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Effects of 7,8-Dihydroxyflavone on ER Stress, TXNIP/NLRP3 and Inflammation Related Markers in Tunicamycin-Treated Mice

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Abstract

Background: Endoplasmic reticulum (ER) stress regulates cell fate through autophagy, apoptosis, and inflammatory pathways. In this study, we investigated the effects of 7,8-dihydroxyflavone (7,8-DHF) on ER stress-related inflammatory responses and examined its association with alterations in proteins involved in the thioredoxin-interacting protein (TXNIP)/NLRP3 signaling pathway. Materials and Methods: Thirty male C57BL/6 mice were divided into three groups (n = 10). ER stress was induced by a single intraperitoneal injection of tunicamycin (TUN, 0.5 mg/kg). A treatment group received 7,8-DHF (5 mg/kg/day) for 21 days following TUN administration, while controls received vehicle. Protein expression of GRP78, CHOP, TXNIP, and NLRP3 was assessed by Western blot. Serum IL-1β and IL-18 levels were measured by ELISA, and oxidative stress markers (SOD, CAT, and MDA) were evaluated spectrophotometrically. Results: TUN significantly increased GRP78, TXNIP, NLRP3, IL-1β, IL-18, and MDA levels. Treatment with 7,8-DHF significantly reduced CHOP, TXNIP, NLRP3, IL-1β, IL-18, and MDA levels compared with the TUN group. In addition, CAT activity was significantly increased following 7,8-DHF treatment. Conclusion: Overall, these findings suggest that DHF attenuated TUN-associated alterations in ER stress, oxidative stress, and inflammatory markers. However, further molecular studies are required to elucidate the underlying mechanisms.

Keywords: ER stress; NLRP3; TXNIP; 7,8-dihydroxyflavone

1. Introduction

Disruption of endoplasmic reticulum (ER) proteostasis can initiate cellular responses that extend beyond protein quality control and contribute to oxidative and inflammatory signaling. The ER normally supports protein folding and processing, Ca^{2+} handling, and lipid and steroid synthesis [1]. When its functional capacity is challenged by factors such as altered nutrient supply, hypoxia, oxidative imbalance, increased secretory demand, or mutations affecting ER function, unfolded or misfolded proteins accumulate and ER stress develops [2].

The principal adaptive response to this disturbance is the unfolded protein response (UPR). By coordinating changes in protein synthesis and protein-folding capacity, the UPR attempts to re-establish ER homeostasis. If the stress remains unresolved, however, persistent UPR signaling can engage inflammatory and cell-death pathways [3]. This shift from adaptation to cellular injury is relevant to disorders in which ER dysfunction has been implicated, including obesity, diabetes, inflammatory bowel disease, neurodegenerative diseases, and cancer [4].

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Inflammasome signaling represents one route through which prolonged ER stress may be linked to inflammation. Among these complexes, the nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome responds to several forms of cellular disturbance and participates in the maturation of IL-1β and IL-18 [5]. Potassium efflux, increased reactive oxygen species (ROS), disturbances in intracellular Ca^{2+}, and lysosomal damage are among the signals associated with NLRP3 activation [6]. Previous studies have also connected ER stress with NLRP3 signaling and have implicated the thioredoxin-interacting protein (TXNIP) in this relationship [7,8]. Under oxidative conditions, release of TXNIP from thioredoxin enables its association with NLRP3, providing a potential molecular route from redox imbalance to inflammasome activation [9].

This ER stress–TXNIP–NLRP3 relationship is particularly relevant because ER stress can increase TXNIP expression, while TXNIP-dependent signaling can promote inflammasome activity [10]. Altered TXNIP expression has been described in metabolic, renal, and neurodegenerative disorders, including type 1 and type 2 diabetes [11]. These observations suggest that interventions affecting both ER stress and redox-sensitive inflammatory signaling warrant investigation rather than considering these processes independently.

Bioactive natural compounds have therefore been investigated as potential modulators of pathways involving oxidative stress, inflammation, apoptosis, and ER dysfunction [12]. One such compound is 7,8-dihydroxyflavone (7,8-DHF), a flavonoid reported to act as an agonist of tropomyosin receptor kinase B (TrkB) and to mimic several actions of brain-derived neurotrophic factor (BDNF) [13]. Pharmacological interest in 7,8-DHF partly reflects limitations associated with BDNF itself, including its short half-life and limited blood–brain barrier permeability [14]. Experimental work has attributed neurotrophic, metabolic, antioxidant, and anti-apoptotic effects to 7,8-DHF [15–19]. In our previous cafeteria diet-induced metabolic syndrome study, treatment with 7,8-DHF was associated with attenuation of ER stress and inflammation together with improvement in metabolic alterations [20].

