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Defensive Effect of Quercetin against Tumor Necrosis Factor α-induced Endoplasmic Reticulum Stress and Hepatic Insulin Resistance in HepG2 Cells

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Abstract In order to examine the hypothesis that the treatment of TNF-α can impose inflammation and endoplasmic reticulum (ER) stress in hepatic cells, and that quercetin has a defensive effect against TNF-α-induced ER stress and insulin resistance, we evaluated the effect of quercetin (3 μg/mL and 5 μg/mL) in the TNF-α-induced HepG2 cells. The TNF-α-stimulated control group caused a marked increase in the activation of inflammation and ER stress response. Quercetin, however, caused the interruption of TNF-α-induced inflammation and ER stress. In addition, the treatment of quercetin resulted in significant decreases in serine phosphorylation of IRS-1, phosphorylation of JNK, and the expression of gluconeogenic genes compared with the TNF-α-stimulated control group. In conclusion, we suggest that quercetin can protect hepatic insulin resistance by exerting a protective effect against the ER stress and inflammation induced by TNF-α.

Keywords: *quercetin, TNF-α, ER stress, insulin resistance, inflammation*

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1. Introduction

Tumor necrosis factor (TNF)-α is a pro-inflammatory cytokine produced by various types of inflammatory and non-inflammatory cells. Increased TNF-α levels cause the induction of other pro-inflammatory cytokines, including IL-1β and IL-6 [1,2,3]. Studies have clearly demonstrated that TNF-α is associated with the development of inflammation and insulin resistance in several experimental models of obesity [4,5]. Although the molecular mechanism of the association between obesity and hepatic insulin resistance is unclear, it has been reported that obesity-induced TNF-α leads to hepatic endoplasmic reticulum (ER) stress and insulin resistance, which can accelerate the progression of diabetes in obese subjects [6-11]. In most obese states, the white adipose tissue is characterized by the increased secretion of pro-inflammatory cytokines, which exert negative effects on insulin signaling in the liver through inflammation and ER stress [12,13,14].

Several studies have provided support for the idea that the pathway of TNF-α-induced insulin resistance is directly regulated by insulin receptor substrate-1 (IRS-1) serine phosphorylation, or indirectly by IRS-1 serine phosphorylation via nuclear factor-κB (NF-κB) and the c-Jun N-terminal kinase (JNK) pathway. Other studies have shown that exposure to TNF-α induces ER stress, which induces IRS-1 serine phosphorylation via NF-κB and the JNK pathway [6,7,8,9]. ER stress is also referred to as the unfolded protein response (UPR). The imbalance between the cellular demand for protein folding and the capacity of the ER to promote protein maturation induces an accumulation of unfolded proteins in the ER lumen [15,16].

In order to cope with ER stress and control protein unfolding, cells activate signaling systems that are initiated by three ER transmembrane proteins: PKR-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1) [15,16]. Studies have shown that the activation of the JNK pathway, mediated by IRE1, is associated with the development of insulin resistance [17,18]. Studies have also demonstrated that phosphorylation of eIF2α leads to the activation of C/EBPs, which results in the expression of gluconeogenic genes, such as PEPCK or G6Pase. Thus, the literature has confirmed that hepatic TNF-α-induced ER stress plays a role in hepatic insulin resistance and metabolic dysregulation [19,20,21].

Flavonoids are polyphenolic compounds that are present in a wide variety of plants. Flavonols, the most abundant flavonoids in the diet, exhibit several important biological and pharmacological effects [22]. The main flavonol is quercetin, which is commonly linked to sugars in glycosylated form, such as rutin and quercitrin [23]. Results from

recent studies show that quercetin demonstrates anti-inflammatory effects against TNF-α in vitro and in vivo [24-27]. According to Ruiz et al. [26], quercetin inhibits the expression of macrophage inflammatory protein-2 gene and cofactor recruitment at the chromatin of pro-inflammatory genes in TNF-induced cells and mice.

In order to examine the hypothesis that the treatment of TNF-α can induce inflammation and ER stress in hepatic cells, and that quercetin has a protective effect against TNF-α-induced ER stress and insulin resistance via its anti-inflammatory effect, we evaluated the effect of quercetin on TNF-α-stimulated HepG2 cells.

