Role of MAPK in apolipoprotein CIII-induced apoptosis in INS-1E cells
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Abstract

Background: Individuals with type 2 diabetes mellitus (T2DM) have elevated levels of circulating apolipoprotein CIII (apoCIII). ApoCIII plays an important role for plasma triglyceride levels and elevated levels of the apolipoprotein have been connected with dyslipidemia in T2DM subjects. In addition, apoCIII has been linked to enhanced β-cell apoptosis. The present study was undertaken to investigate apoptotic mechanisms induced by the apolipoprotein.

Results: ApoCIII (10 μg/ml) enhanced apoptosis 2-fold in insulin-producing INS-1E cells after 24 hours exposure to the apolipoprotein. At this time point phosphorylation of mitogen activated protein kinase (MAPK) p38 had doubled but ERK1/2 and JNK were not activated. Instead, ERK1/2 showed rapid and transient phosphorylation (2-fold after 0.5 hour). No JNK phosphorylation was observed. In support of a role of activation of not only p38 but also ERK1/2 in apoCIII-induced apoptosis, inclusion of p38 inhibitor SB203580 (10 μM) or ERK1/2 inhibitor PD98059 (100 μM) normalized apoptosis. Whereas influx of Ca²⁺ was linked to apoCIII-induced ERK1/2 activation, pro-apoptotic protein CHOP/GADD of the unfolded protein response (UPR) was not affected by apoCIII.

Conclusion: It is suggested that elevated circulating apoCIII levels may contribute to β-cell apoptosis via activation of p38 and ERK1/2 in individuals with T2DM. Therapies aiming at normalizing levels of apoCIII could be beneficial not only for the function of the β-cell but also for cardiovascular protection.
[11,12]. To this aim serum samples from subjects with newly diagnosed diabetes with family history of diabetes (FHD) and impaired β-cell function, and from healthy individuals with no FHD and well functioning β-cells were protein profiled. Several circulating proteins were differentially displayed in the newly diagnosed T2DM individuals [9]. In the present study we have investigated mechanisms by which one of the identified proteins, apolipoprotein CIII (apoCIII), affects the insulin-producing cell. The choice of protein was based on the observed up-regulation of apoCIII in the circulation in individuals with T2DM [9,13,14] and enhanced apoptosis in an insulin-secreting cell line exposed to the apolipoprotein [15]. In the present study we hypothesized that apoCIII-induced β-cell apoptosis was connected to activation of mitogen-activated protein kinases (MAPKs) and/or induction of the pro-apoptotic protein CCAAT/enhancer-binding protein homologous protein/growth arrest and DNA-damage 153 (CHOP/GADD153) of the unfolded protein response (UPR), mechanisms which have been demonstrated to be activated in β-cell apoptosis [7,16-19]. The results indicate that MAPKs p38 and extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), but not c-Jun NH2-terminal kinase (JNK) or CHOP/GADD153 play a role in apoCIII-induced β-cell apoptosis.

**Methods**

**Chemicals and reagents**

Reagents of analytical grade and MilliQ water were used. ApoCIII was purchased from Meridian Life Science (Saco, MA). PD98059, SB203580, verapamil, tolbutamide, thapsigargin and protease inhibitory cocktail (Sigma P-8340) were obtained from Sigma (St. Louis, MO). RPMI 1640 culture medium, fetal calf serum (FCS), sodium pyruvate, glutamine, penicillin and streptomycin were purchased from Invitrogen (Carlsbad, CA). Culture flasks and plates were obtained from Cell Signaling (Beverly, MA). The CHOP/GADD153 and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The immuno-reactive bands were visualized by chemiluminescence (‘ECL Plus’ or ‘ECL Advance’, GE Healthcare, Uppsala, Sweden) according to the manufacturer’s protocol, imaged with Fluor-S Multimager MAX (Bio-Rad, Hercules, CA). After imaging, the PVDF membranes were quantified with Quantity One software (BioRad). The expression level of each protein was normalized to unphosphorylated MAPK and/or β-actin.

**Apoptosis measurements**

DNA fragmentation of INS-1E cells was evaluated by determining oligonucleosome formation using the Cell Death Detection ELISAPLUS kit (Roche Diagnostics, Manheim, Germany). Measurements were related to DNA content and expressed as fold of optical density obtained for control cells cultured in the absence of apoCIII.

**Statistical analysis**

Results are presented as means ± SEM (apoptosis) or representative blots (MAPKs and CHOP/GADD153) for four independent experiments. Western blots were densitometrically analyzed. Differences between groups are assessed by ANOVA followed by Tukey’s post hoc test for apoptosis measurements and Student’s t-test for immunoblots. A probability level of p < 0.05 was considered to be statistically significant.

