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Role of translationally controlled tumour protein in palmitate-induced beta-cell lipotoxicity in type 2 diabetes mellitus

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Summary

The aim of the present study was to investigate the role of translationally controlled tumour protein (TCTP) in type 2 diabetes mellitus (T2DM). The work was based on the observations that TCTP showed reduced expression in pancreatic β-cells when exposed to elevated levels of saturated fatty acids such as palmitate for extended time periods (lipotoxic conditions) and that TCTP has anti-apoptotic properties. The underlying hypothesis of this work was that TCTP expression regulates β-cell apoptosis in lipotoxic conditions. The hypothesis was addressed by over-expressing the TCTP in lipotoxic β-cells.

Cloning of the TCTP encoding gene *tpt1* was successful into the pCDNA3.1(-) vector, which was confirmed by restriction analysis and sequencing. Recombinant plasmid (pCDNA3.1(-)+*tpt1*) transfection was employed to deliver the *tpt1* gene into the pancreatic β-cell line MIN6. TCTP expression was analyzed by western blotting and expression of TCTP was highest 48 hours of transfection. MIN6 cells expressing different TCTP levels were exposed to increased levels of palmitate (0.25 mM) for 48 hours. TCTP levels in non-transfected MIN6 cells were reduced by 25% in presence of palmitate. At the end of the culture period the extent of apoptosis in the cells containing different TCTP levels was determined. In non-transfected cells palmitate induced a 1.5-fold increase in apoptosis. Although all transfected cells and the control cells treated with palmitate showed increased apoptosis, this rise in apoptosis was attenuated in cells over-expressing TCTP.

The palmitate-induced decrease in TCTP expression in non-transfected cells is in agreement with previous findings showing similar reductions. The accentuated apoptosis evident in transfected cells calls for optimizing the transfection procedure to bring down apoptosis. The observed decrease in the levels of apoptosis in TCTP over-expressing cells implies a role of TCTP in the regulation of apoptosis in lipotoxic β-cells and that the present cellular system can be used to address the hypothesis of a role of TCTP in maintaining normal β-cell physiology.

Introduction

Diabetes mellitus

Diabetes mellitus is a complex disease resulting from inadequate production of insulin in the β-cells of the islets of Langerhans present in the pancreas. Insulin is involved in the regulation of glucose levels in the body and is the only glucose-lowering hormone. It is estimated that more than 180 million people are suffering from diabetes and that this number will double by 2030 (Zimmet *et al.* 2001). Diabetes mellitus occurs in two kinds, type 1 and type 2 diabetes mellitus.

Type 1 diabetes mellitus (T1DM) is an autoimmune disorder involving the destruction of β-cells, which results in absolute insulin deficiency and is therefore also called insulin-dependent diabetes mellitus (Atkinson and Eisenbarth 2001). This aspect of the disease is more prevalent in children and has high incidence among people in developed countries (Gale 2002). The disease is known to cause cardiovascular, renal and nervous system disorders because it often appears early in life leading to long-term consequences (Daneman 2009).

Type 2 diabetes mellitus (T2DM) is the most common type of diabetes mostly found in developed countries and with an expected growing incidence in the near future in developing countries such as India and China (King *et al.* 1998). T2DM is a complex disorder involving both environmental (diet, chemical agents etc) and genetic aspects (McCarthy and Zeggini 2007). It is characterized by increased levels of glucose and free fatty acids (FFAs), where insufficient release of insulin is a major contributing factor (Del Prato and Tiengo 2001). The present work focuses on causes underlying β-cell dysfunction in T2DM.

Glucolipotoxicity

T2DM becomes evident as a result of insufficient release of insulin, which is precipitated by dysfunctional β-cells, eventually leading to apoptosis (Butler *et al*. 2003). Thus, β-cell apoptosis plays a significant role for the occurrence of T2DM just like in T1DM, where high levels of β-cell death due to apoptosis are observed (Butler *et al*. 2003). Individuals with T2DM show elevated levels of glucose and FFAs in the circulation (Dimitriadis *et al*. 2004). The long-term consequences of T2DM have been connected with these rising levels of glucose (hyperglycemia) and FFAs (hyperlipidemia) in the body through their deleterious effects on β-cell function and mass which are collectively termed as glucolipotoxicity (Poitout and Robertson 2008). Negative effects resulting from high glucose or FFAs alone are called glucotoxicity or lipotoxicity, respectively. Glucotoxicity is manifested in dysfunctional β-cells, which ultimately leads to β-cell death through apoptosis (Ubeda *et al.* 2006). The decreased ability to respond to insulin levels (insulin resistance) is also a characteristic feature of T2DM, which is aggravated in obese T2DM individuals (DeFronzo 2004). The rising levels of obesity and the connected increased levels of non-esterified fatty acids in the circulation are major contributing factors for the development of T2DM (Carpentier 2008). Lipotoxic conditions, especially when levels of saturated fatty acid are elevated, cause dysfunctionality of β-cells, which include glucose insensitivity, impaired glucose-stimulated insulin secretion (GSIS) and ultimately leads to β-cell death (Newsholme *et al.* 2007). Therefore, elevated levels of FFAs observed in T2DM patients (Dimitriadis *et al.* 2004) can promote β-cell loss and contribute to glucose

intolerance (Poitout and Robertson 2008).