2. Materials and Methods

2.1. Cell culture and Treatments

HepG2 cells were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). HepG2 cells were maintained in Dulbecco's minimal essential medium (DMEM; Hyclone Laboratories, Logan, Utah, USA) containing 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, Utah, USA), 100 mg/L penicillin-stereptomycin, and 2 mmol/L glutamine (Hyclone Laboratories, Logan, Utah, USA). Cells were maintained at 37°C under a humidified atmosphere of 5% CO2. The medium was refreshed approximately three times a week. HepG2 cells were seeded at a concentration of $3*10^5$ cells/well in 6-well tissue culture dishes and incubated to proliferate for 24 h. Then the cells were treated with quercetin (3 μg/mL and 5 μg/mL), immediately followed by stimulation with 1 ng/mL TNF-α for inflammation induction for 24 h.

2.2. Protein Extraction and Western Blot Analysis

Cells were harvested, lysed in a CelLytic ™ MT cell lysis reagent (Sigma Aldrich, Sigma, St. Louis, MO, USA), and centrifuged at 12,000 g for 20 min at 4°C. The protein content of the clear lysates was estimated by the Bradford method using a protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts (100 μg protein/lane) of total protein were dissolved in NuPAGE® LDS sample buffer 4X (Life Technologies, Gaithersburg, MD, USA). Protein samples were separated on 5% or 10% SDS-polyacrylamide gel, and were transferred to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). Membranes were incubated for 1 h in a blocking solution containing 5% nonfat milk in Tris-buffered saline, and then incubated for 12 h at 4°C with antitotal-eIF2α (1:500), antiphospho-eIF2α (1:500), antitotal-IRE1α (1:1,000, Novus Bio, Littleton, CO, USA), antiphospho-IRE1α, antitotal JNK (1:1,000), antiphospho-JNK (1:1,000), antitotal NF-κB (1:1,000), antiphospho-NF-κB (1:1,000), antitotal IRS-1 (1:1,000), and antiphospho-IRS-1 (serine, 1:1,000) antibody. Except for the antitotal-IRE1α antibody, all other antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). After incubation with the primary antibody, membranes were incubated with a secondary antibody (antirabbit IgG HRP-linked antibody, 1:5,000, Cell Signaling Technology, Inc., Beverly, MA, USA) for 1 h at room temperature. Protein bands were developed using the SuperSignal™ West Dura Extended Duration Substrate (Pierce, Milwaukee, WI, USA) and visualized with the ChemiDoc imaging system from Bio-Rad Laboratories (Hercules, CA, USA).

2.3. Isolation of total RNA and real-time PCR

Cells were lysed in the presence of buffer RLT (lysis buffer, Qiagen, Valencia, CA, USA) including 1% β-mercaptoethanol. Total RNA was extracted from the cells' lysate using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). Complementary DNA was synthesized from 1 μL purified total RNA in 20 μL of reaction buffer using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). Real-time PCR (Applied Biosystems, Foster City, CA, USA) was performed on triplicate samples using the 1 μL cDNA with the SYBR Green PCR Master Mix (iQ SYBR Green Supermix, Bio-Rad Laboratories, Hercules, CA, USA). The cDNA was amplified for 45 cycles of denaturation (95°C for 30 s), annealing (58°C for 30 s), and extension (72°C for 45 s) with specific primers (Table 1). The data of the real-time RT-PCR results and calculation of the relative quantitation were performed using 7500 System SDS software version 1.3.1 (Applied Biosystems, Foster City, CA, USA).

