to a reduced body weight and, most importantly, an improved glucose tolerance (Fig. 2E and F). Immunostaining of islets from two healthy human subjects (Fig. 2G 1 and 2) and one T2DM patient (Fig. 2G 3) showed a higher degree of apoCIII positive cells in the T2DM islets, which is in accordance with the findings in the ob/ob mice.

**ApoCIII overexpression increases [Ca^{2+}]_{i} in MIN6 cells.** To corroborate the negative role of locally produced apoCIII on β-cell function, this apolipoprotein was overexpressed in the insulin-secreting cell line MIN6. Hampered by the ability of an antibody to recognize apoCIII in mouse liver sections and lysates, we resorted to a c-Myc tagged mouse apoCIII construct that allowed us to explore the localization of Myc-apoCIII within β-cells. Myc-apoCIII was
detectable in media of the Myc-apoCIII-transfected cells after a 24-hour culture period, indicating that the synthesized apoCIII is being readily released into the extracellular medium (Fig. 3A). No co-localization between Myc-apoCIII and insulin C-peptide could be detected in the transfected cells (Fig. 3B). MIN6 cells transfected with either Myc-apoCIII or a control vector were loaded with Fura-2AM to determine the effect of mouse apoCIII on [Ca\(^{2+}\)]\(_i\) upon maximum activation of the voltage-gated Ca\(^{2+}\) channels by KCl stimulation. A significantly higher increase in [Ca\(^{2+}\)]\(_i\), after KCl exposure, was observed in MIN6 cells expressing Myc-apoCIII as compared to the control transfected cells (Figure 3C). Compared to control, a higher increase in [Ca\(^{2+}\)]\(_i\) was also seen in nearby non-transfected cells in the same culture dish upon depolarization, which is likely to be explained by, in addition to the autocrine effect on the transfected cell itself, a paracrine effect of apoCIII.

**Reduced endogenous islet apoCIII in vitro and in vivo results in improved [Ca\(^{2+}\)]\(_i\) handling and less apoptosis.** Next we investigated how a specific lowering of apoCIII in the islet affected function and survival of the β-cells, here depicted as changes in [Ca\(^{2+}\)]\(_i\) and apoptosis. Expression of apoCIII in islets exposed to antisense oligonucleotides was 20% compared to control islets (Fig. 4A). Islets were loaded with Fura-2AM to determine the effect of apoCIII on [Ca\(^{2+}\)]\(_i\), subsequent to both high glucose and KCl exposure. As previously noted (17), ob/ob islets had a significantly higher peak amplitude of [Ca\(^{2+}\)]\(_i\) compared to ob/lean islets (Fig. 4B). The peak amplitude of [Ca\(^{2+}\)]\(_i\), upon stimulation with either glucose or KCl, was reduced in ob/ob islets where apoCIII had been lowered (Fig. 4B and C). Although ob/ob islets are hyperplastic with massive β-cell proliferation, it does not rule out the presence of apoptosis in these islets. We determined the level of apoptosis in freshly isolated islets by measuring the activity of caspase 3/7. Apoptosis in islets from 12-weeks old ob/ob mice was significantly higher compared to islets from 4 weeks old ob/ob mice (Fig.
This is consistent with a previous finding where reduced levels of phospho-FoxO1 in islets of New Zealand obese mice resulted in higher levels of apoptosis (27). Lowering endogenous apoCIII in islets from 12-weeks old ob/ob mice, when apoCIII levels are the highest, decreased caspase 3/7 activity by 31%, compared to islets treated with scrambled antisense (Fig. 4E right panel). Since our previous results have shown that apoCIII increases the activity of the voltage-gated Ca\(^{2+}\)-channel (28), we exposed \(\beta\)-cells to the Ca\(^{2+}\)-channel blocker verapamil and this reduced caspase activity by 15% (Fig. 4E left panel). Together, these results suggest that increased FoxO1-induced expression of apoCIII within the ob/ob pancreatic islet leads to deranged [Ca\(^{2+}\)]\(_i\), and an increase in \(\beta\)-cell apoptosis.