Glucose-stimulated insulin secretion

Insulin secretion varies with changes in the levels of blood glucose in the body. The ability of the β-cells to secrete insulin in response to rising glucose levels is called glucose-stimulated insulin secretion (GSIS). When GSIS is determined, the β-cell is exposed to a low glucose concentration ranging from 2 to 5 mM and a higher glucose concentration ranging from 11 to 25 mM. The resulting insulin secretion is called basal and stimulatory insulin secretion, respectively. In primary β-cells GSIS results in 10-fold elevated insulin secretion (Bergsten and Hellman 1993) where as in insulin-producing cell line MIN6 it is slightly less (Ishihara *et al.* 1993).

Free fatty acids

Chronic exposure of β-cells to FFAs leads to increase in insulin secretion observed at non-stimulatory conditions with a resulting decrease in GSIS (Zhou and Grill 1995). Some FFAs, in particular saturated species, also induce apoptosis in β-cells when the exposure time is prolonged (El-Assaad *et al.* 2003). The exact mechanisms underlying β-cell death caused by saturated FFAs have not been clearly defined. Toxic effects of FFAs on the β-cell depend on their chain length and degree of saturation (Stein *et al.* 1997).

Palmitic acid or palmitate, which is a long chain saturated fatty acid and one of the abundant FFAs in plasma, causes damage to the β-cell during long periods of exposure and promotes β–cell death by apoptosis (Karaskov *et al.* 2006). In general, saturated fatty acids such as palmitate and stearate are more toxic in comparison with their mono-unsaturated counterparts e.g. oleate, which shows low or no toxicity (El-Assaad *et al.* 2003). Possible reasons why saturated but not mono-unsaturated fatty acids cause β-cell death have been studied intensely. Several mechanisms have been proposed including the development of endoplasmic reticulum (ER) stress and the unfolded protein response (UPR).

Endoplasmic reticulum stress and the unfolded protein response

The ER is one of the largest cell organelles and is involved in synthesis and folding of cellular proteins (Oyadomari *et al*. 2002). The pancreatic β-cell is responsible for insulin secretion in response to variations in the blood glucose level in the body. Thus, the ER of the β-cell is a highly dynamic organelle, as it has to adapt to increasing demands of insulin synthesis and folding (Baumann *et al*. 2001). ER stress is a condition when the ER fails to correctly fold increasing amounts of newly synthesized native proteins (Oyadomari *et al.* 2002). A series of concerted cellular responses called the unfolded protein response (UPR) are initiated (Rutkowski and Kaufman 2004). The UPR involves activation of three different pathways, which arise from the activation of different ER stress sensors present in the membrane of the ER. The sensors are inositol requiring ER to nucleus signal kinase 1 (IRE1), activating transcription factor 6 (ATF6) and PKR like ER kinase (PERK) (Zhang and Kaufman 2004). The overall aim of the UPR is to alleviate the cell from the ER stress, which involves attenuation of protein synthesis thereby decreasing the amount of the unfolded protein in the cell. This action is primarily performed by the PERK pathway through phosphorylation of translational elongation factor alpha (eIF2α) (Harding *et al.* 2001). The UPR and in particular the ATF6 pathway also increases the folding capacity of the ER by up-regulating synthesis of chaperones

such as immunoglobin heavy chain binding protein (BiP) (Haze *et al.* 1999). Activation of IRE1 also increases synthesis of chaperones and also stimulates degradation of the unfolded proteins (Lee *et al.* 2003). When these rescue mechanisms fail to restore ER function cells undergo apoptosis. Underlying mechanisms for apoptosis triggered by the UPR are under investigation. One of the suggested mechanisms is activation of pro-apoptotic factor C/EBP homologous protein (CHOP) by phoshorylation of eIF2α, which in turn is activated by PERK (Harding *et al.* 2001). Also, down-regulation of the anti-apoptotic protein Bcl2 and activation of Bax, which causes activation of mitochondrial apoptotic pathways (Eizirik *et al.* 2008), may contribute to mediate the observed apoptosis. Whereas palmitate but not oleate causes ER stress (Karaskov *et al.* 2006), UPR-related apoptosis could be a mechanism of palmitate-induced β-cell lipotoxicity.