Table 1. Primer sets used for real-time PCR

Gene	Accession number		Sequence
GAPDH	M33197	F	5'- ATG GAA ATC CCA TCA CCA TCT T -3'
		R	5'- CGC CCC ACT TGA TTT TGG-3'
TNF-α	NM000594	F	5'- CCA CTT CGA AAC CTG GGA TTC-3'
		R	5'- TTA GTG GTT GCC AGC ACT TCA-3'
IL-1β	NM000576	F	5'- TTA AAG CCC GCC TGA CAG A-3'
		R	5'- GCG AAT GAC AGA GGG TTT CTT AG-3'
IL-6	M54894	F	5'- AGG GCT CTT CGG CAA ATG TA-3'
		R	5'- GAA GGA ATG CCC ATT AAC AAC AA-3'
CHOP	S40706	F	5'- CTC TGA TTG ACC GAA TGG TGA A-3'
		R	5'- GGG ACT GAT GCT CCC AAT TG-3'
XBP-1	NM005080	F	5'- CCT GAG CCC CGA GGA GAA-3'
		R	5'- GGC AGT CTG AGC TGC TAC TCT GT-3'
GRP78	X87949	F	5'- TGG CGG AAC CTT CGA TGT-3'
		R	5'- GCC ACA ACT TCG AAG ACA CCA T-3'
PEPCK	NM002591	F	5'- TGG GCT CGC CTC TGT CA-3'
		R	5'- CCA CCA CGT AGG GTG AAT CC-3'
G6Pase	U01120	F	5'- GAG TGG AGT GGC ACG ATC TTG-3'
		R	5'- GAC ATG AGA ATC GCT TGA ACC A-3'

2.4. Statistical Analysis

All experimental data were expressed as mean ± standard deviation (SD). The significance of the treatment effects was analyzed by Duncan's multiple range tests after one-way ANOVA using SPSS statistical procedures for Windows (SPSS PASW Statistic 20.0, SPSS Inc., Chicago, IL, USA). Statistical significance was considered at the $p < 0.05$ level.

3. Results

3.1. Effect of Quercetin on Inflammation in TNF-α-induced HepG2 Cells

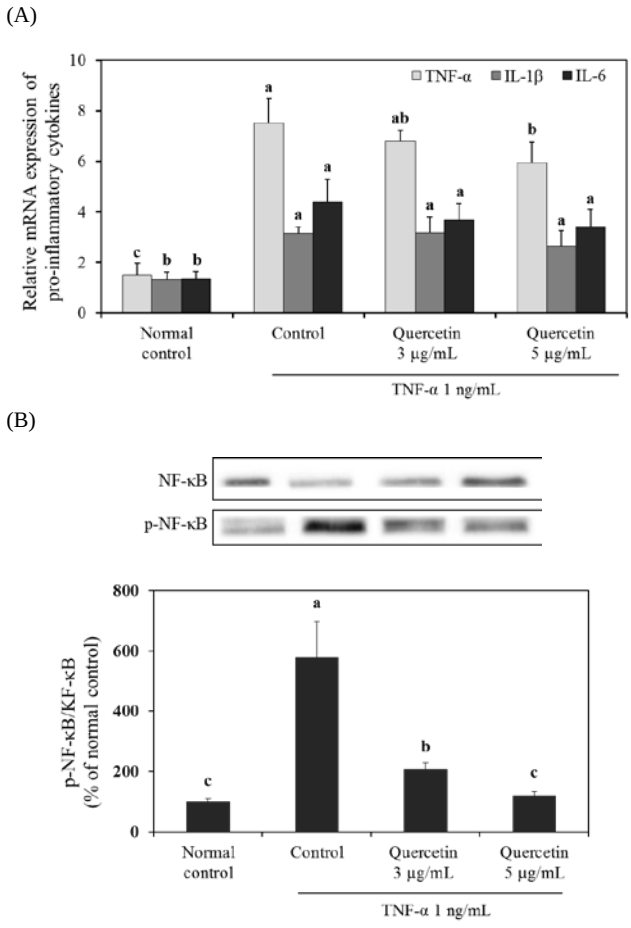

Figure 1. Effect of quercetin on inflammation in TNF-α-induced HepG2 cells. (A) mRNA expression of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in TNF-α-induced HepG2 cell. (B) Representative Western blots for total protein and phosphorylation of NF-κB in TNF-α-induced HepG2 cells. The data are expressed as the mean ± standard deviation (n=3). The different letters show a significant difference at $p < 0.05$ as determined by Duncan's multiple range test.

