RESEARCH ARTICLE

Transient Knock Down of Grp78 Reveals Roles in Serum Ferritin Mediated Pro-inflammatory Cytokine Secretion in Rat Primary Activated Hepatic Stellate Cells

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Abstract

Chronic liver diseases, including cancer, are characterized by inflammation and elevated serum ferritin (SF). However, the causal-relationship remains unclear. This study used primary rat hepatic stellate cells (HSC) as a model to investigate effects of physiological SF concentrations (10, 100 and 1000 pM) because HSCs play a central role in the development and progression of liver fibrosis. Physiological concentrations of SF, either horse SF or human serum, induced pro-inflammatory cytokine IL1β, IL6 and TNFα secretion in rat activated HSCs (all $p<0.05$). By contrast, treatment did not alter activation marker αSMA expression. The presence of SF markedly enhanced expression of Grp78 mRNA ($p<0.01$). Furthermore, transient knock down of Grp78 by endotoxin EGF-SubA abolished SF-induced IL1β and TNFα secretion in activated HSCs (all $p<0.05$). In conclusion, our results showed that at physiological concentrations SF functions as a pro-inflammatory mediator in primary rat HSCs. We also provide a molecular basis for the action of SF and identified Grp78-associated ER stress pathways as a novel potential therapeutic target for resolution of fibrosis and possible prevention of liver cancer.

Keywords: Serum ferritin - hepatic stellate cells - liver fibrosis - Grp78 - endoplasmic reticulum stress

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Introduction

Hepatic fibrosis is the result of the wound-healing response of the liver to repeated injury. The activation of hepatic stellate cells (HSC) in response to liver injury is considered to be a key cellular event that drives the liver fibrosis (Bataller and Brenner, 2005). HSC activation refers to the trans-differentiation of quiescent HSC into proliferative and contractile myofibroblast. In general, the process of HSC activation involves: (1) decreased intracellular vitamin A stores; (2) increased production of extracellular matrix (ECM) proteins and activation marker α-smooth muscle actin (α-SMA); (3) increased production of pro-inflammatory mediators (e.g. IL1β, TNFα, IL6) and anti-inflammatory cytokine (e.g. TGFβ); and (4) acquired highly proliferative and myofibroblast-like (Bataller and Brenner, 2005).

Substantial evidences support the view that dysregulated iron homeostasis is associated with liver fibrogenesis (Batts, 2007). Though the underlying mechanisms associated with iron overload and iron toxicity of the liver is still unknown. Ludwig and colleagues studied tissue iron in 447 cirrhotic livers and reported 32% cases with positive iron staining and 8.5% cases with hepatic iron overload in the hemochromatosis range (Ludwig et al., 1997). The amount of stored vitamin A decreases is a key feature which associated with the activation of quiescent hepatic stellate cells to myofibroblasts. Early study showed that vitamin A is involved in the regulation of iron release from the liver (Staab et al., 1984). Therefore, the amount of stored vitamin A decreases in activated HSC may contribute to hepatic iron overload. Tissue ferritin is the major iron storage molecules in the liver. However, circulating ferritin concentrations are elevated in inflammation and are associated with chronic liver injury (Lin et al., 2006; Manousou et al., 2011). Ramm and colleagues first showed that activated HSC expressed high affinity ferritin receptor (Ramm et al., 1994) and internalization of ferritin by activated HSC reduced α-SMA expression (Ramm et al., 1996). Recently, they showed iron-bound ferritin or iron free molecules (apofferitin or recombinant human H- or L-subunits) activates IL1β and inducible nitric oxide synthase (iNOS) synthesis via NF-kB signaling pathways in activated HSC (Ruddell et al., 2009). Iron is an important mediator of oxidant stress, with reactive oxygen species (ROS) known to induce NF-kB activation. The fact that iron free apoferritin molecules induced similar levels of NF-kB activation compared with iron-bound ferritin suggesting iron-dependent and iron-independent effects on the

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pathogenesis of hepatic fibrogenesis (Ruddell et al., 2009).