Palmitate-regulated β-cell proteins controlling apoptosis

Palmitate-induced β-cell apoptosis is a complex phenomenon involving several processes in addition to the UPR (Lai *et al.* 2008). Indeed, recent reports show that palmitate-induced apoptosis and development of the UPR are not always correlated (Cunha *et al.* 2008). When β-cells that had been exposed to FFAs were protein profiled, several proteins including carboxypeptidase E were found to be inhibited by palmitate (Jeffrey *et al.* 2008). Another protein specifically inhibited by the fatty acid palmitate was translationally controlled tumor protein (TCTP) (Hovsepyan. and Bergsten 2007). Whereas palmitate reduced the expression by 70%, oleate did not affect the expression.

Translationally Controlled Tumor Protein

Translationally controlled tumor protein is encoded by the *tpt1* gene, ubiquitously expressed and has a molecular mass of approximately 23 kDa (Chitpatima *et al.* 1988). As the name suggests the protein is expressed in tumor cells. The protein can bind calcium and has anti-apoptotic characteristics (Xu et al., 1999).The high conservation of the protein among all organisms (Thaw *et al.* 2001) and its high expression in the cancerous state (Chung *et al.* 2000) has promoted the concept that it is a house-keeping protein essential for cell growth and division. TCTP is highly regulated by a wide variety of external stimuli including calcium stress and pro-apoptotic factors (Bommer and Thiele 2004). Although TCTP known to involve in variety of cellular functions such as cell cycle, preventing apoptosis etc, definite physiological role has not been defined yet (Bommer and Thiele 2004).

TCTP is regulated both at the transcriptional and the translational level (Xu *et al.* 1999). The protein was demonstrated to have anti-apoptotic properties and its down-regulation or silencing led to apoptosis (Yang *et al.* 2005). The anti-apoptotic nature of TCTP has been ascribed its prevention of the dimerization of pro-apoptotic Bax (Bcl associated x protein) which (Susini *et al.* 2008). TCTP known to bind with other anti-apoptotic proteins such as Bcl-xL and Mcl1 and mediate the prevention of dimerization of Bax (Susini *et al*. 2008). Over-expression of this protein could bring down the apoptosis rate (Li *et al.* 2001) and increasing apoptosis rates were observed when its expression was reduced (Yang *et al.* 2005).

Aim

The aim of the present work was to determine the role of TCTP in type 2 diabetes mellitus, more specifically, to check whether the TCTP expression is regulated by

palmitate in insulin secreting pancreatic β-cell line MIN6 and to examine whether the expression of TCTP affected apoptosis in the MIN6 cells exposed to elevated levels of palmitate for extended time periods.

Results

TCTP levels in palmitate-treated β–cells

TCTP levels were measured in insulin-producing MIN6 cells cultured in the presence and absence of 0.25 mM palmitate. Whereas TCTP levels were not affected by palmitate after culture for 24 hours, levels of TCTP were reduced by 25% when the MIN6 cells were cultured in the presence of the fatty acid for 48 hours (Fig 1).

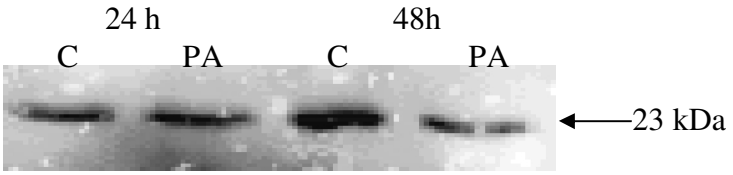

Figure 1. Western-blot analysis of TCTP expression in MIN6 cells. The expression was determined using 15 µg of total protein extracted from MIN6 cells after 24 h and 48 h without (C) palmitate treatment or with 0.25mM palmitate (PA) treatment. The expression of TCTP was detected using an anti-TCTP antibody. The 23kDa band correspond to the TCTP. Representative blot of two independent experiments.

TCTP cloning

In order to restore palmitate-induced reduction in TCTP, over-expression of *tpt1* was attempted. To this end *tpt1* was cloned into pCDNA3.1(-) which has size of 5400 bp. *tpt1* has a coding sequence (CDS) of around 519 bp. The construction of pKDK01 (pCDNA3.1(-)+*tpt1*) was confirmed by PCR analysis by employing the same primers used to synthesize the CDS of *tpt1*. A broad band around 550 bp, which corresponds to the CDS of *tpt1*, was observed (Fig 2). Also, a narrow band around 3300 bp could be resulting from unspecific amplification.