Since liver is the predominant source of apoCIII it is necessary to clarify the extent to which an increased local production of the apolipoprotein within the islet will have an effect on the \(\beta\)-cell on top of that already provided by the liver. For this purpose we took the advantage of a global apoCIII\(^{-/-}\) mouse (29) and the ACE as an in vivo experimental platform (21). By transplanting islets from either knock-out or ob/ob mice into the eye of ob/ob mice with high serum levels of apoCIII, we created an in vivo model where both grafts were subjected to high systemic levels of the apolipoprotein, but only the ob/ob islets maintained a local production of apoCIII (Fig. 5A). The islets were allowed to be in the eye for two weeks and during this time they developed a normal vascularization and hence were exposed to high systemic levels of apoCIII (Fig. 5B). Thereafter the islets were dissected out of the eye and subjected to measurements of [Ca\(^{2+}\)]\(_i\), subsequent to depolarization with KCl, to maximally activate the voltage-gated Ca\(^{2+}\)-channels. Islets from the apoCIII\(^{-/-}\) mice demonstrated a lower increase in [Ca\(^{2+}\)]\(_i\), suggesting that the local islet production of apoCIII indeed has a detrimental effect on \(\beta\)-cell [Ca\(^{2+}\)]\(_i\) handling (Fig. 5C and D).

To further substantiate the significance of intra-islet levels of apoCIII for pancreatic \(\beta\)-cell function and survival, islets from apoCIII\(^{-/-}\) mice and C57Bl/6 (B6) mice, as control, were
transplanted into the eyes of B6 mice. Islets from apoCIII/− mice were transplanted into the right eye and control islets from B6 mice were transplanted into the left eye of the same recipient mouse (Fig. 6A). The islets were allowed to be vascularized and thereafter the transplanted mice were fed a HFD until they showed an impaired glucose tolerance. The levels of apoCIII were determined in serum and they were increased in B6 mice on HFD compared to mice fed normal chow (Fig. 6B). In this unique experimental setup the very same animal served both as a control and an islet cell specific apoCIII knock out. It was thus possible to expose the two types of islets to exactly the same high systemic serum levels of apoCIII, originating from the liver, and the only difference was that the islets in the right eye were not expressing apoCIII whereas those in the left eye did. The islets were imaged over time from 1 to 3 months post-transplantation (Fig. 6C). As expected, the islets from the control B6 mice increased in size with time. In contrast, the apoCIII/− islets had a smaller increase in size compared to the control islets, despite being exposed to the same high systemic levels of apoCIII (Fig. 6C and D). Importantly, vessel density, number of macrophages and NAD(P)H/FAD ratio were lower in the transplanted apoCIII/− islets (Fig. 6E,F,G, H and I), suggesting that the lack of intra-islet apoCIII production prevents the HFD-induced inflammatory reaction.

**Discussion**

Diabetes is a severe, multifactorial and life-long disease spreading like an epidemic worldwide and where available treatment strategies are inadequate. T2DM is characterized by two major defects namely insulin resistance and β-cell failure, although the direct link between the two is not known. In the present study we report on apoCIII as the long sought for factor linking insulin resistance to β-cell failure in T2DM. The mechanistic explanation is that islet insulin resistance can promote local apoCIII production that negatively affects
function and survival of the pancreatic β-cell, on top of the already high levels of the apolipoprotein promoted by the liver. This points to much wider negative effects of apoCIII in the pathogenesis of diabetes than previously assessed from cardiovascular studies.

T2DM develops as a consequence of the pancreatic β-cell no longer being able to cope with increased insulin resistance in the peripheral insulin target tissues, like the liver (30). It is well-known that the expression of apoCIII is increased in the liver under conditions of insulin resistance (5). We and others have previously shown that also the pancreatic β-cell is equipped with insulin receptors and thereby applies insulin-induced signal-transduction for function and survival (15). Importantly, we now demonstrate that the pancreatic islet can be subjected to insulin resistance leading to a progressive change in FoxO1-regulated gene expression in ob/ob islets over time. Hence, with prolonged hyperinsulinemia, insulin signaling at the islet level was compromised, similar to what has been reported for the β-cell-specific insulin receptor knockout mouse (βIRKO) and the β-cell-specific PI3K subunit p85α knockout mouse (βPik3r1) (14, 31). In diabetic ob/ob mice there is therefore not only insulin resistance in the peripheral target tissues, leading to increased serum levels of apoCIII, but also islet insulin resistance, resulting in increased local production of the apolipoprotein.

Interestingly, we also found an increased number of apoCIII positive cells in islets from a patient with T2DM compared with islets from healthy individuals. In line with the overall negative effects reported for apoCIII (9, 10), our findings that systemically decreasing the levels of this apolipoprotein in vivo improves the diabetic phenotype are logical.

