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ARTICLE

Hesperidin Upregulates Heme Oxygenase-1 To Attenuate Hydrogen Peroxide-Induced Cell Damage in Hepatic L02 Cells

MING-CANG CHEN,[†] YI-YI YE,[†] GUANG JI,*[,‡] AND JIAN-WEN LIU*[,†]

[†]State Key Laboratory of Bioreactor Engineering and Shanghai Key Laboratory of Chemical Biology, School of Pharmacy, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China, and [‡]Laboratory of Liver Disease, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China

Hesperidin, a naturally occurring flavonoid presents in fruits and vegetables, has been reported to exert a wide range of pharmacological effects that include antioxidant, anti-inflammatory, antihypercholesterolemic, and anticarcinogenic actions. However, the cytoprotection and mechanism of hesperidin to neutralize oxidative stress in human hepatic L02 cells remain unclear. In this work, we assessed the capability of hesperidin to attenuate hydrogen peroxide (H_2O_2)-induced cell damage by augmenting the cellular antioxidant defense. Real-time quantitative polymerase chain reaction, Western blot, and enzyme activity assay demonstrated that hesperidin upregulated heme oxygenase-1 (HO-1) expression to protect hepatocytes against oxidative stress. In addition, hesperidin also promoted nuclear translocation of nuclear factor erythroid 2-related factor (Nrf2). What's more, hesperidin exhibited activation of extracellular signal-regulated protein kinase 1/2 (ERK1/2). Besides, ERK1/2 inhibitor significantly inhibited hesperidin-mediated HO-1 upregulation and Nrf2 nuclear translocation. Taken together, the above findings suggested that hesperidin augmented cellular antioxidant defense capacity through the induction of HO-1 via ERK/Nrf2 signaling. Therefore, hesperidin has potential as a therapeutic agent in the treatment of oxidative stress-related hepatocyte injury and liver dysfunctions.

KEYWORDS: Hesperidin; heme oxygenase-1 (HO-1); mitogen-activated protein kinases (MAPK); nuclear factor erythroid 2 related factor (Nrf2); hydrogen peroxide (H_2O_2)

INTRODUCTION

Nutritional studies recommend the regular consumption of fruits and vegetables to favor a healthy quality of life. Epidemiological studies have shown that these foods may reduce the risk of death from coronary heart diseases and cancer (*1*, *2*). Citrus fruits and products are important sources of health-promoting constituents and are widely consumed around the world (*3*). Flavonoids are one of the most important compounds present in the genus *Citrus*. Recently, the health effects of citrus flavonoids have been attracting attention. These compounds have shown potential beneficial properties against several diseases, as they have shown biological activity on experiments with animals, cell lines, and in vitro assays associated with carcinogenic, cardiovascular, inflammatory, allergic, and bleeding disorders (*4*, *5*). As the most abundant flavonoids in *Citrus*, flavanones not only play an important physiological and ecological role but also are of commercial interest because of their multitude of applications in the food and pharmaceutical industries (*3*, *6*−*8*).

Heme oxygenase-1 (HO-1), one component of endogenous antioxidative defenses, catalyzes the breakdown of heme to iron, carbon monoxide, and biliverdin (*9*). It has attracted particular interest since it is finely upregulated upon oxidative stress. HO-1 and the subsequent metabolites of heme catabolism play vital roles in regulating important biological responses in liver injury, including oxidative stress, inflammation, cell survival, and cell proliferation (*10*, *11*). It has been demonstrated that flavonoids upregulate HO-1 expression by activating nuclear factor erythroid 2 related factor (Nrf2) translocation (*12*). Many researches have examined that mitogen-activated protein kinases (MAPKs) are involved in the protective mechanisms of hepatocyte damage and liver dysfunction (*13*).

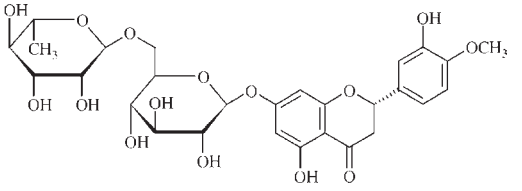

Figure 1. Chemical structure of hesperidin (MW, 610.57 Da).