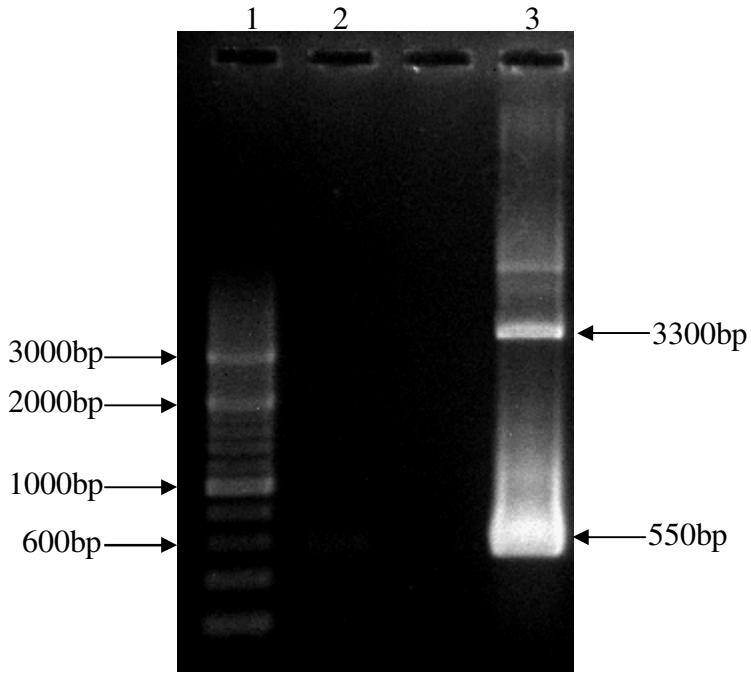

Figure 2. PCR analysis of pKDK01. Lane 1: DNA ladder; lane 2: Negative control; lane 3: PCR sample containing pKDK01. Here the same primers were employed which were used to synthesize the *tpt1* gene. The gel is representative of two independent experiments.

Construction of pKDK01 was also confirmed with restriction analysis using restriction enzymes Apo1 and Dde1 (Fig 3). One extra band resulting from digestion by restriction enzyme Dde1 around 1500 bp appeared (Lane 6) in comparison to the digestion of control plasmid (Lane 4). With the Apo1 enzyme a band around 1400 bp appeared (Lane 5) in comparison to digestion of control plasmid (Lane 3).