In order to clarify, at the molecular level, how local islet production of apoCIII affected the β-cell we took the advantage of MIN6 cells overexpressing the apolipoprotein. Interestingly, these cells released apoCIII that promoted both autocrine and paracrine Ca²⁺ influx. This is consistent with previously observed effects of exogenously added human apoCIII on both rat insulinoma cells (25), primary mouse β-cells (32) and human β-cells (Juntti-Berggren et al
unpublished observations). Next we investigated how a specific lowering of apoCIII in the islet affected β-cell $[\text{Ca}^{2+}]_i$ and apoptosis. Subsequent to antisense treatment, stimulated $[\text{Ca}^{2+}]_i$ increase was reduced. This effect on $[\text{Ca}^{2+}]_i$, was paralleled by a decrease in apoptosis and explained by decreased activity of voltage-gated Ca$^{2+}$-channels.

Finally, to prove that local islet production of apoCIII *in vivo* makes a difference for β-cell function and survival, on top of the already high systemic levels produced by the liver, we took the advantage of a global apoCIII$^{-/-}$ mouse (29) and the anterior chamber of the eye as an *in vivo* experimental platform (21). In this way we could create a situation where the same animal served both as a control and a β-cell specific apoCIII$^{-/-}$, which should be the ultimate experimental system compensating for any unspecific and/or compensatory effects.

Consequently, transplanting apoCIII$^{-/-}$ islets into one eye and control islets from ob/ob or B6 mice into the other eye of an ob/ob mouse or a HFD treated mouse, thereby exposing both types of islets to the same high systemic apoCIII levels originating from the liver, enabled us to show that islets lacking local production of apoCIII were protected and maintained normal function compared to islets expressing the apolipoprotein. In the latter case islets not only demonstrated unphysiological increases in $[\text{Ca}^{2+}]_i$ and apoptosis but also much higher *in vivo* proliferative activity, paralleled by an increase in vessel density, islet macrophages and NAD(P)H/FAD ratio, as a marker of β-cell metabolism. These changes are typical of an inflammatory milieu provided by apoCIII released locally within the islet as a consequence of insulin resistance (33-35).

In the present study we have demonstrated that apoCIII can serve as a link between insulin resistance and β-cell failure in T2DM. Preventing insulin resistance at the islet level is thus crucial to preserve β-cell mass, especially during the late phase of T2DM where β-cells are progressively lost. Hence, local islet production of apoCIII may become a drugable target and thereby form the basis for a novel diabetes treatment regimen. It is of interest to note that
there are human data from subjects with a genetic mutation in the apoCIII gene and thereby life-long reduced levels of apoCIII, demonstrating that these persons are overall healthier and have increased insulin sensitivity (11).

**Material and Methods**

**Animals and islets.** Age-matched ob/ob \((\text{Lep}^{\text{ob}}/\text{Lep}^{\text{ob}})\) and ob/lean \((\text{Lep}^{\text{ob}}/\text{Lep}^{+})\) on a C57BL/6J background were obtained from our own breeding colony at Karolinska Institutet, Stockholm, Sweden. Mice used in experiments were genotyped and they were between 4 to 12 weeks of age. ApoCIII/ mice were originally generated at The Jackson Laboratories, USA. The C57Bl/6 mice were purchased from Harlan laboratories, USA. Animal care and experiments were carried out according to the Animal Experiment Ethics Committee at Karolinska Institutet. Human islets were obtained within the Nordic Network for Islet Transplantation and were approved by the Regional Ethical Review Boards in Uppsala and Stockholm.

**Pancreatic islet isolation.** Animals were killed by cervical dislocation and the pancreas perfused with 3 ml of 1 mg/ml collagenase A (Roche, USA) in Hank’s balanced salt solution (HBSS) (Sigma, Sweden) buffer supplemented with 0.2 % BSA and 25 mM HEPES. Pancreas was thereafter extracted and digested at 37°C for 20 min. Islets were handpicked and were either immediately used for mRNA/ protein analysis or were cultured overnight in RPMI 1640 medium supplemented with 10 % FCS, 2 mM glutamine as well as 100 U/ml and 100 µg/ml of penicillin and streptomycin, respectively.