**Results**

**ApoCIII-induced apoptosis and MAPKs**

ApoCIII was increased by 2-fold when INS-1E cells were exposed to apoCIII for 24 hours (Fig 1). To investigate underlying mechanisms of the apoCIII-induced rise in apoptosis, activation of MAPKs p38, ERK1/2 and JNK in INS-1E cells were measured at 0, 0.5, 1, 2, 4, 8, 12 and 24 hours after introduction of the apolipoprotein. Levels of p-p38 were low in the cells at onset of the apoCIII-exposure and were then progressively increasing for the dura-
tion of the experiment manifested as 2-fold rise (p < 0.05) after 24 hours (Fig 2A). To determine if activation of the MAPK was causally related to apoCIII-induced apoptosis, cells were exposed to the inhibitor of p38 phosphorylation SB203580 (SB) or 100 μM of the ERK1/2 inhibitor PD98059 (PD) during 0.5 hours prior to apoCIII treatment as indicated. After culture, apoptosis was measured as DNA fragmentation and normalized to DNA content. *P < 0.05 denotes effect of apoCIII.

Involvement of ERK1/2 in apoCIII-induced apoptosis in INS-1E cells was also investigated. Levels of p-ERK1/2 were elevated 2-fold (p < 0.05) already after 0.5 hour and declined thereafter (Fig 2B). After 24 hours, when the apoCIII-induced rise in apoptosis was determined, ERK1/2 levels had returned to control levels. To investigate whether the early rise in p-ERK1/2 was contributing to apoCIII-induced apoptosis, cells were exposed to the inhibitor of ERK1/2 phosphorylation PD98059 prior to introducing apoCIII. Pre-exposing the cells to the inhibitor prevented the apoCIII-induced rise in p-ERK1/2 (not shown) and reversed apoptosis in the presence of the apolipoprotein (Fig 1). The inhibitor alone had no effect on apoptosis.

Thirdly, phosphorylation of JNK was measured in INS-1E cells exposed to apoCIII. No activation of the MAPK by the apolipoprotein was observed during the 24-hour exposure time (Fig 2C).

ApoCIII-induced ERK1/2 activation and calcium
The role of Ca²⁺ influx for ERK1/2 activation was next investigated. INS-1E cells treated with apoCIII were exposed to L-type Ca²⁺ channel blocker verapamil prior to apoCIII-treatment. ApoCIII-induced ERK1/2 activation observed after 0.5 hour was reversed by treatment with the channel antagonist (Fig 3). Further support of a role of Ca²⁺ influx as activator of ERK1/2 was provided when INS-1E cells were exposed to tolbutamide. After treatment with the K⁰₆₆ channel blocker, elevated levels (p < 0.05) of p-ERK1/2 were observed (Fig 3).

ApoCIII-induced apoptosis and CHOP
Lastly, we investigated if apoCIII-induced elevated apoptosis in INS-1E cells (Fig 1) involved enhanced expression of pro-apoptotic protein CHOP/GADD153. In the apoCIII-exposed cells CHOP/GADD153 protein levels
ApoCIII-induced ERK1/2 activation. Levels of phospho-
ylated ERK1/2 in INS-1E cells treated or not with apoCIII 
were measured after 0.5 hours by immunoblotting. Cells 
were exposed to L-type Ca²⁺ channel blocker verapamil 
(Verap) or K₅ᵦᵢ channel blocker tolbutamide (Tolb) as indi-
cated.

Discussion

The present study indicates that elevated levels of the 
apolipoprotein apoCIII could promote apoptosis in insu-
lin-producing β-cells via activation of MAPK p38 and 
ERK1/2. The study is a continuation of our previous study 
in which apoCIII was identified as an up-regulated pro-
tein by proteomic methodology when serum obtained 
from individuals with newly diagnosed T2DM and docu-
mented impaired β-cell function was compared to serum 
from control individuals with normal β-cell function [9].

ApoCIII is an 8.8 kDa polypeptide synthesized by the liver 
and playing an important role in controlling catabolism 
of triglyceride-rich lipoproteins by inhibiting the activity 
of lipoprotein lipase thereby inducing hypertriglyceri-
demia [21,22]. The apolipoprotein is mainly associated 
with HDL but also with LDL and VLDL [23,24]. In indi-
viduals with T2DM the levels of the apolipoprotein are ele-
vated [9,13,14]. To what extent the elevated apoCIII levels 
in T2DM are accounted for by rise in a particular lipopro-
tein particle class is less clear, however. Although the lev-
els of VLDL in T2DM individuals are increased 
substantially compared to non-diabetic subjects, apoCIII 
was not increasing in concert with the increased VLDL 
concentration and core lipids in these individuals [14]. 
Instead increased apoCIII content was observed in small 
dense LDL (sdLDL) in T2DM patients compared to the 
corresponding fraction from healthy individuals [25]. The 
potential role of the sdLDL, which is a lipoprotein sub-
class, was further emphasized by the strong association 
with coronary disease progression. Based on these results 
sdLDL and apoCIII have been suggested as markers of the 
atherogenic dyslipidemia of insulin resistance and type 2 
diabetes [25-27]. In addition, connection between genetic 
alterations in apoCIII and T2DM was recently evidenced 
by the finding of strong association between changes in 
ApoC3 with lipid derangements in individuals with the 
disease [28]. When over-expressing apoCIII in transgenic 
mice, hypertriglyceridemia follows [29]. Conversely, dis-
ruption of ApoC3 in mice reduced triglyceride levels [30]. 
Despite the strong correlation relationship between 
apoCIII and triglyceride levels [13], very little is known 
about direct effects of the apolipoprotein on the insulin-
producing cell.