Hesperidin (**Figure 1**), a naturally occurring flavonoid present in fruits and vegetables, has been reported to exert a wide range of pharmacological effects including antioxidant, antihypercholesterolemic, anti-inflammatory, anticarcinogenic actions, and antimicrobial activity (*14*, *15*) . Hesperidin is effectively used as a supplemental agent in the treatment protocols of complementary settings. Many research studies have focused on the potential uses of hesperidin as free radical scavengers and inhibitors of lipid

*To whom correspondence should be addressed. (J.L.) Tel/Fax: (+86)21-64252044. E-mail: liujian@ecust.edu.cn. (G.J.) Tel/Fax: (+86)21-64252044. E-mail: jiliver@vip.sina.com.

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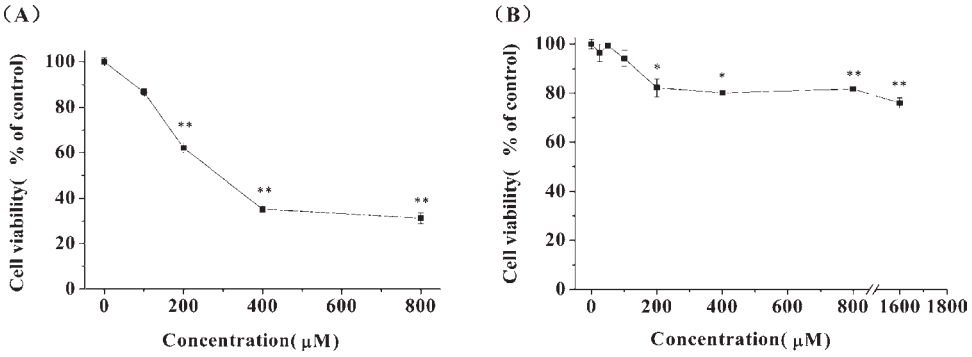

Figure 2. Effects of H_2O_2 and hesperidin on the proliferation of L02 cells. (**A**) L02 cells were treated with different concentrations of H_2O_2 for 1 h. After the medium was removed, cells were incubated with RPMI 1640 medium (with 10% fetal bovine serum) for a further 24 h and prepared for measurement by MTT analysis. (**B**) L02 cells were treated with different concentrations of hesperidin for 24 h and then measured by MTT analysis. $^*p < 0.05$ and $^{**}p < 0.01$ represent significant differences as compared with the control group.

peroxidation to prevent oxidative damage (*16*, *17*). Besides direct hydrogen-donating properties to quench reactive oxygen species (ROS), more attention has recently been focused on the influence of flavonoids on the signaling pathway and its indirect interaction with the endogenous antioxidative defense system (*18*). However, whether hesperidin can alleviate liver injury induced by hydrogen peroxide (H_2O_2) and the related molecular mechanisms remains unclear. Therefore, we designed the present work to study the potential protective effect of hesperidin against H_2O_2-induced oxidative stress in hepatocytes and to characterize the underlying mechanisms of HO-1 regulation by hesperidin via the activating MAPK/Nrf2 pathway.

MATERIALS AND METHODS

Chemicals. Hesperidin (purity ≥ 99%, high-performance liquid chromatography grade) was purchased from Shanxi Sciphar Biotechnology (Xi'an, Shanxi, China). RPMI 1640 medium and fetal bovine serum were obtained from Gibco (Grand Island, NY). Extracellular signal-regulated protein kinase 1/2 (ERK1/2) inhibitor, PD98059, p38 inhibitor, SB203580, c-Jun N-terminal kinase (JNK) inhibitor, and SP600125 were obtained from Calbiochem (San Diego, CA). Antibodies against HO-1, Nrf2, phosphor ERK1/2, ERK1/2, phosphor JNK, JNK, phospho-38 MAPK, p38 MAPK, β-actin, lamin B, and AP-labeled goat antirabbit immunoglobulin were purchased from Bipec Biopharma (Cambridge, MA). Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) color development substrate were obtained from Promega Biotech (Madison, WI). The other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

Cell Culture. The normal human hepatic cell strain, L02, was purchased from Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). L02 cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a 5% CO_2-humidified environment. The culture medium was changed every other day, and the cells were usually split 1:4 when they reached confluence. Cells were plated onto appropriate multiwells plates or dishes, and experiments were performed when cells reached about 70% confluence.