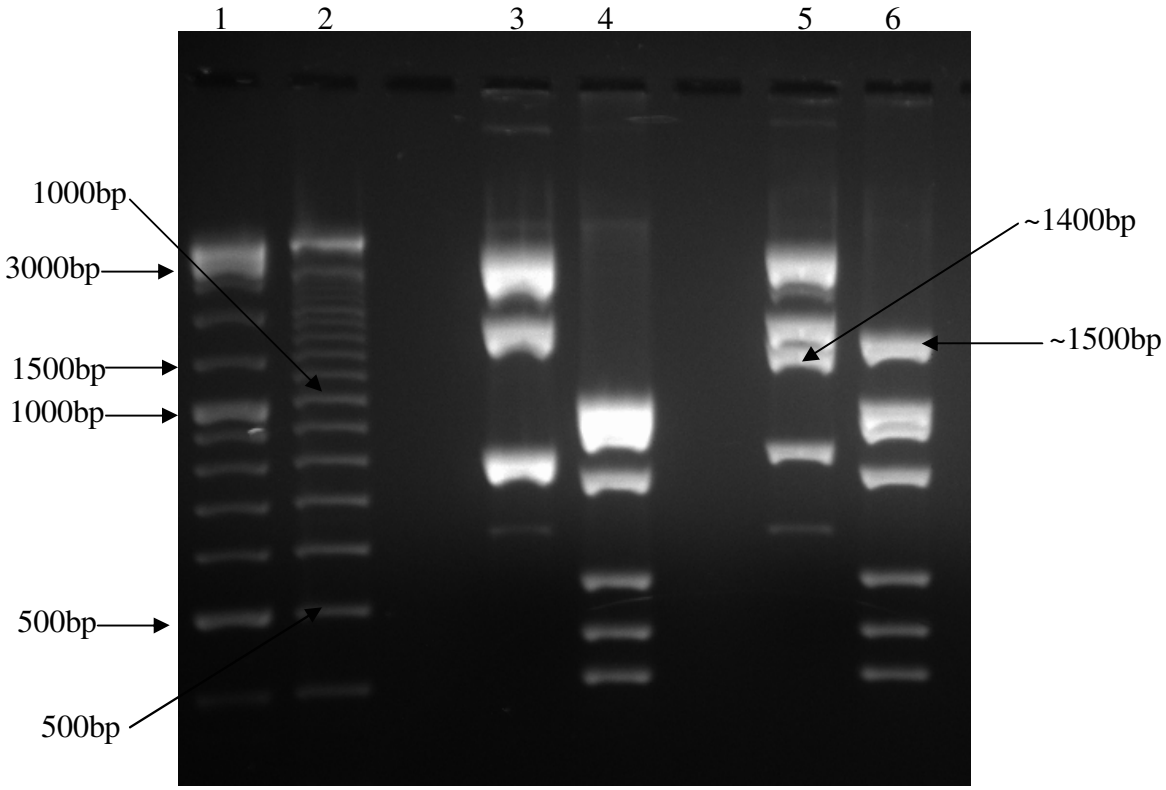

Figure 3. Restriction analysis of pKDK01. Lane 1 and 2: DNA ladder; lane 3 and 4: restriction digestion of empty vector with Apo1 and Dde1 respectively; lane 5 and 6: restriction digestion of pKDK01 with Apo1 and Dde1 respectively. The extra bands resulting from digestion of pKDK01 with Apo1 and Dde1 are at around 1400bp and 1500bp respectively. The gel is representative of two independent experiments.

TCTP over-expression

Insulin-producing MIN6 cells were transfected with pKDK01 plasmid or with the empty vector. Expression of *tpt1* was analyzed by western blotting of TCTP in cells 24, 48 or 72 hours after transfection with empty vector (C) or pKDK01 (T) (Fig 4). Whereas no induction of TCTP was observed 24 hours after transfection, TCTP levels were 4-fold higher in pKDK01-transfected cells after 48 hours, TCTP levels declined to 2-fold 72 hours after transfection.

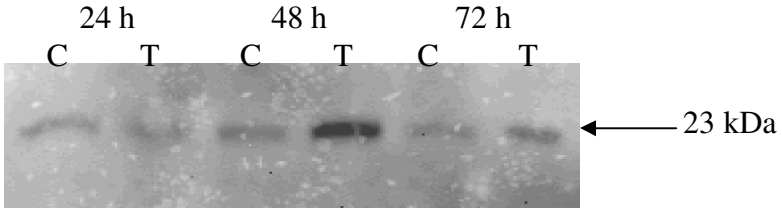

Figure 4. Western-blot analysis of TCTP expression in MIN6 cells. The expression was determined using 15 µg of total protein extracted from MIN6 cells after 24h, 48h and 72h after transfection with empty control plasmid (C).or *tpt1* over-expressing plasmid (T). The expression of TCTP was detected using an anti-TCTP antibody. The 23kDa band correspond to the TCTP. Representative blot of two independent experiments.

TCTP and palmitate-induced apoptosis

MIN6 cells were transfected with pKDK01 or empty vector and treated with palmitate for 48 hours. After the culture period apoptosis was measured. In non-transfected cells palmitate raised apoptosis almost 1.5-fold (Fig 5). In plasmid containing cells apoptosis increased considerably irrespective of whether the plasmid contained *tpt1* or not. Interestingly, in MIN6 cells over-expressing *tpt1*, apoptosis was found to be reduced in cells exposed to palmitate.

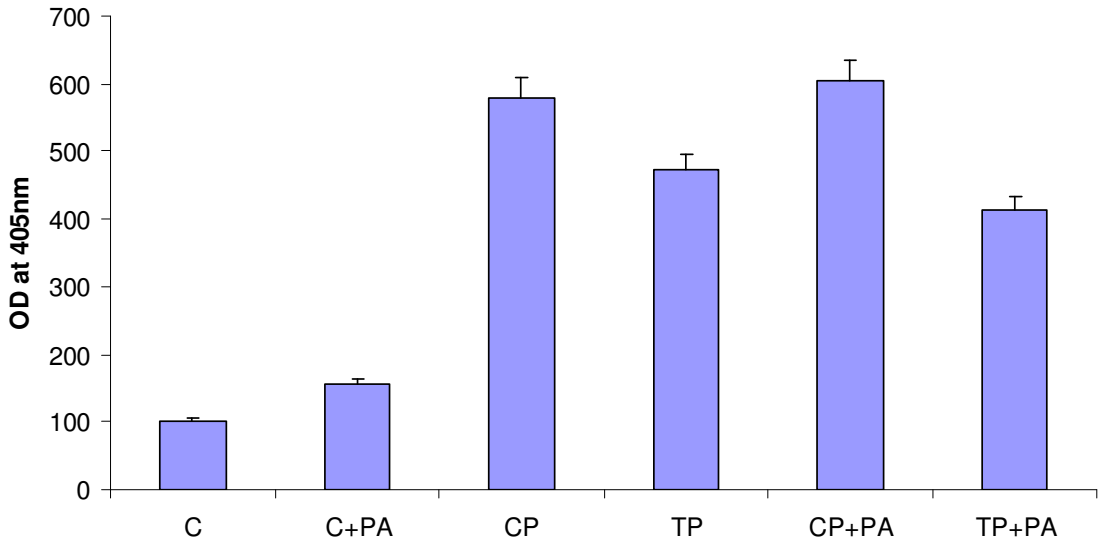

Figure 5. TCTP over-expression and apoptosis in MIN6 cells. Cells were transfected with empty control plasmid (CP) or pKDK01 (TP) in the absence or presence of palmitate (PA) for 48 hours. Control cells (C) were not transfected. The apoptosis rate was measured as OD at 405nm. Results are means ± SEM for two independent experiments.