We found that the TNF-α-stimulated control group resulted in marked increases in the level of mRNA expression of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and the expression of NF-κB phosphorylation compared with the normal control (Figure 1). The group of HepG2 cells treated with quercetin caused no significant difference in the expression of IL-1β and IL-6 compared with the TNF-α-stimulated control group. In contrast, the TNF-α expressions in quercetin 5 μg/mL were decreased compared with the TNF-α-stimulated control group (Figure 1A). The groups of HepG2 cells treated with quercetin caused a significant decrease in the expression of NF-κB phosphorylation compared with the TNF-α-stimulated control group, which were dose-dependent (Figure 1B) ($p < 0.05$).

3.2. Effect of Quercetin on the Expression of eIF2α and CHOP in TNF-α-induced HepG2 Cells

The expression of eIF2α phosphorylation and CHOP in the TNF-α-stimulated control group was significantly increased compared with the normal control group (Figure 2). Quercetin caused a significant decrease in the expression of eIF2α phosphorylation compared with the TNF-α-stimulated control group, however, which were dose-dependent (Figure 2A). In addition, the group of TNF-α-induced HepG2 cells treated with quercetin 5 μg/mL resulted in significant decreases in the expression of CHOP compared with the TNF-α-stimulated control group (Figure 2B) ($p < 0.05$).

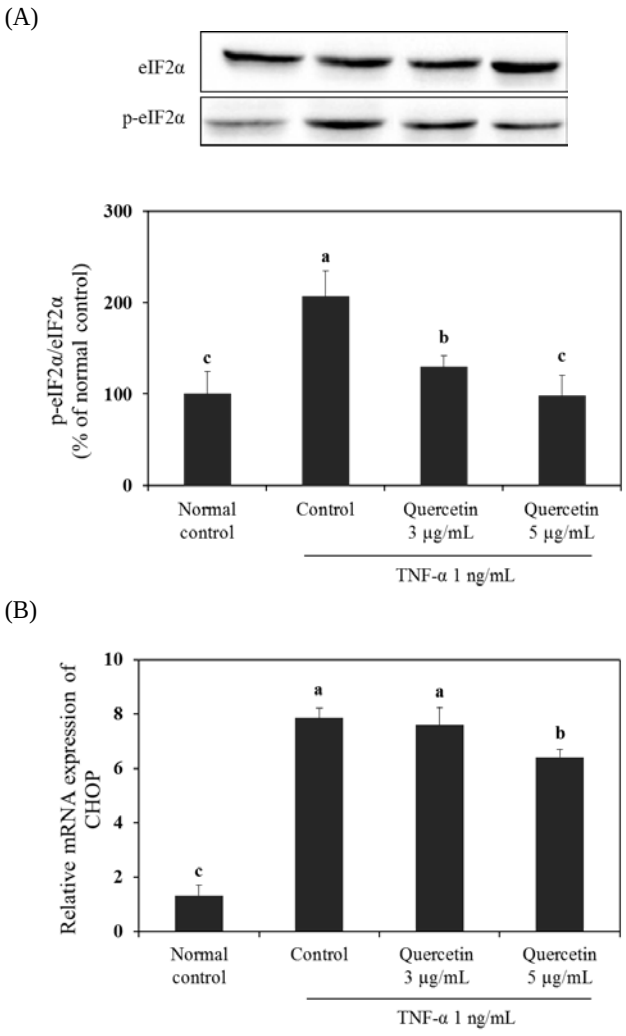

Figure 2. Effect of quercetin on the expression of eIF2α and CHOP in TNF-α-induced HepG2 cells. (A) Representative Western blots for total protein and phosphorylation of eIF2α in TNF-α-induced HepG2 cells. (B) mRNA expression of CHOP in TNF-α-induced HepG2 cell. The data are expressed as the mean ± standard deviation (*n*=3). The different letters show a significant difference at $p < 0.05$ as determined by Duncan's multiple range test