Cell Viability Assay. After hesperidin treatment, the cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tetrazolium dye assay according to our previous work (*19*).

Cell Morphological Assessment. At the end of hesperidin incubation, the morphology of cells was monitored under an inverted light microscope and stained with hematoxylin and eosin (H&E) according to standard protocols.

Determination of HO-1 Activity. Cellular HO-1 activity was detected described previously (*20*) with little modification. Briefly, 20 μL aliquots of cell suspension (2×10^6 cells) were reacted with 20 μL of hemin (150 μM) and 20 μL of NADPH (4.5 mM) in subdued lighting at 37 °C for 15 min. Blanks were cell sonicates reacted with hemin only. The reaction was stopped with dry ice (−78 °C).

Real-Time Quantitative Polymerase Chain Reaction (RT-PCR). Total RNA was isolated by Total RNA (Mini) kit (Watson Biotechnologies, Shanghai, China). RNA was quantitated by optical density measurement at 260 and 280 nm using a spectrophotometer (all RNA samples had an A_{260}/A_{280} ratio > 1.8), and integrity was confirmed by running RNA on a 1.2% agarose gel. Primers were obtained from Shanghai Sangon Biological Engineering Technology & Services (Shanghai, China), and their sequences were as follows: 5′-GGTGATAGAAGAGGCCAAGACTGC-3′ (sense) and 5′-TGTAAGGACCCATCGGAGAAGC-3′ (antisense) for HO-1 and 5′-GGTCGGAGTCAACGGATTTG-3′ (sense) and 5′-ATGAGCCCCAGCCTTCTCCAT-3′ (antisense) for GAPDH. Equal quantities (2.0 μg) of total RNA were converted to first-strand cDNA using Revert Aid First Stand cDNA Synthesis kit (Fermentas Life Sciencs, Lithuania, EU) for RT-PCR. RT quantitative PCR and melt−curve analyses were performed with the SYBR Green Realtime PCR kit (TOYOBO, Osaka, Japan) and an iCycler machine (Bio-Rad, Hercules, CA). Amplification was comprised of 40 cycles (95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s for HO-1 and 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s for GAPDH). Relative quantities of expression of the genes of interest in different samples were calculated by the comparative Ct (threshold cycle) value method (*21*).

Western Blot Assay. Cells were seeded in 10 cm dishes. After hesperidin treatment, total protein extracts were isolated according to our previous work (*19*). The extraction and isolation of nuclear fraction were performed according to previous work (*22*). Protein concentrations were determined using the BCA assay (Pierce, Rockford, IL). The Western blot assay was performed according to our previous work (*19*) with little modification. Briefly, equal amounts of proteins (30−50 μg) were separated by soidum dodecyl sulfate−polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were blocked with 2% bovine serum albumin and then incubated with appropriate primary antibodies overnight at 4 °C. Expression of protein was detected by staining with NBT and BCIP. β-Actin and Lamin B were used as loading controls for total/cytoplasm fraction extracts and nuclear fraction extracts, respectively.

Statistical Analysis. All results shown represent as means ± standard deviations from triplicate experiments performed in a parallel manner unless otherwise indicated. Significance was tested among and between groups using the one-way analysis of variance (ANOVA) followed by Dunnett's posthoc test.

RESULTS

Effects of H_2O_2 and Hesperidin on the Proliferation of L02 Cells. To assess the effects of H_2O_2 and hesperidin on the cell viability, we used the MTT tetrazolium dye assay. As shown in **Figure 2A**, H_2O_2 significantly inhibited L02 cell proliferation. However, hesperidin had no noticeable effect on the viability of L02 cells under experimental conditions shown in **Figure 2B**. In subsequent experiments, we used 200 μM H_2O_2, as an oxidative stress model, to study the effect of hesperidin on L02 cells damaged by oxidative stress.