**Cell Culture.** MIN6 cells, between passage 35 and 42, were cultured in DMEM containing 11.1 mM glucose and supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 10% FCS, and 75 µM β-mercaptopethanol at 5% CO\(_2\) and 37°C. MIN6 cells were
transfected using Lipofectamine (Invitrogen, USA) with the control expression construct containing tdTomato or c-Myc tagged mouse apoCIII construct for 24 h. All experiments were performed 48 h after transfection.

**Biosensor construction and transduction of islets.** pENTR2A.RIP1.FoxO1GFP was generated by replacing the cDNA for EGFP in pENTR2A.RIP1.EGFP with that of FoxO1-GFP obtained from pEGFP.N1.hFOXO1 (36) IRES-3Tomato was generated by introducing a cDNA encoding three copies of the red fluorescent protein dTomato (37) downstream of the IRES sequence in pIRES (Clontech, USA). The IRES-3Tomato cassette was then introduced into pENTR2A.RIP1.FoxO1GFP thus creating pENTR2A.RIP1.FoxO1GFP-IRES-3Tomato. pENTR2A.RIP1.FoxO1(H215R)GFP-IRES-3Tomato was generated by replacing histidine (CAT) 215 by arginine (CGT) by site-directed mutagenesis by employing the QuikChange mutagenesis kit (Stratagene, USA) and respective oligonucleotides purchased from Sigma. All constructions were verified by DNA sequencing. The expression cassette was transferred into the promoterless adenovirus plasmid pAd/PL-DEST (Invitrogen, USA) by the Gateway technique. The ViraPower Adenoviral Expression System (Invitrogen, USA) was used to generate a replication-deficient adenovirus, which was used for transduction of cells and islets. Islets from ob/lean and ob/ob mice were transduced with 10^7 pfu/ml of the biosensor encoding adenovirus.

**RNA isolation and quantitative RT-PCR analysis.** Total RNA was isolated from sorted cells or isolated pancreatic islets using the RNAeasy Micro Kit (Qiagen, Germany). Briefly, cells were lysed by first using RLT lysis buffer followed by a spin Qiashredder lysis. For liver and intestine RNA isolation, tissue/cell disruption was first carried out by a hand-held rotor-stator homogenizer in RLT lysis buffer followed by 1000 x g centrifugation for 5 min. The supernatant was subsequently transferred to a Qiashredder column to complete the lysis prior
to the RNAeasy Mini Kit application (Qiagen, Germany). All lysates were applied to the RNAeasy spin column and the subsequent RNA isolation and on column DNase treatment were carried out according to manufacturer recommendations. Total RNA was reverse transcribed at 37° C with Multiscribe (Applied Biosystems, USA). The expression of all genes was measured by real-time quantitative PCR with Taq SYBR Supermix with ROX (Invitrogen, USA) on an ABI7900HT instrument (Applied Biosystems, USA). β-actin was used as an endogenous control with 1 cycle at 95° C for 10 min followed by 40 cycles of tandem 95° C for 30 sec and 60° C for 1 min. In all cases, unless otherwise stated, gene-specific intron spanning primers were used and the PCR melting curve produced one single peak corresponding to a specific single amplified product.

**Metabolic Studies.** Non-fasted glucose was measured with a glucose meter (Accu-Chek Advantage, Roche Diagnostics, USA) and serum insulin by the ArcDia 2-photon fluorescence excitation microparticle fluorometry (TPX) assay for insulin (ArcDia Group, Finland). Intraperitoneal glucose tolerance test (IPGTT) was performed in mice fasted for 6 or 12 hours. Blood glucose was measured at 0, 15, 30, 45, 60, 90 and 120 min after an i.p glucose injection of 2 g/kg body weight. C57Bl/6 mice (Harlan laboratories, USA) were fed a high fat diet (HFD) (Open Source Diets D12492, research diets inc, USA) consisting of 60 % kcal from fat from the age of 4-9 months.