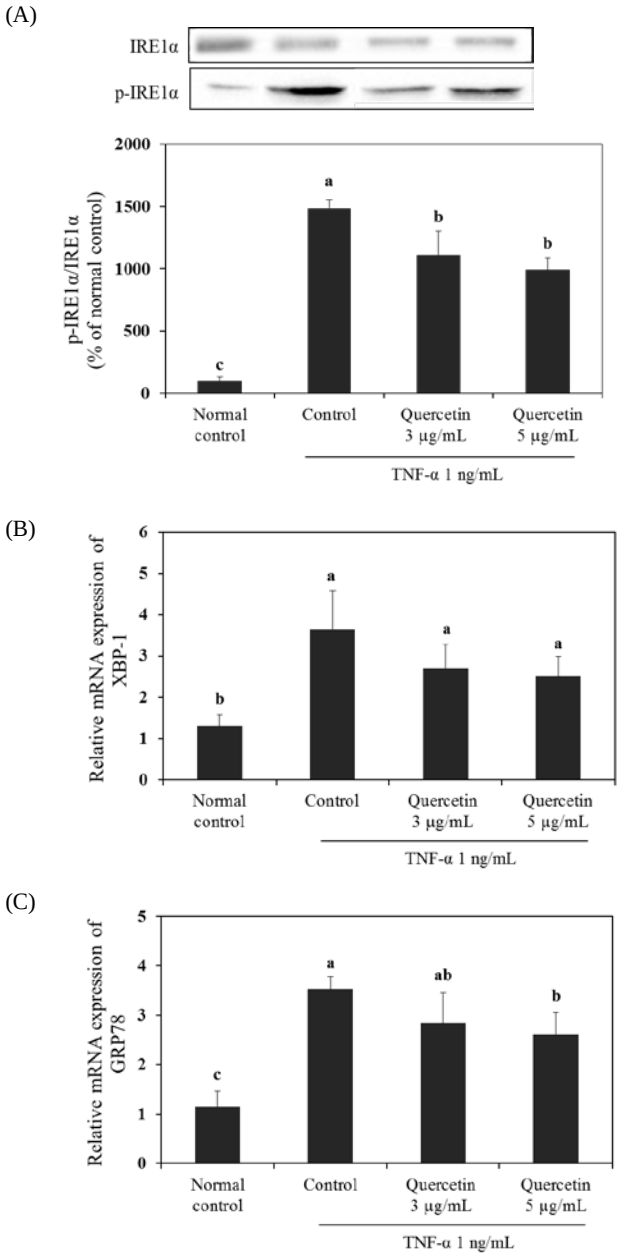

Figure 3. Effect of quercetin on IRE1α, XBP-1, and GRP78 expression in TNF-α-induced HepG2 cells. (A) Representative Western blots for total protein and phosphorylation of IRE1α in TNF-α-induced HepG2 cells. (B) mRNA expression of XBP-1 in TNF-α-induced HepG2 cell. (C) mRNA expression of GRP78 in TNF-α-induced HepG2 cell. The data are expressed as the mean ± standard deviation (n=3). The different letters show a significant difference at $p < 0.05$ as determined by Duncan's multiple range test

3.3. Effect of Quercetin on IRE1α, XBP-1, and GRP78 Expression in TNF-α-induced HepG2 Cells

The TNF-α-stimulated control group resulted in a marked increase in the expression of IRE1α phosphorylation compared with the normal control. The treatment of quercetin caused a statistically significant decrease in the level of expression of IRE1α phosphorylation compared with the TNF-α-stimulated control group (Figure 3A). The expression of XBP-1 and GRP78 of the TNF-α-stimulated control group was significantly increased compared with the normal control group (Figure 3B and Figure 3C). We found no significant difference in the level of mRNA expression of XBP-1 between the TNF-α-stimulated control group and the groups of TNF-α-induced HepG2 cells treated with quercetin (Figure 3B). In addition, the group of HepG2 cells treated with quercetin 5 μg/mL caused significant decreases compared with the TNF-α-stimulated control group (Figure 3C) ($p < 0.05$).