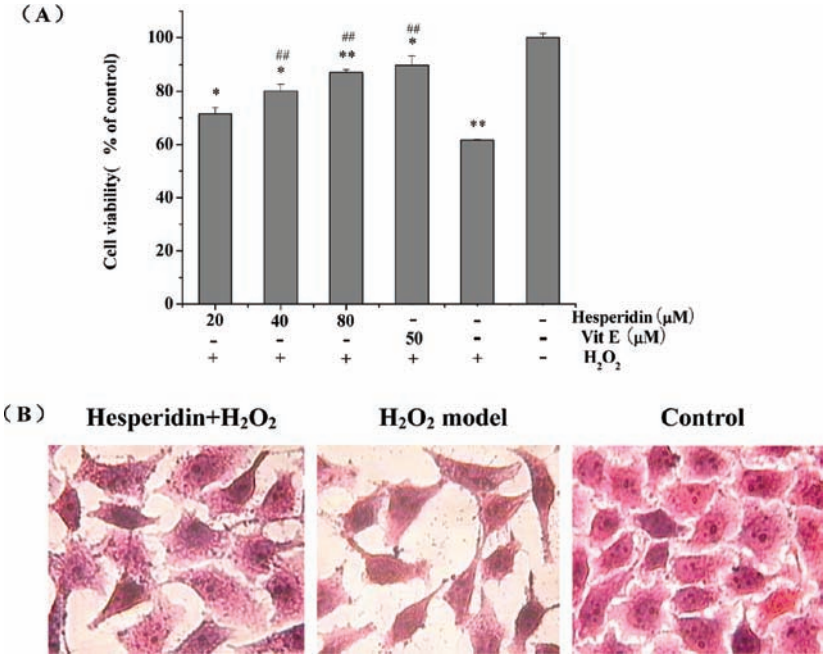

Figure 3. Hesperidin attenuated H_2O_2-induced cell injury. (**A**) Cell viability was measured by MTT assay. Cells were treated with H_2O_2 for 1 h and then washed with cold phosphate-buffered saline (PBS) twice and then fresh medium containing different concentrations of hesperidin (20, 40, and 80 μM) or Vit E (50 μM) for 24 h for measurements. (**B**) Morphologic changes of cells were observed under an inverted light microscope (250× H&E). Cells were treated with H_2O_2 for 1 h and then treated with or without 80 μM hesperidin for a further 24 h. Cells were stained with H&E as described in the Materials and Methods. $^{*}p < 0.05$ and $^{**}p < 0.01$ represent significant differences as compared with the control group. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ represent significant differences as compared with the H_2O_2 model group.