Endoplasmic reticulum (ER) unfolded protein responses (UPR) are involved in iron metabolism (Oliveira et al., 2011) and chronic inflammation (Aragon and Younossi, 2010). The liver is a major site of iron storage and is particularly susceptible to injury from iron overload. The ER stress response is an important homeostatic device for the liver during the stresses of metabolic process. It has become clear that obesity and metabolic stress are associated with the activation of cellular stress signaling and inflammatory pathway (Hotamisligil, 2005). Like other secretory cells, the liver cells such as hepatocytes and HSC, are rich in ER. Because of their high protein synthesizing capacity, it is easy to postulate that UPR/ER stress response plays an important role in chronic liver injury. When cells experience cellular stress, triggered by viral/intracellular bacterial replication, energy deficiency or overload, Ca^{2+} depletion and misfolded protein accumulation, the glucose-regulated protein 78 (GRP78) dissociates from ER resident receptors IRE1, PERK and ATF6 (Aragon and Younossi, 2010). Dissociation of GRP78 leads to the activation of the UPR. Therefore, the ER resident chaperone GRP78 is considered as a hallmark of ER stress regulation.

Our previous study showed GRP78 is the molecular target of ferric iron in the animal model of inflammatory bowel diseases (Werner et al., 2011). Transient knock down Grp78 protein abolished TNFα and Granzyme B secretion in cytotoxic CD8αβ T cells (Chang et al., 2012). We hypothesized that ER stress-associated pathways are the molecular targets of serum ferritin and Grp78-associated ER stress mechanisms may influence activation status of rat primary HSC through regulation of pro-inflammatory cytokine production.

Materials and Methods

Primary Hepatic Stellate Cells (HSC) isolation

Primary HSC were obtained from Spraque-Dawley rat. Briefly, rat HSC were isolated from normal male Sprague-Dawley rats (600 ± 50 g) by sequential pronase/collagenase perfusion. Cells were purified by density gradient centrifugation and HSC were carefully harvested from the upper phase after centrifugation. Isolated primary cells were cultured on plastic 12-well plates and grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY), with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY)-2mMglutamine -100M nonessential amino acids (NEAAs) -penicillin/streptomycin (100 IU/ml and 100 g/ml) for 24 hours. After cells were adhered, culture mediums were changed to 10% FBS-DMEM. Cells were cultured for further 7 to 14 days to induce an activated phenotype. Phonotypical changes of quiescent HSC to activated HSC were confirmed by the immunofluorescent staining of α-smooth muscle actin (α-SMA) or by quantitative PCR. Activated HSC were routinely cultured in 10 cm^2 uncoated plastic tissue culture dishes (Corning Glass Works, Corning, NY) and passage when cells reach 80 % to 90 % confluences. Cells were maintained at generation 12 to 20. Cell culture medium contained ingredients of 10 % FBS. Cells incubated in an atmosphere of 5 % CO_2 /95% air at 37°C incubator.

Table 1. Gene Names, Primer, Amplified Pcr Products and Annealing Temperature

Gene name	Primer sequences	Amplicon (bp)	Tm (°C)
GAPDH	Sense-GGGTTCCTATAAATACGGACTGC	112	60
	Anti sense-CCATTTTGTCTACGGGACGA		60
Grp78	Sense-CTGAGGCGTATTTGGGAAAG	120	60
	Anti sense-TCATGACATTCAGTCCAGCAA		60
IL-6	Sense- GTCAACTCCATCTGCCCTTCAG	102	60
	Anti sense- GGCAGTGGCTGTCAACAACAT		60
TNF-α	Sense- ACAAGGCTGCCCCGACTAT	88	60
	Anti sense- CTCCTGGTATGAAGTGGCAAATC		60
IL-1β	Sense-ATCCCAAACAATACCCAAAGAAGAA	100	60
	Anti sense-TGGGGAACTGTGCAGACTCAAAC		60
iNOS	Sense- AGGGAGTGTTGTTCCAGGTG	81	60
	Anti sense- TCTGCAGGATGTCTTGAACG		60
α-SMA	Sense- GGCCACTGCTGCTTCCTCTTCTT	78	60
	Anti sense- TGCCCGCCGACTCCATTC		60

Activation and Grp78 knock down using EGF-SubA fusion protein

0.5×10^5/well activated HSC were seeded on plastic 12-well plate and cells were allowed to adhere to the plate for overnight. After adherence, cells were stimulated with or without horse SF (10 pM, 100 pM, 1000 pM) (Sigma). Cells were harvested at the end of 24 hours post activation. In another experiment, a total of $0.5x10^6$/well activated HSC was pretreated with 500 pM bacterial endotoxin EGF-SubA (SibTech, Brookfield, CT) for 3 hours then 100 pM serum ferritin were added to cells for further 21 hours. Cells were harvested at 24 hours post-stimulation. Effects of Grp78 knock down were evaluated by real-time PCR and 2% agarose gel electrophoresis.

