Characterization of Tunicamycin as Anti-obesity Agent

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Abstract - Adipocytes undergo adipocyte stress in the excessive presence of lipid. Adipocyte stress accompanies the typical signs of endoplasmic reticulum (ER) stress: unfolded protein response and overexpression of molecular chaperones. Apoptotic induction in adipocytes is known as a good strategy for treating obesity. The drug “tunicamycin” was tested for its therapeutic potential in inducing apoptosis on differentiating adipocytes of 3T3-L1. When the 3T3-L1 cells, stimulated for adipogenesis, were treated with tunicamycin, they showed typical ER stress symptoms. Despite progression in ER stress, however, the differentiated 3T3-L1 hardly proceeded to apoptosis based on the CHOP protein expression and FACS analysis. This is very different from C2C12, the myogenic counterpart of 3T3-L1, which showed significant apoptosis along with ER stress. This study also characterizes a potential mechanism whereby adipocyte may avoid apoptosis to sustain the pathological state of obesity. The level of GRP94 expression significantly upholds in 3T3-L1 under tunicamycin treatment compared to preadipocytes and C2C-12. When GRP94 expression was inhibited by siRNA, 3T3-L1 showed a higher level of CHOP expression compared to C2C12 cells. In conclusion, adipocytes exert an anti-apoptotic mechanism under ER stress caused by tunicamycin; thus, apoptotic induction in adipocyte is not a viable anti-obesity option. The unusual level of GRP94 may serve as a key role whereby adipocytes reach to the obesity level circumventing the apoptosis.

Keywords: Adipogenesis, Apoptosis, ER stress, Myogenesis, Obesity, Tunicamycin

INTRODUCTION

The endoplasmic reticulum (ER) complex serves as a manufacturing factory of protein where nascent polypeptides finish their functional structure through folding and oligomerization. ER is also the biosynthetic manufacturing plant of lipid, such as cholesterol and fatty acids, whose regulation is governed by sterol regulatory element-binding proteins (SREBPs). In addition to regulation of genes involved in lipid biosynthesis, transport and storage of lipid are under the tight control of SREBP (Oyadomori et al., 2006; Puthalakath, 2007). For adipogenic induction, SREBP regulates C/EBP β and δ which are essential for adipogenic clonal expansion (Fu et al., 2005). At the early clonal expansion, cells intermittently divide but further cell division is completely inhibited by PPARγ and C/EBPα factors. Without the assistance of the SREBP cleavage-activating protein (SCAP), however, SREBPs remain as non-functional membrane attachments in the ER. SCAP mobilizes SREBP into the Golgi complex and activates SREBP by cleavage before SREBP’s nuclear transport (Ron and Walter, 2007; Rutkowski et al., 2008).

Adipogenic differentiation in vitro goes through various post-confluence stages: hormonal induction, clonal expansion, growth arrest, and terminal differentiation. At the final stage of adipogenesis, the terminal differentiation normally undergoes a 2-3 weeks of growth arrest. At the terminal stage, adipocytes do not follow a feedback inhibition and lipid storage exceeds the optimal cellular capacity (Lane and Tang, 2005). At this stage, lipid storage droplets appear throughout the cell culture and the droplets increase in mass as fresh media is supplied. From the evolutionary perspective, this is often considered an adaptation mechanism to maximize energy reserve in the face of unstable food supply. However, the lack of feedback inhibition on lipid deposition serves as pathogenic and excessive lipid deposition in adipocytes. Under excess lipid biosynthesis and deposition, cells show adipocyte stress which is virtually ER stress (Li and Lee, 2006). Indeed molecular chaperones enhance their expression and trans-
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MATERIALS AND METHODS

Cell culture and adipogenesis or myogenesis under tunicamycin treatment

The 3T3-L1 preadipocytes were cultured in DMEM containing 10% bovine calf serum (BCS) in 5% CO2 at 37°C. When 3T3-L1 reached a full confluence, the preadipocytes were induced for adipogenesis with MDI cocktail in DMEM containing 10% fetal bovine serum (FBS). The MDI cocktail was added into the culture media as in isobutyl-1-methylxanthine (0.5 mM), dexamethasone (1 μM), and insulin (1 μg/ml). These three components in MDI were purchased from Sigma (St. Louis, USA). Two days after the initial treatment, the preadipocytes were newly cultured with 1 μg/ml insulin in a 10% FBS DMEM. For C2C12 cells, myogenesis was induced by lowering FBS content to 2% in a DMEM after a 24 hour culture in a 10% FBS media. The culture media was replenished with a fresh DMEM with 2% FBS every other day. For both types of cells, ER stress was induced by treating cells with tunicamycin at 2.5 or 5.0 μg/ml.