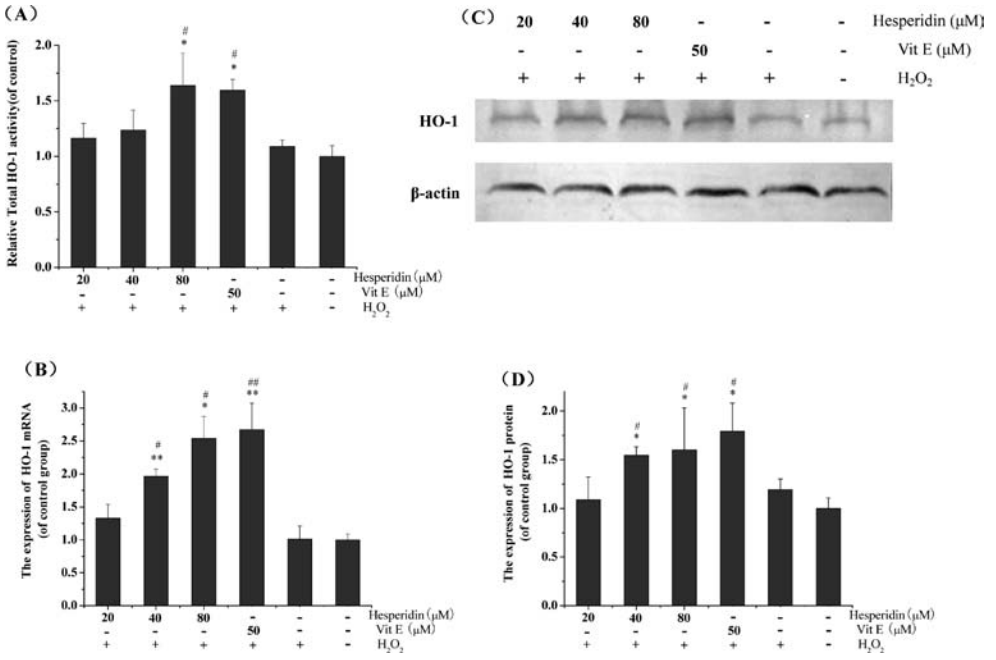

Figure 4. Effects of hesperidin on HO-1 expression. Cells were treated with H_2O_2 for 1 h and then treated with indicated concentrations of hesperidin (20, 40, and 80 μM) or Vit E (50 μM) for 24 h for measurements. (**A**) Effects of hesperidin on HO-1 enzyme activity. HO-1 activity was carried out as described in the Materials and Methods. (**B**) Effects of hesperidin on HO-1 mRNA expression. HO-1 mRNA expression was analyzed by RT-Q-PCR. (**C**) Effects of hesperidin on HO-1 protein expression. HO-1 protein expression was analyzed by Western blot. Equal amounts of proteins (30 μg) were prepared for Western blot assay. Data shown are representative of twice independent experiments. (**D**) Scanning densitometry was used for semiquantitative analysis as compared to control group levels. $^{*}p < 0.05$ and $^{**}p < 0.01$ represent significant differences as compared with the control group. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ represent significant differences as compared with the H_2O_2 model group.

Hesperidin Attenuated H_2O_2-Induced Cell Death. To assess the ability of hesperidin to protect against H_2O_2-induced cell death, L02 cells were treated with 200 μM H_2O_2 for 1 h, and then, different concentrations of hesperidin (20, 40, and 80 μM) or 50 μM vitamin E (Vit E) were added for 24 h. Just like antioxidant Vit E, hesperidin exerted a dose−response effect on the protection against H_2O_2-induced cell death, shown in **Figure 3A**. This suggested that hesperidin could attenuate oxidative injury