Human serum stimulation experiment

Fasting serum samples were collected from young male adolescents (ages 13.33±0.21 years). Informed parental written consent was obtained for enrollment into the study. The study was approved by the Research Ethics Committee of Taipei Medical University (201204011). Serum ferritin (SF) was determined using a commercially available electrochemiluminescence immunoassay and was quantitated by the Roche Modular P800 analyzer. Young male adolescents were grouped according to the concentrations of SF: low SF group [n=7; SF=19.5 ng/ml (13.0-26.0)] and high SF group [n=7; SF=230 ng/ml (106.0-616.0)]. Activated HSC (0.5×10^5/ well) were starved for 12 hours without the presence of fetal bovine serum (FBS). After 12 hours, cells were washed twice with 1xPBS. A total of 10% human serums with different concentrations of serum ferritin were added to cells for 16 hours. Control cells were cultured without human serum.

RNA isolation and quantitative reverse transcription-PCR (qPCR)

Total RNA was extracted using an RNeasy Mini Kit (QIAGEN, USA). RNA yield and quality were assessed by absorbance using a Nanodrop BD-1000 spectrophotometer (LabTech International). 1 μg total RNA was used for reverse transcription using the M-MLV reverse transcriptase (Invitrogen). Primers were designed using the universal probe library (Roche, Germany). Details

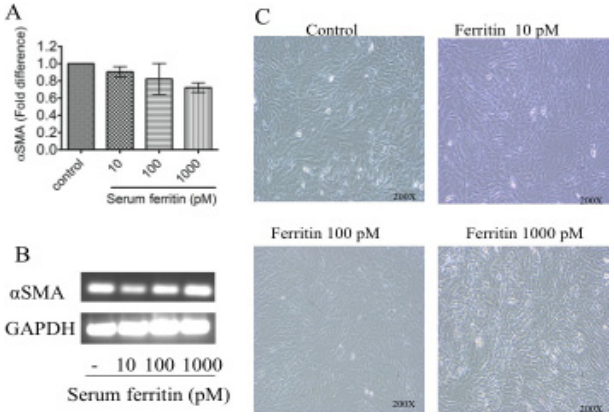

Figure 1. Effects of Serum Ferritin on Primary Rat Activated Hepatic Stellate Cells (HSC). HSC were cultured with or without serum ferritin (10, 100, 1000 pM) for 24 hours. The addition of serum ferritin did not alter α-SMA mRNA (A, B) nor did cell morphology (C). Gene expression data were normalized to the expression level of GAPDH and are presented as fold difference. Data are expressed as mean±SEM (n=4-6 wells/treatment)

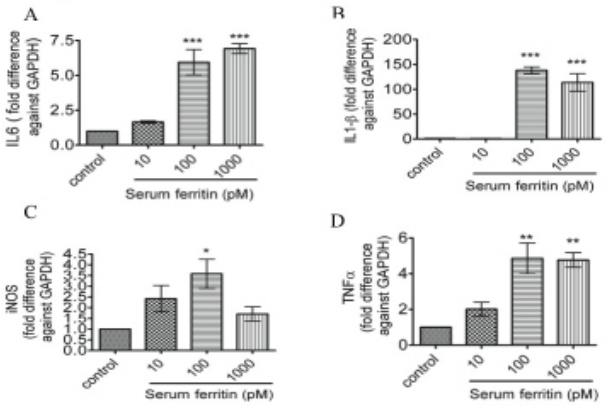

Figure 2. Serum Ferritin Triggered Transcriptional Activation of Pro-inflammatory Cytokine Genes in Activated HSC. The presence of SF triggered transcriptional activation of IL6 (A), IL1β (B), iNOS (C) and TNFα (D) gene expression compared to control. Gene expression data were normalized to the expression level of GAPDH and are presented as fold difference. Data are expressed as mean±SEM (n=4-6 wells/treatment). Significant differences are shown with *p<0.05, **p<0.01, ***p<0.001 compared with control

of primer concentration and sequences were shown in Table 1. Amplicon sizes ranged between 50-150 base pairs (bp) to ensure high amplification efficiency. RNA expression profiles of both target and reference genes were performed using the LightCycler 480 (Roche, Germany) as 10 µl per PCR reactions (400 nM primers, 14.3 ng of RNA converted cDNA, LightCycler® 480 SYBR Green I Master). Reporter was the non-specific DNA binding dye using the LightCycler® 480 SYBR Green I Master (Roche). The relative induction of mRNA expression was calculated using the following equation ECp (control–treatment) and normalized for the expression of GAPDH.