The ER stress markers of GRP78, GRP94, and CHOP were identified by immunoblotting using respective polyclonal antibodies (Santa Cruz). After treatment with tunicamycin, cleared cell lysates were electrophoresed on a 10% polyacrylamide gel. The electrophoresed gel was blotted onto a patch of nitrocellulose membrane at room temperature. The membrane was incubated in TBS-T containing 5% non-fat milk. Each antibody, against the three markers, was diluted as 1:1,000 in 3% non-fat milk. Following incubation with antibodies, the membranes were thoroughly washed in TBS-T and incubated in horse radish peroxidase conjugated secondary antibody. Signals were detected in Enhanced Chemiluminescence reagent (Roche Diagnostics, USA) and quantitated using the image analysis function of Adobe Photoshop 9.0.

Quantitative PCR and microscopy on differentiating cells

After cells were induced for adipogenic or myogenic differentiation, they were treated with tunicamycin. Adipogenic progression was assayed by quantitative PCR targeting C/EBPδ, C/EBPβ, PPARγ and PPARδ genes. After initial incubation for 5 minutes at 95°C, the polymerization reaction was carried out in 30 cycles at 72°C for one minute (95°C for 10 sec for denaturation and 55°C for 10 sec for annealing). The effect of tunicamycin on differentiation was monitored under microscope (200X). In the differentiating analysis, 3T3-L1 was stained for microscopy using the standard Oil Red procedure (Fu et al., 2005) while C2C12 was examined without staining.

FACS analysis for annexin V

Following treatment, the cells were loosened in 0.25% trypsin, washed copiously in FACS buffer (PBS containing 0.1% sodium azide and 2% FCS) and collected by centrifugation at 500 g for 5 min. The cells were resuspended to 10^7 cells/ml in 100 μl while the blocking antibody was added at 5 μl per tube. Primary antibody was added and FACS reaction was incubated in a buffer that included ice for 30 minutes. At the termination of incubation, the cells were gently vortexed and incubated in the dark for 30 min on ice. The annexin V-staining reagent was added and the FACS samples were incubated on ice in the dark for one hour. Using the FACS buffer, the cells were washed twice before undergoing FACS analysis.
GRP94 siRNA inhibition experiment

For the GRP94 inhibition experiment, the siRNA and transfection kit were purchased from Santa Cruz Biotech. The solution of anti-GRP94 siRNA and transfection was mixed using mild vortexing and then incubated for 30 minutes at room temperature. The cells were treated with 2 ml of siRNA media and approximately $10^6$ cells were placed in 1 ml of siRNA transfection media. The siRNA transfection reaction continued for 6 hours at 37°C in a CO₂ incubator. The cells, transfected with GRP94 siRNA, were placed in appropriate culture dishes and treated using various experimental conditions. Protein expression was quantitated by the image analysis of Adobe Photoshop 9.0.

RESULTS

Expression of ER stress markers under tunicamycin treatment

The effect of tunicamycin on the expression of ER stress markers, during adipogenesis or myogenesis, was analyzed by immunoblotting. After induction for differentiation, 3T3-L1 and C2C12 cells were treated with tunicamycin. Fig. 1 shows that the two cell lines displayed an elevated level of ER stress markers compared to its non-treated counterpart (i.e., at t=0 min). Before tunicamycin treatment, both cell lines did not show any significant expression of GRP78 and CHOP (Fig. 1A and C). However, GRP94 was already present in the differentiating adipocytes and myocytes (Fig. 1B). This observation indicates that adipogenic differentiation accompanies a significant level of ER stress in the absence of tunicamycin. When tunicamycin treatment continued up to 3 hours, 3T3-L1 cells demonstrated a higher level of GRP94 expression compared to C2C12 cells. GRP 94 expressed higher approximately 2 to 7 times in 3T3-L1 cells than in C2C12 depending on the time points but its expression patterns were relatively similar in both cell lines. In terms of CHOP expression, C2C12 cell showed a surge at the three hour after tunicamycin treatment (Fig. 1C). The 3T3-L1, however, showed a basal level of expression throughout the tunicamycin treatment. This expression pattern was also very different from the typical expression pattern in HEK293 cells which showed a steady increase in GRP78 and GRP94 (Fig. 1D). In summary, a significant difference was evident in molecular chaperone expression in differentiating myocytes and adipocytes during ER stress conditions. In developing myocytes, the GRP94 expression rapidly decreased but CHOP expression increased during ER stress.

