TMF protects chondrocytes from ER stress-induced apoptosis by down-regulating GSK-3 β

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Abstract: Endoplasmic reticulum (ER) stress-induced chondrocyte apoptosis plays a critical role in osteoarthritis cartilage degeneration. Previous studies showed that 5,7,3',4'-tetramethoxyflavone (TMF) exhibited chondroprotective activity through inhibiting PGE2-induced ER stress and down regulating the expression of GSK-3 β . To further investigate the role of GSK-3 β in ER stress-induced chondrocytes apoptosis and the protective role of TMF, GSK-3 β siRNA and pcDNA3.1-myc-GSK-3 β were employed to knock down and overexpress GSK-3 β , respectively, in chondrocytes. Results showed that TM-induced ER stress significantly promoted chondrocytes apoptosis. These could be effectively reversed by GSK-3 β deficiency, while GSK-3 β overexpression significantly up regulated ER stress and increased chondrocytes apoptosis. In addition, TMF down regulated the expression of GSK-3 β and inhibited ER stress-induced chondrocytes apoptosis. Collectively, TMF is a potential natural compound with chondroprotective property through inhibition of ER stress-induced apoptosis with down regulation of GSK-3 β .

Keywords: TMF; ER stress; GSK-3 β ; chondrocytes apoptosis

Introduction

5,7,3',4'-tetramethoxyflavone (TMF), one of the mainly active constituents derived from *Murraya exotica*, has been reported to demonstrate anti-inflammatory, anti-fungal, anti-mycobacterial, and anti-malarial activities [1]. Previously, we have demonstrated that TMF exhibits chondroprotective activity through inhibiting chondrocytes apoptosis induced by endoplasmic reticulum (ER) stress and through down regulating the expression of GSK-3 β [2]. In addition, TMF has been showed to protect cartilage in a rat model of osteoarthritis (OA) through reduction of the concentration of inflammatory cytokines TNF α , IL-1 β , and PGE2 in the knee synovial fluid and down regulation of EP/cAMP/PKA and Wnt/ β -catenin signaling pathways [3]. However, the underlying mechanism of the TMF protective activity against chondrocyte apoptosis is still unknown.

ER is the membrane organelle dealing with the folding and maturation of protein, the biosynthesis of lipid, and the balance of calcium. In order to maintain cellular homeostasis, the unfolded protein response (UPR), a unique self-protecting mechanism, has been developed to adapt to new micro-environments produced by detrimental stimuli. Three ER transmembrane sensors including inositol-requiring enzyme-1 (IRE1), PKR-like ER kinase (PERK), and activating transcription factor-6 (ATF6) are linked to UPR [4]. The persistence of homeostatic imbalance leads to ER stress, characterized by accumulation of unfolded or mis-folded proteins in ER. However, once these stimuli are overwhelming and ER stress persists, cells are programmed to switch from pro-survival to pro-apoptosis [5], through activating the downstream factors such as C/EBP-homologous protein (CHOP) and c-Jun N-terminal kinase (JNK) [6].

GSK-3 β is the multifunctional Ser/Thr kinase to degrade β -catenin/APC/Axin complex and phosphorylate β -catenin to prevent its nuclear translocation and transcriptional activity. Tight regulation of β -catenin signaling is required for a healthy articular cartilage [7], which indicates the critical role of GSK-3 β . Recently, GSK-3 β has been demonstrated to be an important mediator of inflammatory activity and increase the production of inflammatory cytokines in animal models [8]. In addition, GSK-3 β is also an obligatory factor in ER stress-triggered apoptosis [9]. However, the interaction mechanism between GSK-3 β and ER stress is still unclear. In this study, we focused on determining whether GSK-3 β regulated ER stress-induced chondrocytes apoptosis. We also tested the protective role of TMF in ER stress-induced chondrocytes apoptosis and regulation of GSK-3 β .

Method

Material

Fetal bovine serum (FBS), Dulbecco's modified Eagle's minimum essential medium (DMEM), penicillin, and streptomycin were obtained from Gibco (Life technologies, NY, USA). GSK-3 β , GSK-3 β -Tyr216-p, phosphorylated glycogen synthase (p-GS), GRP78, IRE1 α , p-IRE1 α , XBP1S, PERK, p-PERK, CHOP, p-JNK, ATF6, and GAPDH monoclonal antibodies and the peroxidase-conjugated secondary antibody were purchased from Abcam (Cambridge, UK). Tunicamycin (TM) was bought

from Sigma Chemicals (Louis, United States). TMF was obtained as described previously [2].