Elevated apoCIII levels have in one previous study been 
associated with enhanced apoptosis in insulin-secreting β-
cells possibly explained by elevated cytoplasmic Ca²⁺ lev-
els [15]. Since disturbed Ca²⁺ homeostasis of the endo-
plasmic reticulum (ER) would be manifested as elevated 
cytoplasmic Ca²⁺ levels, we examined if apoCIII-induced 
apoptosis was connected to up-regulation of the pro-
apoptotic protein CHOP/GADD153. The protein is a 
down-stream target of the protein kinase R-like ER kinase 
(PERK) signaling pathway of the ER-stress response [31]. 
The pathway is part of an adaptive response, the unfolded 
protein response (UPR), aiming at restoring ER function 
when mis- or unfolded proteins accumulate in the 
organelle as a consequence of disturbances in ER Ca²⁺. 
When the UPR fails, persisting ER-stress follows and 
apoptosis is elevated, where up-regulation of CHOP/ 
GADD153 is a component part [7,19]. The enhanced 
apoptosis induced by apoCIII in INS-1E cells in the 
present study was not associated with enhanced CHOP/ 
GADD153 levels, however.

We also investigated to what extent apoCIII activated 
MAPKs p38, JNK and ERK1/2. Treating INS-1E cells with 
apoCIII almost doubled the amounts of p-p38 after 24 
hours exposure to the apolipoprotein. A role of this activa-
tion in apoCIII-induced apoptosis was supported by the 
abrogation of apoptosis when inhibitor of the kinase was 
administered. In addition, activation of the MAPK has 
been observed in β-cells exposed to elevated levels of fatty 
acids, oxygen radical formation and cytokine, all condi-
tions connected with enhanced β-cell apoptosis [16,17]. 
ERK1/2 activation was also induced by apoCIII but with a 
more rapid and transient phosphorylation pattern. Our 
observations that administration of the L-type Ca²⁺ chan-
nel antagonist verapamil abrogated rise in p-ERK1/2 
duced by apoCIII and that K₅ᵦᵢ channel blocker tolbuta-
mide-induced ERK1/2 activation are supporting a role of 
Ca²⁺ influx, induced by apoCIII, as a component of the 
ERK1/2 activation [15,32]. ERK1/2 activation does not 
seem to be essential for the short-term performance of the 
β-cell since glucose-stimulated insulin secretion (GSIS)
was not affected by presence of an inhibitor of the kinases [32]. In contrast, when the inhibitor was administered to islets exposed to prolonged elevated glucose levels, which in the absence of the inhibitor caused similar rapid and transient ERK1/2 activation and was associated with impaired glucose-stimulated insulin secretion and apoptosis, improved GSIS and reduced apoptosis was observed [18]. The same beneficial effects were obtained by L-type Ca2+ channel antagonist nimodipine. Thus, apoCIII elevates cytoplasmic Ca2+ levels by promoting Ca2+ influx. Although such influx promotes insulin secretion in the short perspective, prolonged elevated Ca2+ levels are associated with enhanced β-cell apoptosis [15,33]. Among the pro-apoptotic signaling pathways ERK1/2 and p38 activation seem to play fundamental roles since their inhibition, without Ca2+ antagonism, independently normalized apoCIII-induced apoptosis. In contrast, JNK was not activated by apoCIII.

Conclusion
In conclusion, the study indicates that elevated levels of the apolipoprotein apoCIII may affect β-cell function via activation of MAPKs p38 and ERK1/2. In addition, negative effects of apoCIII on β-cell function may also be mediated by the rise in circulating triglycerides associated with elevated levels of the apolipoprotein [13,22,34]. In this perspective, therapies aiming at normalizing levels of apoCIII including benzafibrate could prove to be important not only for cardiovascular protection [26] but also to preserve β-cell function.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
EMS was responsible for design, planning, carrying out western blotting of the different MAPK and its linkage to apoCIII-induced apoptosis, calcium dependent ERK1/2 activation, statistical analysis and contributed to write the manuscript. TS carried out the initial experiments of apoptosis-measurements and western blotting of CHOP/ GADD153. PB conceived the study, participated in its design and was responsible for writing the manuscript. All authors read and approved the final manuscript.

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