**Western Blotting.** Islets or cells were washed with PBS, lysed with RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 % NP-40, 0.5 % Na deoxycholate, 0.1 % SDS, EDTA-free protease inhibitor cocktail, 1µg/ml pepstatine and leupeptin). Lysates were passed 5 times through a syringe needle (0.33 x13 mm/29 Gx1/2) followed by 30 min incubation on a rotator at 4° C. Homogenates were spun at 10 000 x g for 10 min and the protein amount was determined in
the supernatants by the BCA method (Thermo Scientific Pierce, USA). Equal amounts of protein (25-50 µg) were separated over a 4-12 % Bis-Tris gel with MES buffering system (Invitrogen, USA). Proteins were subsequently electrotransferred to PVDF membrane. In case of the phosphospecific antibodies, the membranes were probed with the respective antibodies and then stripped and reprobed with antibodies recognizing the respective total protein levels. Rabbit anti-FoxO1, rabbit anti-phospho-FoxO1, Akt and phospho- Akt antibody were purchased from Cell Signaling Technology (Danvers, USA). Further antibodies used were rabbit anti-apoCIII antibody (Santa-Cruz, USA), guinea-pig c-peptide (Abcam, UK), mouse anti-cMyc (Santa Cruz, USA) and mouse anti-tubulin (Abcam, UK). Immunoreactivity was detected with horseradish peroxidase-conjugated secondary antibodies using the ECL system (Amersham, USA). For immunoblotting serum apoCIII, after serum collection (50µl) per animal, samples were albumin-depleted and freeze-dried. Samples were then resuspended in RIPA buffer (50µl) and protein amount was determined by BCA method (Thermo Scientific Pierce, USA). Equal amounts of protein (25µg) were separated over a 4-12 % Bis-Tris gel with MES buffering system (Invitrogen, USA) as described above. Ponceau S (0.1% w/v) in 1% acetic acid (v/v) was used to stain transferred proteins on PVDF blots prior to blocking. After 5 min, blots were rinsed with 5% acetic acid (v/v) with gentle agitation, followed by 3 washes in de-ionized water to allow for visualization of protein bands.

**Immunocytochemistry.** To visualize Myc-tagged apoCIII in MIN6 cells, post-transfected cells were cultured on glass cover slips prior to 3% PFA fixation for 30 min. Subsequently, cells were incubated with antibodies specific for c-Myc (mouse monoclonal, Santa-Cruz, USA) and C-peptide (rabbit polyclonal, Cell Signaling, USA) in 10% goat serum blocking buffer containing 0.25% Triton X-100, overnight at 4°C with gentle shaking. Cells were incubated with Alexa 488 anti-mouse (Santa-Cruz, USA) and Alexa 546 anti-rabbit (Cell
Signaling, USA) in blocking buffer for 1 hour followed by washing and mounting using VectaShield mounting media with DAPI (Vector Labs, USA). Freshly isolated islets were fixed with 4% PFA in PBS for at least 48 h. They were incubated with primary antibodies anti-FoxO1 (rabbit monoclonal, Cell Signaling, USA) and anti-insulin (guinea pig, DAKO/Agilent, USA) in the presence of 0.1% Triton-X100 for permeabilization and 2% BSA for blocking for 48 h at room temperature. Islets were then washed three times with PBS and incubated with the secondary antibodies Alexa 488-labeled anti-rabbit and Alexa 633-labeled anti-guinea pig (Life Technologies, USA) in the presence of 0.1% Triton-X100 and 2%BSA. Human islets were fixed for 30 minutes in 4% PFA and thereafter incubated overnight at 4°C with primary antibodies anti-insulin (guinea pig, DAKO/Agilent, USA) and anti-apoCIII (Santa-Cruz, USA), in 10% goat serum blocking buffer containing 0.25% Triton X-100. After washing the islets were incubated with the secondary antibodies Alexa 488-labeled anti-guinea pig and Alexa 647 anti-rabbit (Life Technologies, USA). The islets were imaged by confocal laser scanning microscopy.

In vivo treatment with antisense to apoCIII. Ob/ob (Lepob/Lepob) mice were treated with apoCIII antisense (ISIS 353982) or an inactive control (ISIS 141923), n= 6/group. 25 mg/kg body weight was administrated i.p twice per week between the ages of 8 to 16 weeks.

Intraocular islet transplantation. Isolated islets from ob/ob, apoCIII −/− and C57Bl/6 mice were transplanted into the anterior chamber of the eye (ACE) of ob/ob (n=8) or C57Bl6 (n=8) mice as described previously (21). Biosensor transduced islets from ob/lean and ob/ob mice were transplanted into the ACE of 8 weeks old syngeneic littermate recipients.