Primary cell isolation and culture

Under sterile conditions, four-week-old rats were sacrificed, and cartilages were isolated from the knee joints. The cartilages were cut into small pieces, and 0.25% pancreatic enzymes were employed to digest for 30 min to remove other tissues and cells, following with employment of 0.2% collagenase II to digest at 37°C for 4h. The chondrocytes cells were isolated and then cultured under the standard conditions, such as DMEM (low glucose) supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C with 5% CO₂. The cultured chondrocytes have been identified as previously mentioned by toluidine-blue staining and immunohistochemical staining [10]. The second passage of chondrocytes was used. The study was approved by the Institutional Animal Care and Use Committee of Gannan Medical University.

Transfection by siRNA or pcDNA3.1-myc

siRNA and pcDNA3.1-myc were purchased from Gene Pharma (Shanghai). Chondrocytes were transfected using optimized concentration of GSK-3 β siRNA. The transfection procedures were carried out according to the manufacturer's instructions. 48 hours after transfection, the chondrocytes cells were lysed. The isolated chondrocytes were transfected with GSK-3 β siRNA. Scrambled siRNA (the sequence is UCACAACCUCCUAGAAAGAGUAGA) was constructed as the control siRNA. qRT-PCR and western-blotting with anti-GSK-3 β were applied to determine mRNA and protein expression to confirm siRNA-mediated knockdown. The plasmid pcDNA3.1-myc-GSK-3 β was also constructed. The procedures included: full length of GSK-3 β was amplified by RT-PCR from mRNA isolated from rat chondrocytes. The product of RT-PCR was cloned into the vector pcDNA3.1-myc and then transfected into chondrocytes. G418 (Sigma) was employed to screen the transfection.

Quantitative analysis of apoptosis

Annexin V-FITC apoptosis assay was employed to quantify the changes of chondrocytes apoptosis by flow cytometry according to the apoptosis detection kit (Nanjing KeyGEN Biological Technology Development Co., Ltd., Nanjing, China) procedures. Chondrocytes were incubated for 48 h, and then treated with 1 μ M TM and TMF. Cells (1 $\times$ 10⁶/mL) were collected and incubated in the buffer containing FITC-annexin V and PI. The apoptosis ratio of chondrocytes was determined by a flow cytometer (FACSCalibur BD, San Jose, CA).

Gene expression analysis

The Easy-spinTM total RNA extraction kit (iNtRON Biotechnology, Seoul, Korea) was employed to extract the total RNA from chondrocytes. According to the standard protocols, the first-strand of cDNA was synthesized by reverse-transcribing 2 μ g of total RNA for each sample using M-MLV (Promega, USA). qRT-PCR was employed to determine the expression levels of *GSK-3 β* , *IRE1 α* , *XBPI*, *CHOP*, *PERK*, *ATF6*, and *GRP78* genes using EzOmics SYBR qPCR kits (Mastecycler, Eppendorf, Germany). Their individual primer sequences were listed in Table 1. The procedure for amplification was: 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 56°C for 45 s, 72°C for 45 s, and finally at 72°C for 10 min. The PCR reactions were performed by using the iCycler iQ real time PCR system (Bio-Rad).

Table 1: Primer sequences for different genes

<i>GSK-3β</i>	forward	5'-GGAAGTCCAACAAGGGAGCA-3'	Ref [11]
	reverse	5'-TTCGGGGTCGGAAGACCTT A-3'	
<i>IRE1α</i>	forward	5'- AGAGAGGCGGGAGAGCCGTG -3'	Ref [12]
	reverse	5'- CGAGGAGGTGGGGGA AGCGA -3'	
<i>XBPI</i>	forward	5'- AGTTAAGAACACGCTTGGAAT -3'	Ref [13]
	reverse	5'- AAGATGTTCTGGGGAGGTGAC -3'	
<i>CHOP</i>	forward	5'- ATATCTCATCCCCAGGAAACG -3'	Ref [13]
	reverse	5'- TCTTCCTTGCTCTTCCTCCTC -3'	
<i>PERK</i>	forward	5'- ATCCCCCATGGAACGACCTG -3'	Ref [14]

<i>PERK</i>	reverse	5'-ACCCGCCAGGGACAAAAATG-3'	Ref [14]
<i>ATF6</i>	forward	5'-CTGGAGGCGCTGGTGAAAAGTG-3'	Ref [15]
	reverse	5'-CCGGACGGGAGATGGGAACA-3'	
<i>GRP78</i>	forward	5'-GCTCGACTCGAATTCCAAAG-3'	Ref [16]
	reverse	5'-TTTGTCAAGGGTCTTTCACC-3'	
<i>GAPDH</i>	forward	5'-CAGTGGCAAAGTGGAGATTG-3'	Ref [17]
	reverse	5'-AATTTGCCG-TGAGTGGAGTC-3'	

Four duplicates were performed for all the PCR reactions. *GAPDH* was used as an internal standard control. For each target, the experimental protocols were designed and optimized for efficiency near one per assumption underlying the $2^{-\Delta\Delta CT}$ method [17].