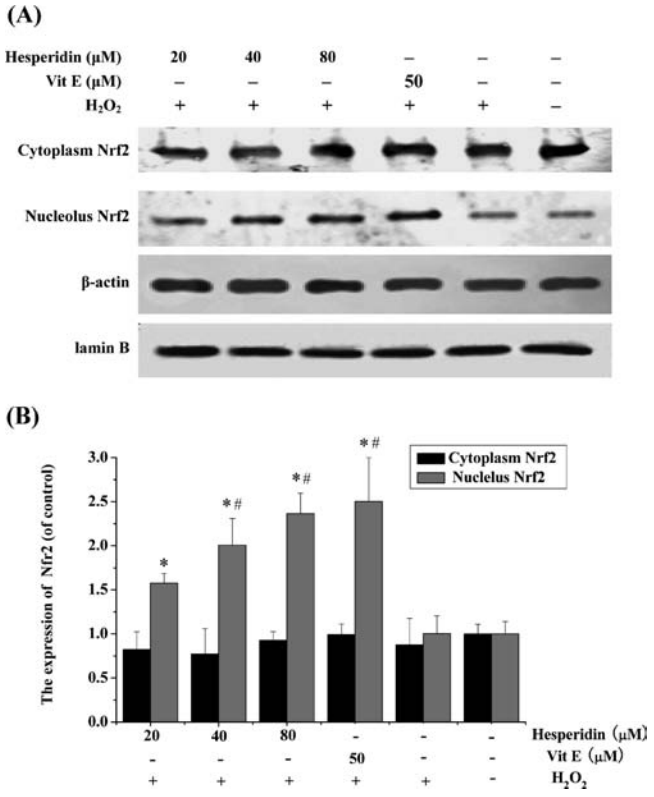

Figure 5. Effects of hesperidin on Nrf2 nuclear translocation. Cells were treated with H_2O_2 for 1 h and then treated with indicated concentrations of hesperidin (20, 40, and 80 μM) or Vit E (50 μM) for 24 h. The extraction of nuclear and cytoplasm fraction was collected and prepared for Western blot. Equal amounts of proteins (30 μg) were prepared for Western blot assay. Data shown are representative of twice independent experiments. (**A**) Effects of hesperidin on Nrf2 protein expression. (**B**) Scanning densitometry was used for semiquantitative analysis as compared to control group levels. $^*p < 0.05$ represents significant differences as compared with the control group. $^\#p < 0.05$ represents significant differences as compared with the H_2O_2 model group.

induced by H_2O_2. We also observed the cell morphology phenomena using H&E staining. As shown in **Figure 3B**, the untreated cells displayed a normal, healthy shape demonstrated by the clear skeletons. On the contrary, cells distorted severely and grew slowly in the H_2O_2 model group. Furthermore, cell microvillus disappeared in the H_2O_2 group. Excitedly, after treatment with hesperidin, L02 cells exhibited considerable improvement in shape as compared to the H_2O_2 group.

Hesperidin Upregulated HO-1 Expression. We further studied the ability of hesperidin to upregulate HO-1 in L02 cells. As shown in **Figure 4**, hesperidin upregulated HO-1 expression at mRNA levels (**Figure 4B**), protein levels (**Figure 4C**,**D**), and HO-1 enzyme activity levels (**Figure 4A**) in a dose-dependent manner. These results indicated that hesperidin enhanced endogenous antioxidative HO-1 gene expression to counteract oxidative damage.

Hesperidin Upregulated HO-1 via Activating Nrf2 Nuclear Translocation. To investigate the mechanism of HO-1 upregulation by hesperidin in L02 cells, we examined the translocation of Nrf2. Using Western blot analysis, we found that hesperidin significantly elevated the nuclear protein levels of Nrf2 but had no remarkable effects on cytoplasm Nrf2 protein levels (**Figure 5**).

Hesperidin Upregulated HO-1 Was Mediated by Activating ERK/Nrf2 Pathway. To investigate the further mechanism of how hesperidin upregulated HO-1 in L02 cells, we examined the