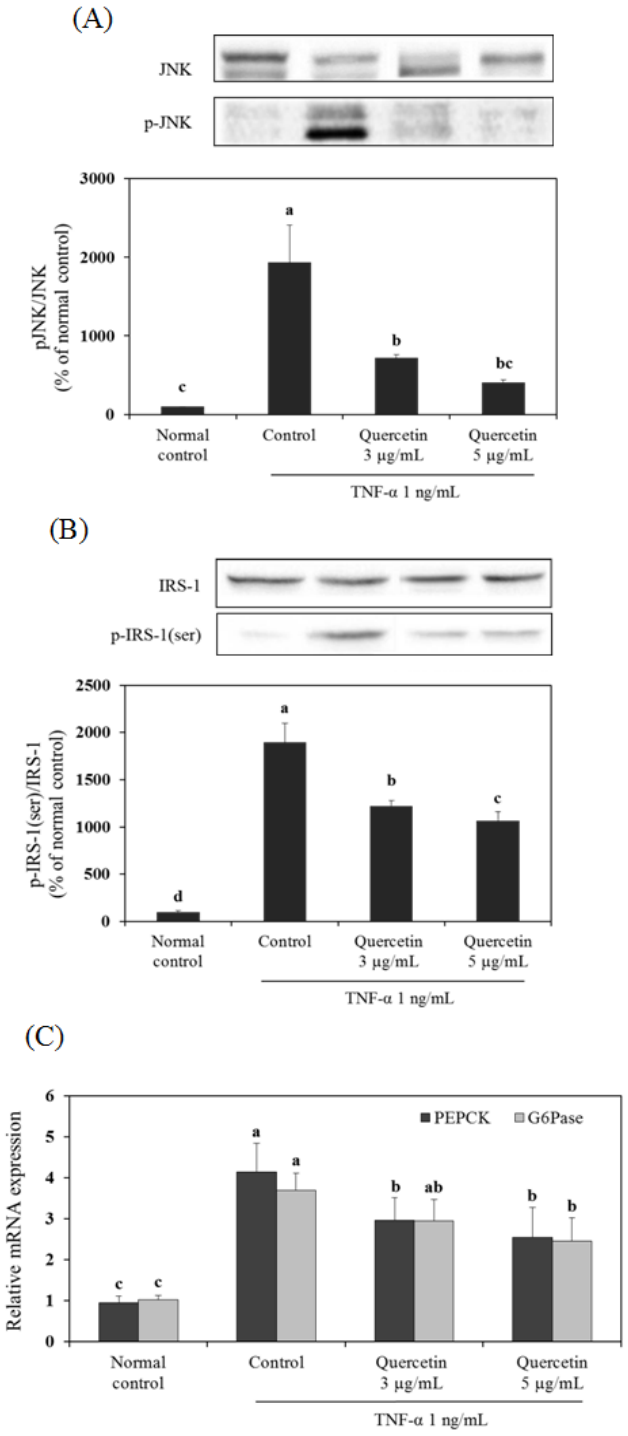

Figure 4. Effect of quercetin on hepatic insulin resistance in TNF-α-induced HepG2 cells. (A) Representative Western blots for total protein and phosphorylation of JNK in TNF-α-induced HepG2 cells. (B) Representative Western blots for total protein and serine phosphorylation of IRS-1 in TNF-α-induced HepG2 cells. (C) mRNA expression of PEPCK and G6Pase in TNF-α-induced HepG2 cell. The data are expressed as the mean ± standard deviation (n=3). The different letters show a significant difference at $p < 0.05$ as determined by Duncan's multiple range test

3.4. Effect of Quercetin on Hepatic Insulin Resistance in TNF-α-induced HepG2 Cells

We showed that the expression of JNK phosphorylation, IRS-1 serine phosphorylation and mRNA of PEPCK and G6Pase of the TNF-α-stimulated control group was significantly increased compared with the normal control group (Figure 4). Compared with the TNF-α-stimulated control group, the groups of quercetin 3 μg/mL and quercetin 5 μg/mL showed significant decreases in the expression of JNK phosphorylation (Figure 4A). In the groups of treatment with quercetin, IRS-1 serine phosphorylation decreased significantly, and in a dose-dependent manner (Figure 4B). In addition, we showed that PEPCK and G6Pase expressions in the group of quercetin 5 μg/mL were significantly decreased compared with the TNF-α-stimulated control group (Figure 4C) ($p < 0.05$).