Discussion

TCTP expression

TCTP levels were found to be going down when the MIN6 cells were exposed to palmitate for 48 h. This reduction correlated to decrease in TCTP levels observed in INS-1E cells when exposed to palmitate (Hovsepyan and Bergsten 2007). The expression of the protein was reduced to 28% in comparison to the cells not exposed to palmitate. It was hard to conclude what happened after 24 h of palmitate exposure as TCTP levels were found to be same in both the control and palmitate exposed cells. But for most of the studies related to fatty acids effects on the pancreatic beta-cell lines 48 h of palmitate treatment has been considered to be a chronic exposure and found be more devastating (Cunha *et al.* 2008).

TCTP over-expression and apoptosis

Cloning of the *tpt1* gene was successful into the pCDNA3.1(-), which has a strong cytomegalovirus (CMV) promoter, that expresses downstream genes (Moeller *et al.* 2005). Transfection efficiency was not possible to determine as the plasmid used is devoid of reporter genes, e.g. encoding fluorescent protein (Moeller *et al.* 2005). In other similar studies the construct has yielded 20-30% transfection rate (Jeffrey *et al.* 2008). Assuming such low transfection efficiency, the observed elevated protein expression after 48 hours of transfection would result from considerable over-expression in successfully transfected cells. The reduction in TCTP levels after 72 hours probably depends upon the transient nature of over-expression using this approach. Also, the observation that mock-transfected cells showed high apoptosis may indicate that despite high expression of the anti-apoptotic protein, the transfection procedure with lipofectamine alone caused considerable apoptosis (apoptosis result not shown). Other methods such as recombinant adenoviral gene transfer may prove superior in yielding high transfection rates and gene expression combined with low rates of apoptosis.

Despite the enhanced apoptosis observed in all transfected cells, apoptosis was reduced in cells over-expressing TCTP. In support of a role for TCTP in palmitate-induced apoptosis, reduction in apoptosis was accentuated in cells exposed to palmitate. Finding that palmitate enhances apoptosis in β cells when exposed for longer periods (El-Assaad *et al.* 2003) corroborates the present finding of increased apoptosis in the presence of palmitate. Less apoptosis was seen in the TCTP overexpressing cells in response to palmitate exposure, which confirms the observation that overexpression of TCTP brings down the incidence of apoptosis (Li *et al.* 2001). Lowering of apoptosis seen in the TCTP over-expressing cells could partially confirm the TCTP regulation in lipotoxic beta-cells since the present findings are from only two independent experiments. To get significant and conclusive data, the present experiments needs to be repeated. Anyhow findings of the present work could be used to address the hypothesis that expression of TCTP is the mechanism by which beta-cell apoptosis is regulated in lipotoxic conditions.

Future plans

The extensive apoptosis observed in transfected cells needs to be reduced. This could be attempted by optimizing the transfection protocol including varying the transfection time and serum concentration during transfection (Dalby et al., 2004). To further verify a role of TCTP in palmitate-induced beta-cell apoptosis TCTP could be silenced by shRNA

technology. Beta cell function can also be assessed by studying their glucose-stimulated insulin secretion. Effects of differential TCTP expression on specific mechanisms including activation of ER stress needs to be examined by studying how proteins associated with ER stress are regulated when TCTP levels are manipulated. Finally, major findings needs to be verified in primary islets by employing the same methodologies which were used in the present work.