In vivo and in vitro imaging of islets and image analysis. The islet grafts were imaged in
vivo as previously described (21) using a TCS- SP5 II laser scanning confocal microscope (Leica microsystems, Germany) with water-dipping objectives (Leica HCX IRAPO L 25.0x0.95 and HXC-APO10x/0.30 NA). Viscotears (Novartis, Switzerland) was used as immersion liquid between the eye and the objective. The mice were anesthetized with Isoflurane (Baxter, USA). Bright field images of islet grafts were obtained by the DFC 295 digital camera (Leica Microsystems). To visualize blood vessels and macrophages 100 µL of 2.5 ng/µL of Texas red labeled Dextran (Life Technologies, USA) was injected into the tail vein. Vessel density was calculated by normalizing the vessel volume to the islet volume. Blood vessels were imaged directly after injection whereas macrophages were imaged 72 hours later.

Islet autofluorescence from NAD(P)H and FAD was determined by 2-photon laser-scanning microscopy using the same microscope setup as described above, but without loading with indicator (38). The redox ratio NAD(P)H/FAD was calculated using LAS AF software (Leica Microsystems). Excitation wavelengths were 760 nm for NAD(P)H and 900 nm for FAD. Fluorescence was detected after band-pass filters 460/50 and 525/50 for NAD(P)H and FAD, respectively. In the transduced islets GFP fluorescence was excited at a 488 nm and fluorescence detected at 505 to 536 nm. Tomato was excited at 561 nm and fluorescence detected at 580 to 650 nm. Backscatter signal from the 561 nm excitation was collected at 555 to 565 nm. Imaging of immunostained mouse islets was performed with an inverted Leica TCS-SP2 with a PL APO CS 20x/0.7NA objective and of human islets with TCS- SP5 II laser scanning confocal microscope (Leica microsystems, Germany) with a water-dipping objective (Leica HCX IRAPO L 25.0x0.95).

Analysis of islet volume, vessel volume and macrophage infiltration were performed with Volocity image analysis software (Perkin Elmer, USA). Vessel density was calculated by normalizing the vessel volume to the islet volume. Image analysis of transduced cells and
islets were performed using LAS AF software. For each cell the central plane was determined using information from each detection channel (GFP, Tomato, backscatter). Three regions of interest (ROI) were drawn manually for each cell, namely, nucleus, cytoplasm and background, respectively. Information from all three detection channels was used to determine the position of the nucleus. To calculate the nuclear/cytoplasm-ratio, only the intensity values of the GFP channel were considered. The ratio for each cell was calculated as follows: ratio= (nucleus-background)/(cytoplasm-background). The ratios were categorized into two groups: 1) Ratios < 1 were considered nuclear FoxO1GFP(HR) negative, since less signal was found in the nucleus than in the cytoplasm. 2) Ratios ≥ 1 were considered nuclear FoxO1GFP(HR) positive, since more (or at least equal) signal was found in the nucleus than in the cytoplasm. For each experiment in vitro or in vivo the percentage of nuclear FoxO1GFP(HR) positive cells was calculated as follows: nuclearFoxO1GFP(HR) (%) = cells with a ratio ≥ 1/total amount of analyzed cells.

Measurements of [Ca\(^{2+}\)]. Changes in [Ca\(^{2+}\)] were recorded in islets after a 16 h incubation period with 0.07 mg/ml apoCIII antisense or a scrambled control (Isis Pharmaceuticals, USA) in RPMI 1640 medium and in islets immediately after micro-dissection from ACE two weeks post- transplantation. The basal medium used for islet perifusion experiments was a HEPES buffer (pH 7.4), containing: 125 mM NaCl, 5.9 mM KCl, 2.6 mM CaCl\(_2\), 1.2 mM MgCl\(_2\), and 25 mM HEPES, 0.1% BSA supplemented with either 3 mM glucose, 11 mM glucose or 25 mM KCl. Islets, loaded with 2 \(\mu\)M fura-2 acetoxymethyl ester (Molecular Probes, USA), were attached to coverslips using Puramatrix Hydrogel (BD Biosystem, USA), and mounted on an inverted epifluorescence microscope (Zeiss, Axiovert 135) connected to a Spex Industries Fluorolog system for dual-wavelength excitation fluorimetry. The measurements were performed as previously described (32).
**Caspase assay.** Activity of capase 3/7 was determined using SensoLyte Homogeneous Rh110 Caspase 3/7 assay kit according to manufacturer’s instructions, with modifications (AnaChem, USA). Briefly, islets or cells were harvested, lysed using lysis buffer (AnaChem, USA) and the protein quantified using BCA method. 10 µg of protein was loaded in a black 384-well plate and topped up with the appropriate amount of assay buffer (AnaChem, USA) containing Rh110 Caspase 3/7 Substrate. Plate was incubated in the dark for 1 hour at 24°C. Fluorescence intensity at Ex/Em = 490/520nm was measured to determine the relative caspase activity.