4. Discussion

TNF-α has been identified as a key regulator of the inflammatory response. The diverse signaling induced by TNF-α leads to several cellular responses, such as cell death, survival, differentiation, and proliferation [1,3]. Signaling from TNF-α receptors on the cell surface can activate NF-κB, transcription factors, and JNK, which promote immunity by controlling the expression of genes, including pro-inflammatory cytokines [28,29]. Many recent reports have demonstrated key roles for the NF-κB signaling pathway and JNK phosphorylation in the development of inflammation-associated metabolic diseases in the liver [18,30,31,32].

The present study showed that the expression of NF-κB phosphorylation and pro-inflammatory cytokines increased significantly in TNF-α-stimulated HepG2 cells compared with that of normal HepG2 cells. In the quercetin-treated groups of ER stress-induced HepG2 cells, the expression of NF-κB phosphorylation and pro-inflammatory cytokines partially decreased (Figure 1).

The development of insulin resistance via the inhibition of IRS signaling and stimulation of the gluconeogenic pathway in the liver is recognized as a target for treating hyperglycemia [33,34,35]. Obesity is a major factor in the development of hepatic insulin resistance, but the molecular mechanisms of the relationship between obesity and hepatic insulin resistance remain in dispute [36,37,38]. Although the molecular mechanisms that link obesity to hepatic insulin resistance are unclear, studies have reported that inflammation and hepatic ER stress induced by pro-inflammatory cytokines lead to the development of hepatic insulin resistance [12,13,14]. We speculated that treatment of TNF-α in hepatic cells can induce inflammation and ER stress, as well as insulin resistance. Therefore, in this study, we investigated the effects of quercetin on TNF-α-induced ER stress and insulin resistance in HepG2 cells.

Studies have shown that the imbalance between the cellular demand for protein folding and the capacity of the ER to promote protein maturation results in the accumulation of unfolded proteins in the ER lumen [15,16]. Denis et al. [6] reported that TNF-α signaling through TNFR1 might be involved in pathways that lead to the induction of ER stress. ER stress triggers the activation of the UPR pathway, including PERK, ATF6, and IRE1. These three ER transmembrane proteins are maintained in an inactive state by binding of the chaperone protein called binding immunoglobulin protein (BiP), also known as 78 kDa glucose-regulated protein (GRP78). The dissociation of the chaperone protein from each ER transmembrane protein during the folding of unfolded proteins can trigger their activation and the induction of the UPR [15,16,39]. The UPR pathway plays dual roles, acting as a positive regulator of ER chaperone proteins and aiding protein folding and ER-associated degradation (ERAD) under normal physiological conditions; they also trigger apoptosis under chronic stress conditions via apoptotic pathways mediated by the activation of C/EBP homologous protein (CHOP) [40,41,42,43].

We found a significant increase in the activation of the ER stress response in the TNF-α-stimulated control group due to increases in the phosphorylation of eIF2α and IRE1α, and increases in the RNA expression of CHOP, XBP-1, and the ER chaperone protein (GRP78) compared with that of the normal control group. These results suggest that the exposure of HepG2 cells to 1 ng/mL of TNF-α for 24 h can exert dual effects on the UPR pathway: ERAD and apoptosis. The quercetin treatment groups significantly reduced the activation of the ER stress response compared with the TNF-α-stimulated control group (Figure 2 and Figure 3).

Elevated levels of TNF-α have been observed in several experimental models of obesity and insulin resistance [4,5,44]. A study by Cai et al. [32] indicated that the production of pro-inflammatory cytokines in the liver increased in mice that were fed a high-fat diet, and that lipid accumulation caused inflammation through NF-κB activation and downstream cytokine production, which caused insulin resistance. Another study reported that the activation of the TNF-α-induced JNK pathway led to IRS-1 serine phosphorylation, followed by the suppression of insulin receptor signaling [18].