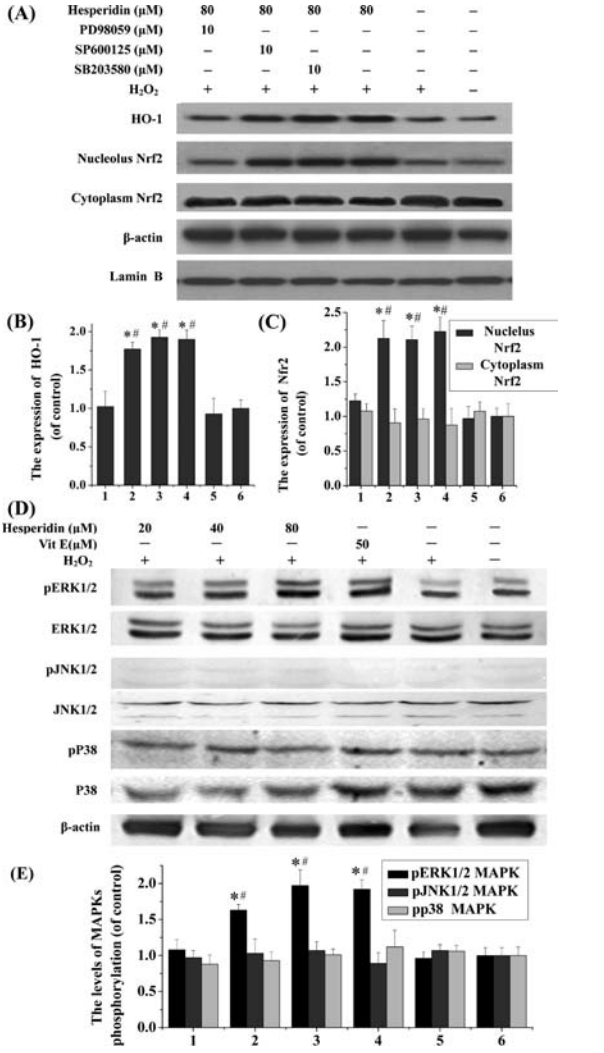

Figure 6. Effects of hesperidin on expression of HO-1 and Nrf2/MAPKs. Data shown are representative of twice independent experiments. (**A**) Effects of MAPK inhibitors on HO-1 protein expression and Nrf2 nuclear translation. L02 cells were treated with 200 μM H_2O_2 for 1 h and then 10 μM ERK1/2 inhibitor, PD98059, JNK inhibitor, SP600125, p38 inhibitor, and SB203580 were added for another 30 min. Twenty-four hours after hesperidin treatment, total protein, nuclear, and cytoplasm fraction extracts were respectively collected, and Western blots were prepared and probed for HO-1 or Nrf2, as described in the Materials and Methods. Equal amounts of proteins (50 μg) were prepared for Western blot. (**B** and **C**) Scanning densitometry was used for semiquantitative analysis as compared to control group levels. Column 1, hesperidin + PD98059 + H_2O_2 group; column 2, hesperidin + SP600125 + H_2O_2 group; column 3, hesperidin + SB203580 + H_2O_2 group; column 4, hesperidin + H_2O_2 group; column 5, H_2O_2 group; and column 6, control group. (**D**) Effects of hesperidin on phosphorylated (p-) and total ERK1/2, p38, and JNK protein expression. Details of each group were carried out the same as in **Figure 4**. Equal amounts of proteins (50 μg) were prepared for Western blot assay. (**E**) Scanning densitometry was used for semiquantitative analysis as compared to control group levels. Column 1, 20 μM hesperidin + H_2O_2 group; column 2, 40 μM hesperidin + H_2O_2 group; column 3, 80 μM hesperidin + H_2O_2 group; column 4, 50 μM Vit E + H_2O_2 group; column 5, H_2O_2 group; and column 6, control group. $^*p < 0.05$ represents significant differences as compared with the control group. $^\#p < 0.05$ represents significant differences as compared with the H_2O_2 model group.

MAPKs signal transduction pathway. As shown in **Figure 6A**,**B**, HO-1 gene expression induced by hesperidin was dependent on

ERK/MAPK activation, as an inhibitor of the ERK/MAPK pathway remarkably abolished the enhancement role of HO-1 induced by hesperidin. We additionally determined the functional significance of ERK/MAPK, p38/MAPK, and JNK/MAPK pathways. Using Western blot analysis, we found that hesperidin markedly activated phosphorylation of ERK1/2 in **Figure 6D**,**E**. Besides, we further investigated the effects of MAPKs inhibitors on Nrf2 nuclear translocation. As shown in **Figure 6A**,**C**, we found that the ERK/MAPK inhibitor significantly blocked Nrf2 nuclear translocation. These suggested that activation of ERK/Nrf2 is critical to the upregulation of HO-1 induced by hesperidin.

DISCUSSION

In the present study, for the first time, hesperidin treatment attenuated H_2O_2-induced human hepatic L02 cell oxidative damage. Interestingly, hesperidin induced HO-1 expression via the activation of the ERK/MAPK and Nrf2 pathways. The upregulation of HO-1, a critical cytoprotective molecule, identified a novel pleiotropic effect of hesperidin on hepatocytes protection to resist oxidant injury by H_2O_2.