The evidence summarized above raises the question of whether 7,8-DHF can also modify inflammatory signaling linked to experimentally induced ER stress. Accordingly, this study evaluated effects of 7,8-DHF on ER stress and inflammation-related biomarkers in tunicamycin-treated mice. We hypothesized that 7,8-DHF would attenuate these ER stress-associated responses and that the changes would be accompanied by altered TXNIP and NLRP3 protein levels.

2. Method

2.1. Study Design

Male C57BL/6 mice aged 6–8 weeks were obtained from the KTU Faculty of Medicine Surgical Research Center and maintained under a 12 h light/dark cycle with free access to food and water. Thirty animals were assigned to three groups (n = 10 each): control, TUN, and 7,8-DHF.

Control group (n = 10): Mice received an equivalent volume of vehicle alone (PBS containing 17% DMSO) intraperitoneally (i.p.) during the experimental period.

TUN group (n = 10): A single i.p. dose of tunicamycin (TUN; 0.5 mg/kg; Sigma, CAS No. 11089-65-9) was used to induce ER stress [21]. Vehicle administration was started 48 h later and continued for 21 days.

7,8-DHF group (n = 10): Following a single i.p. injection of TUN (0.5 mg/kg), 7,8-DHF treatment was started 48 h later. 7,8-DHF (Cayman, CAS No. 38183-03-8) was given i.p. at 5 mg/kg/day for 21 days, in accordance with the dosing regimen used in our previous study [20].

During the experimental period, animals were observed daily for general health status and visible signs of distress. No mortality or overt signs of illness, impaired mobility, abnormal grooming behavior, or reduced food and water intake were observed in any group. These observations were performed as part of routine animal welfare monitoring; however, no formal scoring system or longitudinal records were maintained. All animals were sacrificed during the morning hours to minimize circadian variability and 24 h after the final 7,8-DHF administration. Then serum and liver samples were collected and stored at −80 °C pending biochemical and Western blot analyses. Six representative samples from each group were included in the Western blot analysis. Uncropped original Western blot images with molecular weight markers are provided in the Supplementary Materials.

2.2. Determination of ER Stress and TXNIP/NLRP3 Inflammasome Markers Using the Western Blotting Method

For Western blot analysis, liver tissue was homogenized in RIPA buffer containing protease and phosphatase inhibitors. After centrifugation (15,000× *g*, 15 min), supernatants were recovered and total protein was quantified by the bicinchoninic acid (BCA) assay. Samples were separated on 5–10% SDS-PAGE using the Laemmli procedure [22] and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA, 1620115). Membranes were blocked in 5% non-fat milk for 2 h at room temperature and incubated at 4 °C overnight with antibodies to GRP78 (Elabscience, Wuhan, China, E-AB-40588; 1:1000), CHOP (Elabscience, Wuhan, China, E-AB-68246; 1:1000), TXNIP (Elabscience, Wuhan, China E-AB-17495; 1:1000), NLRP3 (Elabscience, Wuhan, China, E-AB-93112; 1:1000), or β-actin (Cell Signaling Technology, Danvers, MA, USA, 3700S; 1:1000). After washing, the membranes were exposed to the appropriate HRP-conjugated secondary antibody (Bio-Rad, 170-5046). Bands were visualized by incubation with 1 mL ECL reagent (Bio-Rad, 170-5060) for 1–2 min. Densitometric analysis was performed in Image Lab, with each target protein normalized to its corresponding β-actin signal.

2.3. Determination of IL-1β and IL-18 Levels

Serum concentrations of IL-1β and IL-18 were measured with mouse-specific commercial ELISA kits (BT LAB, Shanghai, China; IL-1β, E0192Mo; IL-18, E0044Mo). Measurements followed the manufacturer's protocol, and results were reported as pg/mL.

2.4. Determination of Oxidant–Antioxidant Status

Oxidant–antioxidant status in liver homogenates was evaluated from superoxide dismutase (SOD) and catalase (CAT) activities and malondialdehyde (MDA) levels.