Western blot analysis

The lysis buffer (2% SDS, 10% glycerol, 10 mmol/L Tris, pH6.8, 100 mmol/L DTT) was used to lyse cells to prepare for immunoblotting. A BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA) was employed to determine the protein concentrations with bovine serum albumin as a standard. The samples were then combined with gel loading buffer (50 mmol/L Tris-HCl, pH6.8, 2% SDS, 10% glycerol, and 0.1% bromophenol blue) and denatured for 5 min, following with electrophoresing on 10% SDS-PAGE gel for GSK-3 β , GRP78, IRE1 α , p-IRE1 α , XBP1S, PERK, p-PERK, CHOP, JNK, p-JNK and ATF6. Proteins were western-blotted onto polyvinylidene difluoride (PVDF) transfer membranes. Tris-buffered saline (TBS) containing 5% non-fat milk was used to block blots for 1h, which were incubated with GSK-3 β , GRP78, IRE1 α , p-IRE1 α , XBP1S, PERK, p-PERK, CHOP, p-JNK and ATF6 at 4°C overnight, respectively. After that, the blots were rinsed and incubated with HRP-conjugated goat anti-rat IgG for 1h. The blots were washed and developed by use of a Super Enhanced chemiluminescence detection kit (Applygen Technologies Inc., Beijing, China). Then, the protein bands were visualized after exposure of the membranes to Kodak film (USA). GAPDH was used as the internal control.

Statistical analysis

All data were expressed as mean $\pm$ standard deviation (SD). Statistical analysis of gene expression data were analyzed by a paired t-test. Differences were considered significant at $p < 0.05$.

Results

GSK-3 β facilitated ER stress-mediated chondrocyte apoptosis

To prove the regulatory activity of GSK-3 β in ER stress-induced chondrocyte apoptosis, we knocked down or overexpressed GSK-3 β by transfecting GSK-3 β siRNA or pcDNA3.1-myc-GSK-3 β , respectively, in the second generation of cultured rat chondrocytes. The mRNA and protein expression levels of GSK-3 β in chondrocytes were induced by the ER stress triggering agent tunicamycin (TM) (1 μ M). GSK-3 β siRNA transfection dramatically inhibited GSK-3 β and its downstream factor p-GS expression. In contrast, pcDNA3.1-myc-GSK-3 β significantly facilitated TM-induced GSK-3 β and p-GS expression (Figure 1). TM induced up regulation of GSK-3 β expression could be inhibited by LiCl (Figure 1). TM treatment for 24 h induced chondrocyte apoptosis (14.20 $\pm$ 0.96 %), while GSK-3 β deficiency significantly inhibited TM-induced ER stress chondrocyte apoptosis (3.36 $\pm$ 0.52 %). In contrast, overexpression of GSK-3 β facilitated TM to induced chondrocyte apoptosis (16.00 $\pm$ 1.22 %) (Figure 2). Thus, GSK-3 β is required for ER stress response to promote apoptosis in chondrocytes.

A

B

C

Figure 1. GSK-3β is expressed in chondrocytes transfected with control siRNA (C) for siRNA transfection, GSK-3β siRNA, empty plasmid pcDNA3.1-myc vector (V), and pcDNA3.1-myc-GSK-3β. (A) Analysis of GSK-3β mRNA expression with qRT-PCR. (B) Determination of GSK-3β, GSK-3β-Tyr216-p, and p-GS protein expression with western blot. (C) Determination of GSK-3β-Tyr216-p protein expression with administration of LiCl with western blot. GAPDH was used as internal control. Data were presented by mean ± standard deviation of 4 replicates. * $p < 0.05$ compared with control.