It has also been reported that anti-TNF-α therapy improves insulin sensitivity. Diehl et al. [45] showed that pretreatment with anti-TNF antibodies prevented the regenerative induction of C/EBP expression, which is associated with mRNA levels of PEPCK. Gupta et al. [5] reported that TNF-α preincubation reduced insulin-stimulated Tyr phosphorylation of the insulin receptor (IR-beta) and caused hyperphosphorylation of the IRS-1 serine residue. Thus, TNF-α exposure can play a role in hepatic insulin resistance via inhibition of IRS signaling and the activation of gluconeogenesis.

In the present study, we found a significant increase in serine phosphorylation of IRS-1 and phosphorylation of JNK, as well as the expression of gluconeogenic genes such as PEPCK and G6Pase, in the TNF-α-stimulated control group. The treatment of quercetin in the TNF-α-induced HepG2 cells, however, resulted in significant decreases in serine phosphorylation of IRS-1 and phosphorylation of JNK compared with those in the TNF-α-stimulated control group. PEPCK and G6Pase were significantly different in

the quercetin- and TNF-α-treated HepG2 cells compared with that in the TNF-α-stimulated control group (Figure 4).

Many reports have shown that quercetin has a protective effect on insulin resistance and inflammation in several experimental models [46,47,48]. For example, Vidyashankar et al. [46] showed that quercetin (10 μM) decreases TNF-α gene expression and ameliorates insulin resistance in oleic acid–induced insulin resistance in HepG2 cells. In addition, Rivera et al. [48] showed that the chronic daily administration of quercetin reduces insulin resistance and dyslipidemia in an animal experimental model of metabolic syndrome in obese Zucker rats. These results and our present data suggest that quercetin improves hepatic insulin resistance and suppresses inflammation. We can hypothesize that the protective effect of quercetin in insulin resistance is mediated by the suppression of both ER stress and anti-inflammation. In a study by Suganya et al. [49], quercetin showed a protective effect against ER stress induced in human umbilical vein endothelial cells; they demonstrated that quercetin modulated the expression level of ER stress genes coding for GRP78 and CHOP. Furthermore, it has been reported that quercetin suppresses the activation of IRE1 and PERK in ER stress-induced colonic cells, and protects RAW264.7 cells from apoptosis through its ability to inhibit the ER stress–CHOP signaling pathway [50,51].

5. Conclusion

In summary, we found that TNF-α induced inflammation and ER stress, as well as insulin resistance, in HepG2 cells. The quercetin treatments of the TNF-α-stimulated HepG2 cells ameliorated hepatic insulin resistance by suppressing the inhibition of IRS signaling and the ER stress response. According to the results of the present study, treatment with quercetin improved hepatic insulin resistance by exerting a protective effect against the ER stress and inflammation induced by TNF-α (Figure 5).

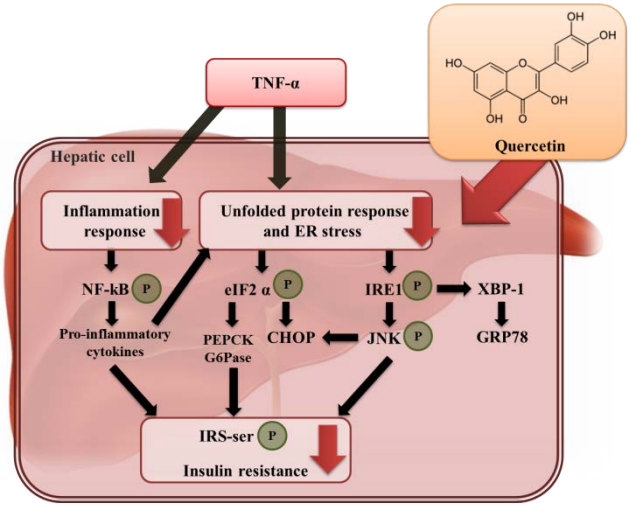

Figure 5. Effect of quercetin on TNF-α-induced ER stress and hepatic insulin resistance in HepG2 cells. TNF-α can induce inflammation and ER stress, as well as insulin resistance, in HepG2 cells. The quercetin treatments in the TNF-α-stimulated HepG2 cells ameliorated hepatic insulin resistance by suppressing the inhibition of the inflammation and ER stress response

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