2.5. SOD Enzyme Activity Measurement

SOD activity was assayed in liver homogenates using the modified Sun and Oberley procedure [23]. In brief, inhibition of nitroblue tetrazolium reduction in the xanthine–xanthine oxidase system was monitored at 560 nm. Values were normalized to total protein and reported as U/mg protein.

2.6. CAT Enzyme Activity Measurement

CAT activity in liver homogenates was measured with a modified Aebi procedure [24] by following the change in H_2O_2 absorbance at 240 nm. The first-order reaction rate constant (k) was used to calculate activity, which was normalized to protein content and expressed as k/g protein.

2.7. MDA Measurement

Liver MDA was quantified according to Mihara and Uchiyama [25]. After formation of thiobarbituric acid-reactive products under acidic conditions, absorbance was read at 532 nm. Results were normalized to protein content and expressed as nmol/g protein.

2.8. Protein Determination

Total protein in liver homogenates was quantified using the BCA assay and reported as μg/mL.

2.9. Statistical Analysis

Data were expressed as mean ± standard deviation (SD). The normality of data distribution was assessed using the Shapiro–Wilk test. Differences among groups were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test for multiple comparisons. A p value < 0.05 was considered statistically significant. Statistical analyses were performed using IBM SPSS Statistics version 23.0.

3. Results

3.1. Analysis of GRP78-CHOP and TXNIP-NLRP3 Protein Results in Liver Homogenate

The values of GRP78-CHOP and TXNIP-NLRP3 protein results analyzed by the Western blot method in liver homogenate are presented in Figure 1. GRP78 and CHOP proteins were examined as ER stress markers. The GRP78 level was significantly increased in the TUN-treated group compared to the control group (p = 0.033), while the increase in CHOP level was not found to be significant (p = 0.085). 7,8-DHF application reduced GRP78 levels compared to the TUN-treated group, but this reduction was not significant (p = 0.068). The same application was found to significantly reduce CHOP levels compared to the TUN group (p = 0.049). When examining protein levels in the inflammatory pathway, TUN application was found to significantly increase both NLRP3 and TXNIP levels compared to the control group (p = 0.005 and p = 0.022, respectively), while 7,8-DHF treatment significantly reduced the levels of these proteins compared to the TUN-treated group (p = 0.010 and p = 0.009, respectively).

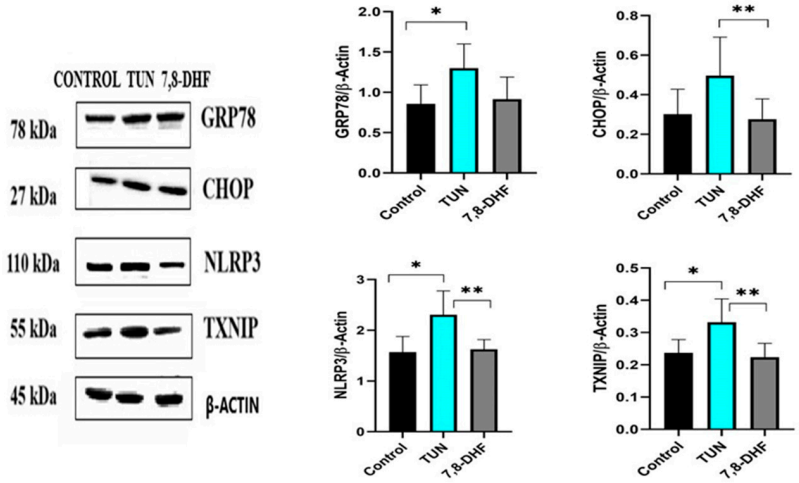


Figure 1. Western blot images and protein levels of GRP78, CHOP, NLRP3, and TXNIP analyzed by Western blot in liver homogenate. Data are presented as mean ± SD (n = 6 animals per group). Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test. * p < 0.05 vs. Control group; ** p < 0.05 vs. TUN group.

3.2. Effect of 7,8-DHF on Inflammatory Conditions

Serum samples were analyzed using commercial ELISA kits to determine whether inflammation developed as a result of the activation of the TXNIP-NLRP3 inflammasome complex and to assess the effect of 7,8-DHF on inflammation. The measurement results are presented in Figure 2. Serum IL-1β and IL-18 levels were significantly increased in the TUN group compared to the control group ($p = 0.006$ and $p = 0.009$, respectively), while in the 7,8-DHF group, these values were significantly decreased compared to the TUN group ($p = 0.027$ and $p = 0.01$, respectively).