Enzyme-Linked ImmunoSorbent Assay (ELISAs)

Measurements of rat interleukin-1β (IL-1β), TNFα and interleukin-6 (IL-6) were performed by Enzyme-Linked Immunosorbent Assay kits (eBioscience or PeproTECH, USA) according to the manufacturer's instructions. As an indicator of nitric oxide (NO) production, nitrite concentration in the serum was determined by Griess reagent. Serum samples were mixed with Griess reagent (50 l, 1% sulphanilamide, 0.1% N-1-naphthyl ethylenediamine) for 10 min, and the absorbance of the chromophoric azo-derivative molecule was measured using a microplate reader at 540 nm.

Statistical analyses

One-way ANOVA with Bonferroni posttests and correction were used to analyze differences between groups. Analysis was conducted using GraphPad Prism 4. Data was presented either as mean ± standard error of the mean (S.E.M.) or median (interquartile range). P<0.05 was considered statistically significant.

Results

The effect of serum ferritin on α-SMA expression and cell morphology

Transdifferentiation of HSC was associated with upregulation of α-SMA and induction of myofibroblas-like morphology. The α-SMA expressed by hepatic stellate cells reflects their activation to myofibroblast-like cell and has been directly related to experimental liver fibrogenesis. Figure 1 showed activated primary rat HSC express high levels of α-SMA and treatment of SF has no effect on α-SMA mRNA expression level (A, B). The presence of 10, 100 and 1000 pM horse SF did not alter cell morphology (Figure 1 C). The cytotoxicity of SF in HSC was also evaluated by the trypan blue exclusion test. HSC cells were exposed to SF (0, 10, 100, 1000 and 10, 000 pM) and cell viability was measured at 24 hours post-stimulation. Treatment of SF did not affect cell viability nor did cell morphology (data not shown).

The effect of serum ferritin on pro-inflammatory cytokine secretion

Emerging evidence suggests that circulating ferritin molecules are marker of inflammation and SF function as pro-inflammatory cytokine mediators. We first investigated effects of SF on function of HSC. Primary rats HSC were treated with 0, 10, 100 and 1000 pM horse serum ferritin and cells were harvested at the end of 24 hours post-stimulation. Figure 2 showed the presence of 100 and 1000 pM SF induced transcriptional activation of pro-inflammatory cytokine genes IL6 (A), IL1β (B) and TNFα (C) compared with control (all p<0.01). A small induction in iNOS mRNA in response to 100pM SF stimulation was also found (Figure 2C; P<0.05).

We next treated rat primary HSC with human serum samples with known serum ferritin concentrations. Cells were first starved for 12 hours without FBS then 10% human serum isolated from adolescents were added to HSC for 16 hours: low SF group [n=7; SF=19.5 ng/ml (13.0-26.0)] and high SF group [n=7; SF=230 ng/ml (106.0-616.0)]. Control cells were treated without human serum. Figure 3 showed high SF groups induced higher IL1β (A), IL6 (B) and TNFα (C) secretion but not NO (D) compared to control.

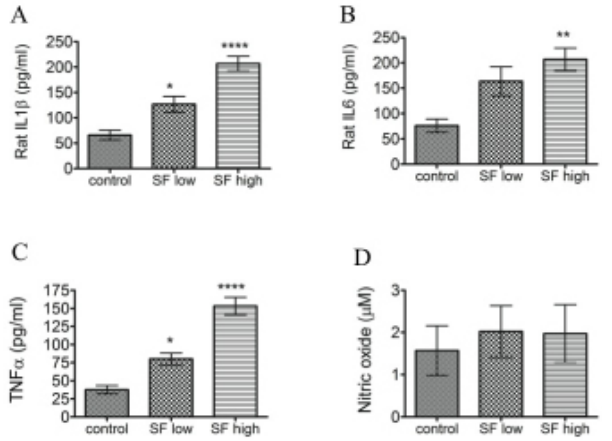

Figure 3. Effects of Human Serum on Primary Rat HSC Cytokine Production. Activated HSC (0.5×10^5/ well) were starved for 12 hours without the presence of fetal bovine serum. Cells were washed twice with 1xPBS then 10% human serum with different concentrations of serum ferritin was added to cells for 16 hours: low SF group [n=7; SF=19.5 ng/ml (13.0-26.0)] and high SF group [n=7; SF=230 ng/ml (106.0-616.0)]. Control cells were treated without human serum. Data are expressed as mean±SEM. Significant differences are shown with *p<0.05, **p<0.01, ****p<0.0001 compared with control