Materials and Methods

Biological material

Mouse MIN6 cells were obtained from professor Jun-Ichi Miyazaki, Osaka University (Sakuma *et al.* 1995). MIN6 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) purchased from Invitrogen (Carlsbad, CA). The medium was supplemented with 25 mM glucose, 10% fetal bovine serum (FBS), 100 units/ml of penicillin, 100 µg/ml streptomycin and 55 µM β-mercaptoethanol (all obtained from Invitrogen, Carlsbad, CA). Cells were maintained at 37^{o}C with 5% CO_2. Passage numbers ranging from 21 to 27 and 50-60% confluency (Zhang *et al.* 2007) was used in all experiments. Expression vector pCDNA3.1(-) and bacterial strain *E.coli* XL1 were obtained from Invitrogen (Carlsbad, CA).

cDNA synthesis

The primers for TCTP were designed with Eco R1 restriction site in the forward primer and BamH1 in the reverse primer and ordered from Invitrogen (Carlsbad, CA).The cDNA was prepared from total RNA obtained from of MIN6 cells by reverse transcription using RT-PCR kit from Invitrogen (Carlsbad, CA), which employs random oligo (dT) primer. The reverse transcription procedure incuded preparing RNA/primer mix by incubating 2 µl of total RNA (5 µg) at 65 ^{0}C for 5 min along with 1 µl of oligo-(dT) primer (50 µ M) making the final volume to 10 µl with distilled water. The cDNA synthesis mix was made by adding 2 µl of 10X RT buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M DTT, 1 µl of RNaseOUT^{TM} (40U/ µl) and 1 µl of Superscript^{TM} III RT (200U/ µl) making total volume to 10 µl. Now both RNA/primer mix and cDNA mix were added and incubated for 50 min at 50 ^{0}C. After this reaction was terminated at 85 ^{0}C for 5 min. Finally RNase H was added to the reaction and incubated at 37 ^{0}C for 20 min to get the pure cDNA.

Polymerase chain reaction (PCR)

The primers for TCTP were designed (table 1) with Eco R1 restriction site in the forward primer and BamH1 in the reverse primer and ordered from Invitrogen (Carlsbad, CA).The *tpt1* gene was amplified from the obtained cDNA using Phusion Flash PCR Master Mix (Finnzymes, Espo, Finland).

Table 1. Primers used in the present study.

Primer	Sequence	Application
TCTP-EcoR1[#] Forward	GGAATTCCACCATGATCATCTACCGGG	Used for amplifying *tpt1* gene.
TCTP-BamH1[#] Reverse	CGGGATCCCTAGATCCAATTTGTTAACATTTCTCA	Used for amplifying *tpt1* gene.

[#] restriction sites are underlined.

Table 2. PCR conditions for the amplification of *tpt1* gene[a]

Component	Volume (µl)
2X Phusion™ Flash PCR Master Mix	10
forward primer (0.5 µM)	2
reverse primer (0.5 µM)	2
template (cDNA)	2
ddH_2O	4

[a]Temperature settings and duration for the PCR: intial denaturation for 30 s at 98 ^{0}C; 35 cycles of denaturation (98 ^{0}C for 10 s), annealing (47 ^{0}C for 30 s) and extension (72 ^{0}C for 30 s); final extension for 10 min at 72 ^{0}C.

Restriction digestion

The obtained PCR product was purified by using Quick Gel Extraction Kit from Invitrogen (Carlsbad, CA) following manufacturer's instructions. Then amplicon and vector were digested with EcoR1 and BamH1, purchased from New England Biolabs (Beverly, MA, USA).

Table 3. Restriction digestion conditions for *tpt1* and pCDNA3.1(-)[d]

Component	Volume (µl)	
	tpt1 digestion	pCDNA3.1(-) digestion
DNA	35 (2 µg)	4 (2 µg)
Eco R1	0.5	0.5
Bam H1	0.5	0.5
Bovine Serum Albumin	5	5
Neb buffer 3	5	5
dd H_2O	4	35

[d]temperature and time duration for the digestions: the reaction was done at 37 ^{0}C for 90 min.

Ligation

After the digestions ligation was set up by taking 1:4 vector DNA: construct DNA ratio. Ligation was done by employing the T4 DNA ligase (Invitrogen, Carlsbad, CA).

Table 4. Ligation conditions for *tpt1* and pCDNA3.1(-)[l]

Component	Volume (µl)
pCDNA3.1(-)	7.5(0.1 µg)
tpt1	18(0.4 µg)
T4 DNA Ligase	0.5
T4 DNA Ligase Buffer	4

[l]ligation conditions: the reaction was carried out at 16 ^{0}C over night.

Transformation and clone confirmation

After the ligation the recombinant plasmids were transformed into *E. coli* XL1 strain by heat shock treatment. For this 5 µl plasmid-DNA was added to 30 µl of *E. coli* XL1 cells and incubated for 30 min on ice. Now heat-shock was given to the cells for 45 s at 42 ^{0}C. Later these cells were placed on ice for 2 min and after which 200 µl of LB medium (Lee *et al*. 1993) was added and incubated for 60 min at 37^{0}C on shaker. Now cells having

recombinant plasmid were selected by plating them on LB agar media with ampicillin (50 µg/ml) over night (Lee *et al.* 1993). Cloning was confirmed by restriction analysis with Apo1 and Dde1 restriction enzymes following the earlier protocol for restriction digestion. PCR analysis was also done on the recombinant plasmid with the same primers used to synthesize the *tpt1* gene by following the earlier protocol used for PCR. Cloning was also confirmed by sequencing.