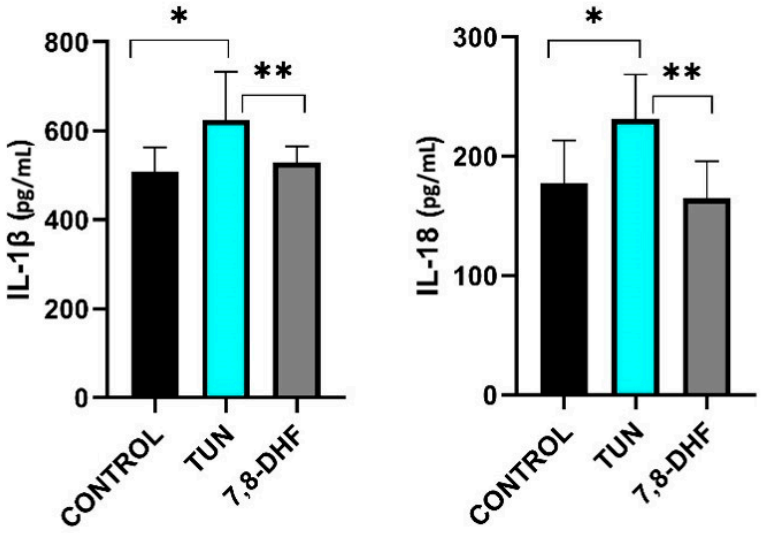


Figure 2. Serum IL-1β and IL-18 levels. Data are presented as mean ± SD (n = 10 animals per group). Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test. * $p < 0.05$ vs. Control group; ** $p < 0.05$ vs. TUN group.

3.3. Effect of 7,8-DHF on Oxidant–Antioxidant Status

In order to investigate whether endoplasmic reticulum stress was accompanied by oxidative stress and the possible therapeutic effect of 7,8-DHF on oxidative stress parameters, the levels of antioxidant enzymes SOD and CAT and the oxidant parameter MDA were measured manually in liver homogenate. A significant difference was observed in MDA levels between the control group and the TUN group ($p = 0.002$), while in the 7,8-DHF group, this value was significantly lower than in the TUN group ($p = 0.035$). When examining the levels of antioxidant enzymes, it was determined that SOD enzyme activity was significantly increased in the TUN group compared to the control group ($p = 0.042$). Although there was a decrease in CAT enzyme activity in the TUN group compared to the control group, this value was not found to be significant ($p = 0.072$). However, CAT enzyme activity in the 7,8-DHF group was significantly increased compared to the TUN group ($p = 0.006$) (Table 1).

Table 1. Data related to oxidative stress parameters.

	SOD (U/mg Protein)	CAT (k/g Protein)	MDA (nmol/g Protein)
Control	1.84 ± 0.50	16.03 ± 3.23	0.78 ± 0.09
TUN	2.60 ± 0.54 *	14.36 ± 1.67	1.15 ± 0.31 *
7,8-DHF	2.45 ± 0.82	19.49 ± 4.10 **	0.89 ± 0.12 **

Data are presented as mean ± SD (n = 10 animals per group). Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test. * $p < 0.05$ vs. Control group; ** $p < 0.05$ vs. TUN group.

4. Discussion

In mammals, ER stress, oxidative stress, and inflammatory responses constitute the main defense networks that help cells adapt to and survive stress conditions caused by biochemical, physiological, and pathological stimuli. However, chronic ER stress, oxidative stress, or inflammation has also been found to be associated with the onset and progression of various human diseases in the modern world [26]. ER stress-induced inflammation underlies the pathophysiology of various diseases, including diabetes, inflammatory bowel disease, cancer, atherosclerosis, chronic nephritis, alcoholic liver disease, hepatitis, pancreatitis, rheumatoid arthritis, and neurodegenerative diseases [27]. TXNIP is a redox sensor and an endogenous antioxidant inhibitor activated by glucose or ROS. During ER stress, TXNIP is rapidly activated by IRE1α, and elevated TXNIPs then trigger the expression of the NLRP3 inflammasome. Thus, TXNIP serves as a link between ER stress and NLRP3 inflammasome activation [11]. A BDNF mimetic and plant flavonoid, 7,8-DHF, has been shown to exert beneficial effects on ER stress and inflammation [20]. The present findings demonstrated concurrent changes in ER stress markers and TXNIP/NLRP3-associated inflammatory proteins in the tunicamycin-treated mice. While these observations support a potential relationship between ER stress and inflammatory signaling, the current experimental design does not allow conclusions regarding causality. This finding suggest that ER stress is not limited to protein folding disorders but also activates inflammatory responses [28,29].