**Statistical analysis.** For individual experiments, the number of animals or islets used (n) is included in each figure legend in parenthesis. All results are expressed as mean ± sem. Statistical analyzes were performed with GraphPad Prism 5, IBM SPSS Statistics 22 and Microsoft Excel 2007. A student’s t-test or one-way ANOVA (Tukey’s post-hoc) were used when appropriate. P values <0.05 were considered statistically significant. In Figure 6E a Fisher’s exact test was performed.

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References

Figure legends

**Fig. 1. Exacerbated hyperglycemia and reduced insulin signaling in ob/ob islets across time.** (A) Average body weight, (B) non-fasted blood glucose levels and (C) non-fasted plasma insulin levels measured in both ob/ob and age-matched ob/lean mice at 4-, 8- and 12-weeks of age (n=8-11). (D) Pancreatic islet gene expression changes between obese and lean controls at 4-, 8- and 12-weeks of age. mRNA levels were determined by real-time qPCR, normalized to β-actin. Expression levels of ob/ob were compared to those of ob/lean, which were set at 1.0. There was no significant change in ob/lean gene expression over time (n=4-9). (E) Western blot analysis of ob/ob islet lysates prepared from animals at 4-, 8- and 12-weeks of age, probed with antibodies specific for pAkt, total Akt, pFoxO1 and γ-tubulin. Bands were quantitated by densitometry. Total FoxO1 immunoblots had poor signal to noise ratios and thus mRNA levels of FoxO1 were measured instead (n=3-4). (F) *In vivo* distribution of FoxO1 in islets from 12-weeks old ob/lean and ob/ob mice transplanted into the ACE of syngeneic mice (n=13). (G) Freshly isolated islets from 12-weeks old ob/lean and ob/ob mice were immunostained for FoxO1 and insulin and the percentage of FoxO1 positive cells were determined (n=3). (H) Representative maximum projection of ob/lean and ob/ob islets (n=10). FoxO1 is labeled with Alexa488 and green is used as digital pseudo-color for fluorescence emitted from Alexa488; red is used as digital pseudo-color for fluorescence emitted from Alexa633, representing insulin. Yellow obtained after overlaying the Alexa488- and Alexa633 signals indicates colocalization. Scale bar 30 μM. Data presented as mean ± sem, *p <0.05, ** p<0.01, ***p<0.001  by student´s t-test or ANOVA (Tukey´s post-hoc).

**Fig. 2. Expression of apoCIII in islets, liver and small intestine across time and the *in vivo* effects of decreasing apoCIII in the ob/ob mouse.** (A) Pancreatic islet, (B) liver and
(C) small intestine apoCIII gene expression was compared between obese and lean controls at 4-, 8- and 12-weeks of age. mRNA levels were determined by real-time qPCR, normalized to β-actin. (n=4-8, 5-7 and 5-6, respectively). (D) Liver mRNA levels of apoCIII after antisense treatment (n=6). (E) Body weight in ob/ob mice treated with scrambled (Src, squares) or apoCIII specific (AS, circles) antisense (n=6). (F) IPGTT after 8 weeks of antisense treatment (n=6). (G) Human islets from two healthy subjects (1 and 2) and one with T2DM (3) were immunostained for apoCIII (red, first panel), insulin (green, second panel) and a merged image (third panel), where yellow indicates colocalization. Scale bar 100 µm. Data presented as mean ± sem, *p <0.05 and **p<0.01.

**Fig. 3. Overexpressing Myc-apoCIII in Min6 cells affects Ca^{2+} handling.** (A) Myc-apoCIII was detectable in media of the Myc-apoCIII-transfected cells after a 24-hour culture period. (B) There was no co-localization of Myc-apoCIII and insulin C-peptide in the transfected cells. (C) Both Myc-apoCIII and control transfected Min6 cells were loaded with Fura-2AM to determine [Ca^{2+}]_i upon membrane depolarization using 25 mM KCl (n=3-4). Data presented as mean ± sem, **p<0.01 by student’s t-test.