Transfection

The TCTP over-expressing plasmid pKDK01 and the empty vector (pCDNA3.1(-)) were transfected to MIN6 cells by means of liposome mediated gene transfer by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The cells were maintained in transfection medium Opti-MEM (Invitrogen, Carlsbad, CA) with lipofectamine for 24 hours and later replaced by complete MIN6 medium without antibiotics. Cells were incubated for 48 hours after transfection and then exposed to palmitate.

Fatty acid preparation

A stock solution of 100 mM palmitic acid (Sigma P-9767, St. Louis, MO) was prepared by in 50% ethanol, heating to 60^{o}C. The stock solution was added to the MIN6 culture medium, which contained 0.5% fatty acid free bovine serum albumin (BSA) (Boehringer Mannheim GmbH, Mannheim, Germany), to get a final concentration of 0.25 mM palmitate. The medium was warmed up to 37^{o}C for 40 min to allow complex formation between palmitate and BSA. The cells were exposed to this fatty acid medium for 48 hours.

Western blot analysis

Samples for western blot analysis were prepared by first washing the cells twice with phosphate buffer saline (PBS) having composition of 0.140 M NaCl, 0.0027 M KCl and 0.010 M phosphate buffer at pH 7.4. Cell lysates with extracted proteins were obtained by lysing the cells with lysis buffer composed of 150 mM NaCl, 10 mM Tris-Cl (pH 7.4), 1% Triton X 100, 5 mM EDTA (pH 8) and 1% protease inhibitory cocktail (Sigma P-8340) on ice for 40 to 60 min. Lysates were centrifuged at 10600 xg for 10 min at 4^{0}C. After centrifugation the supernatant was transferred to a new tube and total protein content was determined by the DC protein assay (Bio-Rad, Hercules, CA). Samples were mixed with sample loading buffer containing 0.313 M Tris-HCl (pH 6.8), 10% sodium dodecyl sulphate (SDS), 0.05% bromophenol blue, 50% glycerol and 15% β-mercaptoethanol. Samples containing the same protein amounts (15 µg) were loaded on 10% SDS-polyacrylamide gel (SDS-PAGE).

Table 5. Composition for 10% SDS-PAGE.

Component	Volume (ml)	
	Resolving gel	Stacking gel
1.5 M Tris-HCl	3.3	2.5
30% acrylamide 37.5:1	4.3	0.4
2% SDS	0.67	0.5
dd H_2O	4.7	0.65

The resolving gel polymerization was done for 1 h by adding 6 µl of N,N,N^1,N^1-Tetramethylethylene-diamine (TEMED) and 60 µl of 10% Ammonium per sulphate (APS). The stacking gel was polymerized for 1 h by adding 5 µl TEMED and 40 µl of 10% APS. Electrophoresis was done at 150V for 1 h in the electrophoresis buffer having composition of 0.25 M Tris, 1.92 M glycine and 1% SDS with final pH of 8.3. After electrophoresis, proteins were transferred to PVDF membrane (Bio-Rad, Hercules, CA) by botting using Mini Trans-Blot apparatus (Bio-Rad, Hercules, CA) at 100V for 1hr at 4^0C. After blotting, PVDF membrane was blocked by incubating in blocking buffer for 1hr having composition of 3% BSA (fraction V, Boehringer Mannheim GmbH, Mannheim, Germany) in PBS with 0.05% Tween. Then immunoblot analysis was carried out by incubating PVDF membrane for overnight at 4 ^{0}C with rabbit anti-TCTP polyclonal antibody (primary antibody) and then incubating with goat anti-rabbit IgG antibody which was conjugated to horse radish peroxidase (secondary antibody) for 1hr at room temperature. Both antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The immunoreactive bands were detected with existing chemiluminescent (ECL) Advance (Amersham Biosciences, Uppsala, Sweden), imaged chemiluminescently by Flour-S MultiImager MAX (Bio-Rad, Hercules, CA) and quantified with Quantity One software (Bio-Rad). Later the PVDF membranes were stained with 0.1% Coomassie Brilliant Blue for 1 hour and destained with 50% methanol for 15 min. The blots were scanned with a table top scanner and quantified with the Quantity One software. The expression level of each protein was normalized to the corresponding Coomassie stained lane.

Apoptosis measurements

Apoptosis was measured using the cell death detection kit ELISA^{PLUS} (Roche Diagnostics, Mannheim, Germany). The assay determines the amount of cytoplasmic histone-associated DNA-fragments (oligonucleosomes).

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