4.1. The Link Between ER Stress and Inflammation

The relationship between ER stress and the inflammatory response can be explained by the fact that UPR signaling pathways increase the production of pro-inflammatory cytokines. In particular, the PERK and IRE1α pathways trigger the activation of the NLRP3 inflammasome by increasing TXNIP expression [7,30]. In Western blot analyses, GRP78 levels, which are sensors and initiators of ER stress, were determined to show whether ER stress occurred with tunicamycin. It was found that TUN application significantly increased ER stress compared to the control group. The significant increase in GRP78 levels indicates that ER stress has begun and that the cell is attempting to mount an adaptive response. To determine whether the cell death pathway was activated as a result of ER stress, CHOP levels were examined. Although tunicamycin administration was found to increase CHOP levels, this increase was not statistically significant (Figure 1). TUN administration was associated with higher mean GRP78 and CHOP expression levels compared with controls; however, the increase in CHOP expression did not reach statistical significance. The lack of statistical significance in the increase may indicate that the stress is directed toward the activation of the inflammatory response rather than cell death [31]. In our previous study, we demonstrated that 21 days of 7,8-DHF administration reduced GRP78 and CHOP levels in ER stress induced by a cafeteria diet [20]. A tendency toward lower GRP78 and CHOP expression was observed following 7,8-DHF treatment, although not all comparisons reached statistical significance (Figure 1).

4.2. Activation of the TXNIP/NLRP3 Pathway

Thioredoxin-interacting protein plays an important role in cellular metabolism by activating inflammatory signaling via NLRP3. The initiation of inflammatory signaling requires the formation of inflammatory sensors [32]. Various studies have reported that TXNIP induction by ER stress supports the activation of the NLRP3 inflammasome [33]. For this purpose, TXNIP and NLRP3 protein levels were determined by Western blot analysis. We found that both TXNIP and NLRP3 levels were significantly increased in the tunicamycin-treated groups compared to the control group, while 7,8-DHF treatment signif-

icantly reduced this increase (Figure 1). TXNIP disrupts cellular redox balance and increases ROS production as a negative regulator of the thioredoxin system [11,34]. This creates the oxidative stress environment necessary for NLRP3 inflammasome activation. In this study, the significant increase in TXNIP and NLRP3 levels in the TUN group indicates that this pathway has been activated and the inflammatory response has begun. The increase in serum IL-1β and IL-18 levels also confirms this activation [5,8]. These cytokines indicate that the inflammatory response is effective at the systemic level and likely contributes to tissue damage. Oslowski et al. [32] showed that ER stress is involved in inflammasome activation and that TXNIP plays an important role in the upregulation and maturation of ER stress-mediated IL-1β. In another study, it was demonstrated that ER stress activates the NLRP3 inflammasome via the ROS/ERK and ROS/TXNIP pathways in non-alcoholic fatty liver disease, and that exogenous hydrogen sulfide application reduces NLRP3-mediated hepatocyte inflammation [33]. Since PERK/eIF2α and IRE1α/XBP1 signaling pathways were not directly evaluated, the present findings do not allow the identification of the specific ER stress branch responsible for TXNIP/NLRP3-associated alterations.