Expression of adipogenic factors affected by tunicamycin treatment

The expression patterns of adipogenic factors were analyzed before and after undergoing tunicamycin treatment, the quantitative PCR analysis. This assay was used to study whether expressions of adipogenic (C/EBPβ, C/EBPδ, PPARγ) and myogenic (PPARγ) markers are modulated in the presence of tunicamycin (Fig. 2). When preadipocytes were treated with tunicamycin, the expression of CEBPβ and PPARγ increased. PPARγ, however, totally disappeared from the tunicamycin-treated samples. This is different from the non-treated control sample which showed a bit of expression (Fig. 2A).
Fig. 2. Expression of differentiation factors was assayed according to the quantitative PCR. The 3T3-L1 cells were treated with tunicamycin (TUN) at three concentrations: 0 [NT], 2.5, and 5.0 μg/ml. Cells were treated with tunicamycin without, before, or after application of methylisobutylxanthine-dexamethasone-insulin (MDI). (A) refers to the expression of differentiation factors under MDI-free (or non-adipogenic) treatment. (B) represents the expression patterns under tunicamycin treatment before (TUN→MDI) or after (MDI→TUN) MDI application (Cells were treated with TUN at 2.5 or 5.0 μg/ml). The MDI column stands for the tunicamycin-free control.

Two days after adipogenic induction, the marker genes were seen to be expressing actively. However, their expression was modulated differently after undergoing ER stress treatment with tunicamycin (Fig. 2B). Overall, their expression decreased compared to the tunicamycin-free treatment and the effect of ER stress treatment led to very different outcomes. PPARγ was most affected by the agent in a dose dependent manner. This result indicates that tunicamycin may exert an anti-obesity effect at the molecular level by down-regulating PPARγ. In addition, tunicamycin’s effect on the expression of PPARδ greatly varied whether the agent was added to the media before or after adipogenic induction. When the tunicamycin treatment preceded the adipogenic induction by methylisobutylxanthine-dexamethasone-insulin (MDI) (TUN→MDI in Fig. 2B), C/EBPβ was negatively affected compared to the reverse tunicamycin treatment (MDI→TUN).

Adipogenic progression less affected by tunicamycin

Tunicamycin treatment produces different effects on cell progression using differentiation analysis. The effect is dependent on the cell lineage, either adipogenic or myogenic. Fig. 3 shows that 3T3-L1 and C2C12, induced for myogenesis and adipogenesis, are differently affected by TUN treatment. Five days into differentiation analysis, the 3T3-L1 cells did not exhibit any disturbance in terms of adipogenic progression both at 2.5 and 5.0 μg/ml. However, myogenic progression was significantly impaired with the tunicamycin treatment of 2.5 μg/ml. The non-treated control (NT4D), however, showed that muscle fibers were formed and fused with each other to form wavy myotubes having elongated multinuclei structures. This observation indicates that adipocytes are less affected by tunicamycin caused ER stress compared to the myocytes.

FACS analysis: apoptosis in adipocytes is less affected by tunicamycin

The extent of apoptotic progression was analyzed by FACS after 3T3-L1 and C2C12 were treated with tunicamycin for the following periods of time: 5 μg/ml for 0, 30, 60, or 240 minutes. They were also incubated in a tunicamycin-free media for one day. Between C2C12 and 3T3-L1 cell lines, the difference in apoptotic progression was evident. After each tunicamycin treatment, the C2C12 cells showed a noteworthy level of early apoptosis compared to 3T3-L1. FACS analysis shows that 3T3-L1 cells did not undergo any significant degree of apoptosis; rather, a significant sign was apparent in the 3T3-L1 compared to C2C12 (Fig. 4).

CHOP expression after inhibition of GRP94 by siRNA

When GRP94 expression was suppressed by using siRNA against GRP94, both 3T3-L1 and C2C12 cells showed a significant level of CHOP protein expression un-
Fig. 4. Apoptotic progression was analyzed by propidium iodide (PI) and annexin-V labeling for apoptotic or necrotic cell death detection. The differentiating C2C12 cells (upper panel) exhibited a significant level of early apoptosis following tunicamycin treatment (5.0 μg/ml) for 0, 30, 60, or 240 minutes. In contrast, 3T3-L1 cells (lower panel) showed a low level of early and late apoptosis under equivalent conditions of tunicamycin treatment.

Fig. 5. Using siRNA, the expression of GRP94 was inhibited in differentiating 3T3-L1 and C2C12. CHOP expression was compared between the two cell types. (A) Inhibition of GRP94 accompanies an enhanced effect on the CHOP expression in both lines of cell. (B) GRP94 and CHOP expressions were quantitated and standardized assuming non-siGRP94 of C2C12 as 1.0. Relative Intensity Difference of (−)GRP 94 or (+)CHOP stands for the reduced or increased expression of each protein after siRNA transfection.