More and more evidence suggests that HO-1 provides cytoprotection in L02 cells to neutralize oxidative stress and that HO-1 gene activation is an important adaptive mechanism to preserve homeostasis at the sites of liver injury (*23*, *24*). The cytoprotective action of HO-1 derives mostly from decreased intracellular prooxidant levels, increased bilirubin levels, and elevated carbon monoxide (CO) production. Bilirubin, as an antioxidant, provides cellular protection against free radical-mediated cell injury (*25*). CO exerts strong antiapoptotic and antiinflammatory effects through the induction of soluble guanylyl cyclase (*26*). Moreover, the iron released during heme catabolism can stimulate ferritin synthesis, and increased levels of ferritin might reduce the cellular concentration of free iron (*27*). HO-1 is induced by a wide range of stimuli, including various antioxidants (*28*). Many studies have demonstrated that hesperidin enhanced the endogenous antioxidative defense activity (*20*). In the present study, hesperidin upregulated HO-1 gene expression in mRNA levels, protein levels, and enzyme activity levels.

Previous reports have shown that Nrf2 plays a key role in regulating HO-1 expression (*12*, *28*, *29*). Nrf2, a basic leucine zipper redox-sensitive transcriptional factor, plays a center role in antioxidant response element (ARE)-mediated phase II detoxifying and antioxidant enzymes and has been reported to be a critical regulator in cell survival mechanisms (*30*). It upregulates the expression of cytoprotective and antioxidant genes that attenuate liver injury (*31*). Under basal conditions, cytoplasmic Nrf2 is bound to the Kelch-like ECH-associated protein 1 (Keap 1), but when cells are exposed to oxidative or xenobiotic stress, Nrf2 dissociates and traverses to the nucleus (*30*). In this study, nuclear Nrf2 was significantly elevated by hesperidin. This observation was consistent with many previous studies. Nrf2 was involved in the upregulation of HO-1 to protect PC12 cells against H_2O_2-induced cell damage (*29*).

To verify the antioxidant effect of hesperidin on the cell signal transduction system, we performed Western blot analysis upon upregulation of HO-1, with a focus on the MAPK pathway. In vertebrates, the three major kinase cascades are represented by ERK, JNK, and p38 MAPK (*32*). All of these kinases appear to be involved to some extent in the upregulation of HO-1 expression in response to diverse stimulus. For instance, in LMH chicken hepatoma cells, arsenite induces HO-1 expression via the ERK/MAPK and p38/MAPK pathway but not the JNK/MAPK pathway (*33*). On the other hand, sodium arsenite regulates HO-1 expression via activation of Ras and the JNK/MAPK pathway in rat hepatocytes (*34*). In the present study, hesperidin dose dependently facilitated phosphorylation of ERK1/2 but neither p38 nor JNK. These indicated that hesperidin could elevate activation of the ERK/MAPK pathway via promotion of the phosphorylation of ERK1/2. In addition, upregulation of HO-1 by hesperidin was remarkably inhibited by PD98059, a highly selective inhibitor of ERK/MAPK pathway, suggesting that hesperidin activation of the ERK/MAPK contributes to HO-1 expression. What's more, the ERK/MAPK inhibitor significantly inhibited hesperidin-mediated Nrf2 nuclear translocation. These data suggested that hesperidin upregulated HO-1 via an activating ERK/Nrf2 pathway.

In conclusion, H_2O_2-induced hepatic L02 cell oxidative injury was attenuated by hesperidin, which was accompanied by upregulation of HO-1. Hepatocytes treated with hesperidin exhibited elevated activation of ERK1/2, which appears to be responsible for nuclear translocation of Nrf2, thereby inducing HO-1 gene expression. As our study was limited to the cell level, further study is needed to confirm that hesperidin has a liver protective effect in organs or humans. However, these antioxidant properties, inhibitory effects against H_2O_2-induced oxidative damage, and promotive endogenous antioxidative defense systems suggested that hesperidin might prove to be a promising therapeutic in the treatment of oxidative stress-related liver injury.

ABBREVIATIONS USED

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HO-1, heme oxygenase-1; Nrf2, nuclear factor erythroid 2 related factor; Vit E, vitamin E; MAPKs, mitogen-activated protein kinases; ERK1/2, extracellular signal-regulated protein kinase 1/2; JNK, c-Jun N-terminal kinase; H_2O_2, hydrogen peroxide; H&E, hematoxylin and eosin; Keap 1, Kelch-like ECH-associated protein 1; ARE, antioxidant response element; NBT, nitro blue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate.

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