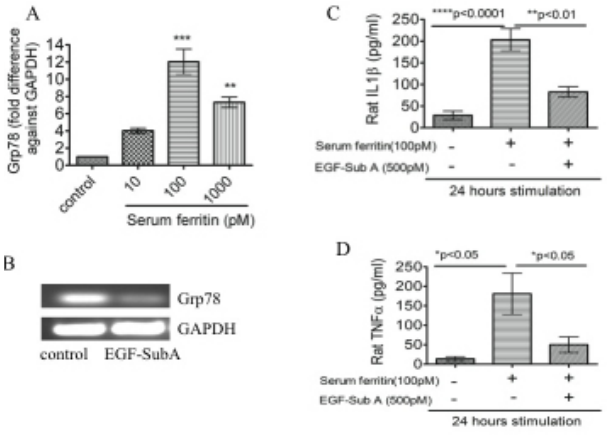

Figure 4. Effects of SF on Grp78-associated ER Stress Pathways. Addition of 100 and 1000 pM serum ferritin induced Grp78 mRNA expression in activated HSC (A). In another experiment, activated HSC were pretreated with 500 pM bacterial endotoxin EGF-SubA for 3 hours then 100 pM serum ferritin were added to cells for further 21 hours. Transient knock down of Grp78 (B) diminished SF-mediated IL1β (C) and TNFα (D) secretion. Gene expression data were normalized to the expression level of GAPDH and are presented as fold difference. Data are expressed as mean±SEM (n=6 wells/ treatment). Significant differences are shown with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared with control or EGFSubA+SF compared with SF treatment

Grp78 pathways is involved in SF-mediated cytokine secretion

Activation of GRP78 gene is considered as a hallmark of ER stress. Increased levels of Grp78 and UPR signal transducers are also associated with cell activation. We next determined the functional effect of serum ferritin on Grp78-related ER stress response. Figure 4A showed the presence of 100 and 1000 pM horse SF induces higher transcriptional activation of GRP78 mRNA expression compared to the control (p<0.001). This data suggests a potential role of Grp78-related ER stress pathways in SF induced HSC activation. We next performed Grp78 knock down experiment to test this hypothesis. A transient knock down of Grp78 by bacterial endotoxin EGF-SubA in rat primary HSC (Figure 4B) resulted in down-regulation of SF-mediated IL1β (Figure 4C) and TNFα secretions (Figure 4D).

Discussion

Chronic liver diseases are characterized by inflammation and elevated serum ferritin, but the causal-relationship remains unclear. Our study used primary rat HSC as a model to investigate effects of SF on liver injury because HSC play a central role in the development and progression of liver fibrosis. We found that physiological concentrations of SF, either horse SF or human serum, trigger pro-inflammatory cytokine release such as IL1β and TNFα. By contrast, treatment of SF in rat primary HSC did not alter HSC activation markers αSMA expression. The presence of physiological SF concentrations also markedly enhanced expression of Grp78 mRNA. The transient knock down of Grp78 by endotoxin EGF-SubA abolished SF-induced IL1β and TNFα secretion in HSC. Our study demonstrated for the first time that SF functions as pro-inflammatory mediators and Grp78-associated ER stress pathways are required for SF-mediated HSC activation.

The precise role of circulating ferritin in HSC activation and liver fibrogensis remains unclear. In experimental models of iron-induced hepatic fibrosis, activated HSC have been shown to be responsible for increased collagen production (Pietrangelo et al., 1994). By contrast, SF was shown to down-regulate the activation marker of αSMA in activated HSC (Ramm et al., 1996). We found no effect of physiological SF concentrations on expression of αSMA or collagenase in activated HSC. Our study was in agreement with a report of Ruddell and colleagues suggesting SF functions as pro-inflammatory modulators and not involve in the activation of quiescent to myofibroblast-like HSC (Ruddell et al., 2009). Interestingly, the authors showed both iron-bound ferritin or iron free apoferritin triggers transcriptional activation of IL1β and iNOS expression in activated HSC via NFkB-dependent signaling pathways. Similar to the report of Ruddell et al, we also observed that apoferritin exerts immunostimulatory effects on activated HSC (data not shown). Our study found SF-or apofferitin mediated HSC activation is not restricted to NFkB pathways but also involve Grp78 associated ER stress mechanisms. A transient knock down Grp78 abolished SF-induced IL1β and TNFα secretion to basal levels, indicating ER stress pathways are critically involve in SF-induce cytokine secretion. Our study demonstrates a potential role of SF in liver fibrogenesis and identify ER chaperone Grp78 response is one of the molecular target of SF.