Under tunicamycin-free treatment. In the presence of tunicamycin, GRP94 siRNA transfected cells did not survive enough for the analysis and no data is shown in Fig. 5. In the differentiating cells, GRP94 showed a significant level of expression while CHOP showed a minimal level. However, the inhibition of GRP94 by siRNA concurred with increase in the pro-apoptotic marker in both types of cell (Fig. 5A). In 3T3-L1, CHOP expression increased more sensitively than C2C12 when GRP94 was down-regulated (Fig. 5B). This indicates that, in differentiating adipocytes, CHOP responded more positively to the reducing level of GRP94 based on the difference in relative expression of GRP94 and CHOP.

DISCUSSION

This study tested tunicamycin as a potential anti-obesity agent to help eliminate excess adipocytes via apoptosis by triggering ER stress. Tunicamycin caused a significant level of ER stress in 3T3-L1 and C2C12 in this study. When ER stress advanced, 3T3-L1 rarely proceed to apoptosis while C2C12 appeared to progress to apoptosis under tunicamycin treatment. This observation strongly implies that adipocytes use an anti-apoptotic means to reduce the effect of ER stress caused by tunicamycin.

When molecular chaperone expressions were compared, no significant difference was found with respect to GRP78. In terms of GRP94 or CHOP expression, however, a major difference was evident between the two types of cell. The differentiating adipocytes showed higher level of GRP94 throughout tunicamycin treatment compared to myocytes. The expression of CHOP, marker for the apoptosis, surged only in myocytes under extended treatment with tunicamycin. This discrepancy, in apoptotic progression, between the two cell lines can be explained by the ample presence of GRP94 in adipocytes.

The anti-apoptotic role of GRP94 was further supported by interference experiments where GRP94 expression was inhibited by siRNA. When the GRP94 gene expression was inhibited, CHOP was the most responsive molecule in both types of cell. The 3T3-L1, however, showed a more sensitive response to the reduced presence of GRP94, compared to C2C12. This strongly implies that the significance of GRP94 is higher in adipocytes than in myocytes. Apoptotic progression therefore is more effectively suppressed in adipocytes by GRP94 and CHOP. The apoptotic factor might be better seen in the absence of GRP94.

A controversy could emerge as to whether CHOP is the molecule that is directly affected by GRP94. Since CHOP can be induced by various conditions such as amino acid deficiency, DNA damage, and hypoxia, GRP94’s knock down may not directly augment the CHOP expression. Based on the siRNA experiment, however, CHOP is the molecule that is consistently responsive in both adipocytes and myocytes. The enhanced CHOP expression, in the absence of GRP94, strongly suggests that the lack of apoptosis results from the prominent presence of GRP94 in adipocytes despite tunicamycin-caused ER stress.

Tunicamycin down-regulated the adipogenic differentiation factor in adipocytes. The C/EBPs and PPARγ factors are believed to play a pivotal role in increasing lipid biosynthesis and its deposition. These factors are also considered to modulate the expression or prolong the half-life
of cyclin-dependant protein kinase (CDK) inhibitor such as p21 and p27. This result implies that tunicamycin can be used to treat obesity by repressing cell cycle by lowering the expression of genes involved in adipogenic progress. However, their modulation may not exert a significant outcome as an anti-obesity agent considering that these genes express during the latter stage of differentiation.

This study shows that tunicamycin is capable of inducing ER stress in adipocytes as well as in myocytes. The ER stress triggered by tunicamycin, however, was insufficient to induce adipocytes to proceed to apoptosis. A different, however, was seen in myocytes. Tunicamycin caused a significant necrosis in adipocytes. This suggests that ER stress might induce necrosis-inducing factors in the adipocytes most likely via inflammatory response. Adipocytes' resistance to apoptosis can be explained by unusual expression of GRP94 in the presence of tunicamycin. Further studies are necessary to investigate a potential crosstalk in adipocytes between inflammatory and apoptotic progression during ER stress.

A study is also necessary to elucidate the potential mechanisms for necrosis and anti-apoptosis which are evident in adipocytes. Another study could also help find a specific agent to inhibit the GRP94 molecule whose presence prevents apoptosis (Sheu et al., 2007). The combined use of multiple ER stress inducers would be a good strategy to treat obesity. Under this combined scheme, tunicamycin may undo the anti-apoptotic machinery in adipocytes, along with other ER stress inducing agents such as thapsigargin or hypoxia.

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