**Fig. 4. Effects of decreasing apoCIII in ob/ob islets.** (A) Isolated islets from 12-weeks old ob/ob mice exposed to apoCIII-specific (AS) or scr antisense oligonucleotides for 18 h. ApoCIII expression was measured by real-time qPCR to determine the efficacy of apoCIII knockdown in cultured islets (n=4). (B) Ob/ob and ob/lean islets exposed to either active apoCIII or scr antisense were loaded with Fura-2AM and exposed to 11 mM glucose to determine glucose-stimulated Ca^{2+} influx (n=4-11). (C) 25 mM KCl was used to induce depolarization and subsequent Ca^{2+} influx (n=9-10). (D) Caspase 3/7 activity in ob/ob islets isolated from animals at 4-, 8- and 12-weeks of age (n=4). (E) Decrease in caspase activity in β-cells from 12-weeks old ob/ob mice either incubated overnight with the Ca^{2+}-channel
blocker verapamil (left column) or exposed to apoCIII antisense (right column) (n=4). *p <0.05, ** p<0.01 by student’s t-test or ANOVA (Tukey’s post-hoc).

Fig. 5. Ob/ob and apoCIII−/− islets transplanted to the anterior chamber of the eyes of ob/ob mice. (A) Islets from ob/ob and apoCIII−/− mice were transplanted into the right, respectively, left anterior chamber of the eye (ACE) of 10 weeks old ob/ob mice. (B) Representative bright field images of islets from ob/ob (left) and apoCIII−/− mice (right). Scale bar=100µm. (C) Representative traces showing the effect of KCl on \([\text{Ca}^{2+}]_{i}\) in islets that have been micro-dissected out from the ACE of an ob/ob mouse with high levels of apoCIII two weeks post-transplantation. (D) Increase in \([\text{Ca}^{2+}]_{i}\) when the islets are depolarized with KCl (n=11 and 9, respectively). *p <0.05 by student’s t-test.

Fig. 6. Local islet production of apoCIII affects islet growth and macrophage infiltration in vivo independent of high systemic levels of apoCIII. (A) Islets from apoCIII−/− and B6 mice were transplanted into the right, respectively, left ACE of B6 mice (n=6). (B) Western blot analysis and quantitation of the band intensities of serum apoCIII from B6 mice fed normal chow (control) or HFD (n=3). (C) The recipient mice were fed a HFD and the transplanted islets were imaged from 1-3 months post-transplantation (scale bar= 100 µM). (D) Fold increase in islet area from 1-3 month post-transplantation (n=8). (E) Texas Red labelled dextran was injected to visualize blood vessels. (F) The vessel density (vessel volume/islet volume) was calculated (n=8). (G) Infiltration of macrophages was evaluated 72 hours after injection of Texas Red labelled dextran. Remnants of the labelled dextran were phagocytized by macrophages that can be observed in red to the right. (H) The volume of macrophages in relation to volume of the transplanted islets. (I) The NAD(P)H/FAD ratio was calculated as a measure of mitochondrial metabolism (n=4). *p <0.05 by student’s t-test (B, D, F and I) or Fisher’s exact test (H).
Fig. S1 Changes in expression of G6pc2 and Mttp1 in ob/ob islets across time
Pancreatic islet gene expression changes between obese and lean controls at 4-, 8- and 12-weeks of age. mRNA levels of (A) G6pc2 and (B) Mttp1 were determined by real-time qPCR, normalized to β-actin (n=4-8). Data presented as mean ± sem, *p<0.05 by ANOVA (Tukey’s post-hoc).

Fig. S2 Serum levels of apoCIII in ob/ob mice
(A) Western blot of serum apoCIII with Ponceau S stain as loading control along with (B) the respective densitometry quantitation (n=3). Data presented as mean ± sem, ** p<0.01 by ANOVA (Tukey’s post-hoc).
ApoCIII Insulin Merge

1

B C

D

E F

G

ApoCIII Insulin Merge

1

2

3
A

Control-tx media
ApoCIII-tx media
Cell Lysate

c-myc
apoCIII

B

CMV
SP
ApoCIII CDS
c-Myc
c-myc tagged ApoCIII
C-peptide

C

Δ 340/390 Fluorescence

Control  ApoCIII

MIN6 cells

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