TMF protects chondrocytes from apoptosis induced by GSK-3β-mediated ER stress

Our previous studies showed that TMF could significant inhibit chondrocytes apoptosis induced by TM using FITC-annexin V and PI staining [2]. To determine whether TMF affected chondrocyte apoptosis induced by ER stress via inhibition of GSK-3β, we employed GSK-3β siRNA and pcDNA3.1-myc-GSK-3β, respectively, to chondrocytes with 1 μM TM for 24 h. Flow cytometry analysis indicated that the apoptosis ratio of chondrocytes transfected with pcDNA3.1-myc-GSK-3β was dramatically elevated. While administration of TMF at the dose of 5 μg/mL and 20 μg/mL in GSK-3β overexpression groups, the apoptosis ratio were significantly decreased (10.08 ± 1.03 % and 6.74 ± 0.68 %, respectively) (Figure 2).

Figure 2. TMF reduced GSK-3 β siRNA-induced apoptosis after treatment with 1 μ M TM for 24 h in chondrocytes. The chondrocytes were incubated for 48 h. Chondrocytes in the negative control group (a) was incubated without adding any medicines. Group (b) was the chondrocytes incubated with 1 μ M TM for 24 h. Group (c) was the chondrocytes transfected with GSK-3 β siRNA alone. Group (d) was the chondrocytes transfected with GSK-3 β siRNA incubated with 1 μ M TM for 24 h. Group (e) was the chondrocytes transfected with pcDNA3.1-myc-GSK-3 β incubated with 1 μ M TM for 24 h. Group (f and g) were the groups that the chondrocytes transfected with pcDNA3.1-myc-GSK-3 β incubated with 1 μ M TM and 5, 20 μ g/mL TMF for 24 h, respectively. Figure (h) was the summarized data indicating the chondrocytes apoptosis ratio, as detected by the flow cytometry. Data were presented by mean $\pm$ standard deviation of 4 replicates. * p < 0.05 compared with control.

TMF decreases the genes expression of ER stress mediated by GSK-3 β

To determine the chondroprotective activity of TMF in ER stress-induced chondrocytes apoptosis mediated by GSK-3 β , we quantified the downstream gene expression of ER stress pathways by real-time qRT-PCR in GSK-3 β siRNA or pcDNA3.1-myc-GSK-3 β transfected chondrocytes. As a result, TM alone significantly stimulated the mRNA expression of GSK-3 β , IRE1 α , CHOP, PERK, and ATF6 (Figure 3). In GSK-3 β overexpression groups, these responses induced by TM were further increased. While in the presence of GSK-3 β siRNA, ER stress responses were dramatically inhibited. In addition, TMF exhibited inhibitory effects on the mRNA expression of GSK-3 β in chondrocytes. Also, TMF treatment significantly down regulated the mRNA expression of ER stress pathways in the GSK-3 β overexpression groups (Figure 3). However, the expression of XBP1 and GRP78 showed in a different pattern, and TMF exhibited slightly stimulatory activity in XBP1 and GRP78 expression (Figure 3).

Figure 3. The mRNA expression changes of *GSK-3β*, *IRE1α*, *XBP1*, *CHOP*, *PERK*, *ATF6*, and *GRP78* in *GSK-3β* siRNA or pcDNA3.1-myc-*GSK-3β* transfected chondrocytes incubated with 1 μ M TM or 5 and 20 μ g/mL TMF for 24 h. Changes in mRNA expression of these genes were detected by qRT-PCR. *GAPDH* was used as internal control. Data were presented by mean $\pm$ standard deviation of 4 replicates. * $p < 0.05$ compared with control.

TMF ameliorates the proteins expression of ER stress mediated by *GSK-3β*

We further determined whether TMF ameliorated the proteins expression of ER stress pathways mediated by *GSK-3β*. The chondrocytes were incubated for 24 h with 1 μ M TM, which significantly induced the activation of PERK, *IRE1α*, and ATF6 signaling pathways. These could be further up regulated in *GSK-3β* overexpression groups. In contrast, *GSK-3β* deficiency effectively exhibited inhibition of PERK, CHOP, p-JNK, *IRE1α*, and ATF6 protein expression. However, the alternative changes of *GSK-3β* expression did not influence the phosphorylation of PERK and *IRE1α* too much (Figure 4). TMF treatment could reverse the up regulation of ER stress, including the pro-apoptotic factors CHOP. In addition, the activity of p-JNK was induced by ER stress and *GSK-3β* while TMF treatment inhibited such induction (Figure 4). However, XBP1S protein expression was negatively modulated by *GSK-3β*. TMF dramatically promoted the protein production of GRP78 and XBP1S, which may be related to its pro-survival activity (Figure 4).