4.3. Oxidative Stress and Antioxidant Response

Under physiological conditions, TXNIP is localized in the nucleus and cannot exit into the cytoplasm. In cases of excessive ROS production, TXNIP expression is upregulated through the inhibition of AMPK phosphorylation and the translocation of TXNIP from the nucleus to the cytoplasm, thereby causing ER and mitochondrial stress. The translocation of TXNIP from the nucleus to the cytoplasm induces interaction between TXNIP and TRX1, leading to the inhibition of TRX1 activity and contributing to the development of various diseases [35]. One of the significant effects of ER stress is an increase in oxidative stress [36]. In the present study, SOD, CAT, and MDA levels were determined using spectrophotometric methods to assess the oxidant–antioxidant status. When MDA levels were examined to indicate oxidative damage, it was shown that TUN application significantly increased MDA levels, while 7,8-DHF application reduced this value (Table 1). The increase in MDA levels after TUN application indicates increased lipid peroxidation and damage to cell membranes. Although the increase in SOD activity indicates that the cell's defense mechanism against oxidative stress has been activated, it is understood that this response is insufficient. The oxidative stress findings should be interpreted with caution. Although tunicamycin increased MDA levels and 7,8-DHF reduced them, the non-significant change in CAT activity and the absence of direct measurements of ROS-related pathways limit mechanistic interpretation. Therefore, the present data suggest an association between 7,8-DHF treatment and reduced lipid peroxidation but do not establish that this effect is mediated specifically through TXNIP signaling. It was demonstrated that ROS causes TXNIP to bind to NLRP3, leading to inflammasome activation [37]. In another study, it was reported that deletion of the TXNIP gene provides protection against spinal neurotoxicity by suppressing oxidative stress and apoptosis [38]. These findings suggest that 7,8-DHF may be a potential therapeutic agent in ER stress-induced inflammatory diseases. The use of 7,8-DHF may be promising in conditions where ER stress and inflammation play a role in pathogenesis, such as metabolic syndrome, diabetes, neurodegenerative diseases, and inflammatory bowel diseases [14,17]. Considering that TXNIP is upregulated in many diseases, the development of compounds that act on this protein is important for targeted treatment strategies [11,12].

5. Limitations

This study has several limitations that should be considered when interpreting the findings. First, a standalone 7,8-DHF-treated group was not included; therefore, the independent effects of 7,8-DHF under basal conditions could not be evaluated. Second, ER stress was induced by a single dose of tunicamycin, and ER stress markers were not assessed at multiple time points. Therefore, the temporal persistence of tunicamycin-induced ER stress throughout the treatment period could not be determined. Third, although the observed changes in TXNIP and NLRP3 protein levels and circulating IL-1β and IL-18 levels support an association between ER stress, inflammatory responses, and the TXNIP/NLRP3 pathway, the present experimental design does not establish a direct causal mechanism. Specific inhibition or genetic manipulation of TXNIP or NLRP3 was not performed. In addition, the study was conducted only in male mice, which may limit the generalizability of the findings. Further studies incorporating additional control groups, time-course analyses, and direct mechanistic approaches are required to confirm the role of the TXNIP/NLRP3 pathway in the effects associated with 7,8-DHF treatment. Mechanistic studies involving ER stress inhibitors (e.g., 4-PBA or TUDCA) or specific TXNIP/NLRP3 pathway inhibitors (e.g., MCC950) are required to confirm the causal role of this pathway in the observed effects of 7,8-DHF. Furthermore, oxidative stress was assessed using the biochemical parameters SOD, CAT, and MDA, whereas oxidative stress-related genes and proteins were not evaluated. Therefore, the present findings provide biochemical evidence of changes in oxidative stress status but do not allow characterization of the underlying molecular oxidative stress pathways. Future studies incorporating gene and protein expression analyses of oxidative stress-related pathways are warranted.

6. Conclusions

The present findings suggest that 7,8-DHF treatment is associated with the attenuation of inflammatory and ER stress-related markers in a tunicamycin-induced mouse model. The reductions in TXNIP and NLRP3 protein levels, together with decreased circulating IL-1β and IL-18 levels and improved oxidative stress parameters, indicate that the effects of 7,8-DHF may be associated with the modulation of the TXNIP/NLRP3 pathway. However, given the limitations of the experimental design, these findings should not be interpreted as establishing a direct causal relationship between 7,8-DHF and TXNIP/NLRP3 inflammasome inhibition. Further mechanistic studies are required to confirm these observations and clarify the pathways underlying the effects of 7,8-DHF.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/cimb48111114/s1.

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Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article or Supplementary Material.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ER, Endoplasmic reticulum; NLRP3, The nucleotide-binding oligomerization domain (NOD)-like receptor protein-3; TXNIP, Thioredoxin-interacting protein; 7,8-DHF, 7,8-dihydroxyflavone; TUN, Tunicamycin; UPR, Unfolded protein response; IRE1, Inositol-requiring enzyme 1; PERK, Protein kinase R (PKR)-like ER kinase; ATF6, Activating transcription factor 6; GRP78, Glucose-regulated protein 78; ROS, Reactive oxygen species; TrkB, Tropomyosin receptor kinase B; BDNF, Brain-derived neurotrophic factor; BCA, Bicinchoninic acid; SOD, Superoxide dismutase; CAT, Catalase; MDA, Malondialdehyde; SD, Standard deviation.

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