Elevated serum ferritin is considered to be a marker of inflammation. Inflammatory cytokines are known to upregulate ferritin expression (Torti and Torti, 2002; Torti et al., 1988), but little is known about the function of SF on inflammation. In this study, we tested hypothesis whether

physiological concentrations of human serum ferritin elicit pro-inflammatory cytokine secretion in activated HSC. We used serum sample from healthy children and not adults because serum sample of children contains lower levels of pro-inflammatory cytokines compared with adults. Mean concentrations of SF in Taiwanese children ages 13.3±1.10 years were 55.9±35.1 ng/mL (men 60.2±55.8 ng/mL and women 54.7±33.0 ng/mL). We treated activated HSC with low [19.5 ng/ml (13.0-26.0)] and high [230 ng/ml (106.0-616.0)] human serum ferritin isolated from male children (n=7/group). The low SF group represents status of iron depletion. High SF group represent physiological SF concentrations in adults and not reflects status of genetic iron overload. In patients with hereditary hemochromatosis, SF is elevated and can range in concentration from 200 to 6500 ng/ml (450-14, 605 pM) (Cragg et al., 1981). Mean concentrations of SF in Taiwanese adults were 173±282 ng/mL (388 pM). Our study found both low and high SF induced pro-inflammatory cytokine secretion compared with control but high SF group induced higher responses. This result is similar to the stimulatory effect of 100 pM horse SF which is in physiological concentration range and is lower than study of Ruddell et al (100 v.s. 10, 000 pM respectively).

UPR pathways are evolutionary conserved mechanisms that allow cells to cope with suboptimal growth conditions. The activation of UPR initiates protective mechanism to maintain ER function which leads to cell adaptation. However, unresolved ER stress can lead to apoptotic cell death (Werner et al., 2011) or abnormal cell growth (Chang et al., 2012; De Minicis et al., 2012; Lim et al., 2011). Activated HSC is known to resistant to apoptosis which represents one of the mechanisms involved in fibrosis promotion. Minicis and colleagues investigated association between HSC survival and ER stress associated mechanisms in experimental animal model and reported calpastatin, which protects HSC from ER stress-induced apoptosis, is highly expressed in activated HSCs (De Minicis et al., 2012). Lim et al reported that activated HSC express a higher basal level of ER stress transducers ATF, ATF6 and CHOP mRNA expression compared to quiescent HSC (Lim et al., 2011). Another report investigated anti-inflammatory properties of cannabinoid in activated HSC and found cannabinoid induces activated HSC cell death through mechanism of IRE1/ASK1/c-Jun N-terminal kinas pathway (Lim et al., 2011). These finding indicates that ER stress associated pathways could exhibit therapeutic potential in the context of liver fibrosis by modulating apoptosis of activated HSC.

The activation of quiescent HSC to myofibroblasts is also associated with increased cell proliferation. Ferritin receptor H subunit was expressed specifically on activated, but not on quiescent, HSC (Ramm et al., 1994). H-ferritin receptor has been shown to down-regulate the proliferation of granulocyte-macrophage and erythroid progenitor cells (Broxmeyer et al., 1986). By contrast, ferritin stimulates the proliferation of the breast cancer cells and antisense ferritin oligonucleotides inhibit growth and induce apoptosis in human breast carcinoma cells (Yang et al., 2002). Our study found both H and L ferritin subunits mRNA were upregulated by the treatment of 100 pM SF compared with control; 1.5 and 4 fold upregulation respectively (Data not shown). In this study, we did not perform cell proliferation assays; therefore, we do not know the effect of SF on HSC proliferation.

In Conclusion: In summary, our results showed that physiological concentrations of SF functions as pro-inflammatory mediators on primary rat HSC. We also provide a molecular basis for the action of SF and identify Grp78-associated ER stress pathways as a novel potential therapeutic target for resolution of liver fibrosis.

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