Figure 4. Protein expression changes of GRP78, IRE1 α , p-IRE1 α , XBP1S, PERK, p-PERK, CHOP, JNK, p-JNK, and ATF6 in IRE1 α siRNA or pcDNA3.1-myc-GSK-3 β transfected chondrocytes incubated with 1 μ M TM and 5 and 20 μ g/mL TMF for 24 h. Changes in protein expression were detected by Western blot. GAPDH was used as internal control. Data were presented by mean $\pm$ standard deviation of 4 replicates. * p < 0.05 compared with control.

Discussion

Chondrocytes are the unique cell type in cartilage to be implicated in multiple stresses, including inflammatory, oxidative, and ER stress. ER stress induced chondrocytes apoptosis has been involved in the pathogenesis of OA [18]. Our previous studies show that TMF could exhibit chondroprotective activity via inhibiting ER stress induced by the pro-inflammatory cytokines, such as PGE2, and via down regulating the expression of GSK-3 β [2]. ER stress has been demonstrated to increase GSK-3 β activity in cultured cells [19], which might be associated with inhibition of PI3K/Akt signaling [20]. On the other hand, GSK-3 β has been identified to be a critical positive mediator of peripheral inflammatory response as revealed by increased induction of cytokines [21] and by activation of nuclear factor- κ B *in vivo* and *in vitro* [22]. To determine whether GSK-3 β is involved in the regulation of ER stress in chondrocytes, we further investigated the pro-apoptotic role of GSK-3 β in ER stress-induced chondrocytes apoptosis and the chondroprotective role of TMF.

Our results demonstrated that TM significantly promoted ER stress pathways, leading to chondrocytes apoptosis. These pro-apoptotic responses were inhibited by GSK-3 β deficiency. Specifically, the activation of GSK-3 β is required for promoting apoptosis in response to ER stress in human neuroblastoma cells [9]. It has been reported that moderate overexpression of GSK-3 β (3.5-fold) is sufficient not to induce apoptosis alone but to facilitate apoptosis induced by other stressors [23]. Activation of GSK-3 β is showed to facilitate K562 cells to be susceptible to TM-induced lysosomal apoptosis, while K562 cells generally show resistance to the apoptotic effects of TM [24]. Consistently, our research results also exhibited that overexpression of GSK-3 β promoted ER stress-induced chondrocytes apoptosis.

During ER stress, IRE1 α regulates the splicing activity of XBP1 mRNA to produce XBP1S. Studies have been showed that IRE1 α -dependent GSK-3 β activation participates to inhibit the endonuclease activity of IRE1 α and suppress the production of XBP1S, leading to inhibition of TNF α transcription in RAW 264.7 macrophages [25]. Consistently, our research found that the protein production of XBP1S was dramatically increased in GSK-3 β -deficient chondrocytes and decreased in GSK-3 β overexpression groups. The phosphorylation of IRE1 α was not greatly influenced by GSK-3 β . However, this regulation was not involved in the gene expression of XBP1. GSK-3 β has been

demonstrated to be a target of PERK signaling. GSK-3 β inhibition significantly blocks the expression of CHOP and ATF4 in atherosclerotic macrophages [26]. Consistently, in our study, GSK-3 β deficiency down regulated PERK and CHOP expression during ER stress in chondrocytes. But the phosphorylation of PERK did not change significantly. The interaction mechanism between GSK-3 β and ER stress is still obscure, and this might be partially related to the uncertainty of downstream targets of GSK-3 β , which is involved in a number of signaling network and modulates many aspects of cell metabolism and physiology.

In our study, we showed that TMF exhibited chondroprotective activity against ER stress-induced apoptosis through down regulating the expression of PERK, p-PERK, CHOP, IRE1 α , p-IRE1 α , ATF6, and p-JNK and, at least in partial, up regulating the expression of the pro-survival factor XBP1S and GRP78. TMF is one of natural flavonoids which have been intensively demonstrated to exhibit many medicinal benefits. Quercetin has been reported to target ER stress signaling pathway and inhibit ADMA-induced GEnC apoptosis [27]. In addition, quercetin also inhibits Pb-induced ER stress by mediating the PI3K/Akt and IRE1 α /JNK signaling pathways [28]. TM could significantly promote the activation of GSK-3 β , which positively regulated the response of ER stress. TMF was demonstrated to down regulate the activation of GSK-3 β , leading to protection of chondrocytes from ER stress-induced apoptosis. The chondroprotective property of TMF raises the possibility of developing TMF as an ER stress resistant and counter-apoptosis therapeutics for joint degenerative diseases.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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