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Troxerutin Protects the Mouse Liver against Oxidative Stress-Mediated Injury Induced by D-Galactose

Zi-feng Zhang,† Shao-hua Fan,† Yuan-lin Zheng,* Jun Lu, Dong-mei Wu, Qun Shan, and Bin Hu

Key Laboratory for Biotechnology on Medicinal Plants of Jiangsu Province, School of Life Science, Xuzhou Normal University, Xuzhou 221116, Jiangsu Province, People's Republic of China.
† These authors contributed equally to this work

Troxerutin, a trihydroxyethylated derivative of rutin, has been well-demonstrated to exert hepatoprotective properties. In the present study, we attempted to explore whether the antioxidant and anti-inflammatory mechanisms were involved in troxerutin-mediated protection from D-gal-induced liver injury. The effects of troxerutin on liver lipid peroxidation, antioxidant enzymatic activities, and the expression of inflammatory mediator were investigated in D-gal-treated mice. The results showed that troxerutin largely attenuated the D-gal-induced TBARS content increase and also markedly renewed the activities of Cu, Zn-SOD, CAT, and GPx in the livers of D-gal-treated mice. Furthermore, troxerutin inhibited the upregulation of the expression of NF-κB p65, iNOS, and COX-2 induced by D-gal. D-Gal-induced tissue architecture changes and serum ALT and AST increases were effectively suppressed by troxerutin. In conclusion, these results suggested that troxerutin could protect the mouse liver from D-gal-induced injury by attenuating lipid peroxidation, renewing the activities of antioxidant enzymes and suppressing inflammatory response. This study provided novel insights into the mechanisms of troxerutin in the protection of the liver.

KEYWORDS: Troxerutin; D-gal; oxidative stress; inflammatory response; mouse liver

INTRODUCTION

Many investigations have demonstrated that oxidative stress was a common mechanism of hepatoxin-induced liver injury (*1*−*3*). Hepatoxins catabolize free radical-attacked essential cell constituents, such as nucleic acids, proteins, or lipids, and also induce lipid peroxidation, damage the membranes of liver cells and organelles, cause the swelling and necrosis of hepatocytes, and ultimately result in liver injury. D-Galactose (D-gal) is a normal substance in the body. However, at high levels, D-gal could cause the metabolism of sugar in disorder and lead to the accumulation of reactive oxygen species (ROS) (*4*−*6*). It has been well-established that D-gal overload could induce changes that resemble observations in the normal aging process in rodents. The animal model of accelerated aging induced by D-gal has been widely utilized for aging research and antiaging pharmacology studies (*7*−*10*). Most of the related studies are concerned with neurotoxicity and cognitive dysfunction (*4*−*6*, *8*, *9*), and some of them refer to hepatotoxicity (*7*, *8*) . There is increasing evidence that subcutaneous (s.c.) injection of D-gal at high dose could induce severe oxidative stress, as indicated by elevated levels of malondialdehyde (MDA) and decreased levels of antioxidant enzymes in murine liver (*7*, *8*, *11*). The recent research has demonstrated that D-gal causes damage to the mitochondrial integrity and the efficiency of ATP production, which contributes to more ROS generation in mitochondria and results in cellular oxidative stress in mouse liver (*12*).

There is growing interest in the pharmacological potential of natural products such as flavonoid compounds. Rutin is a well-known flavonoid glycoside identified as vitamin P with quercetin and hesperidin. Rutin is widely present in many plants, especially the buckwheat plant, and has been shown to exert multiple pharmacological activities including antiallergic, anti-inflammatory, antiviral, antiproliferative, and anticarcinogenic activities (*13*−*15*). Troxerutin, a trihydroxyethylated derivative of rutin, has been traditionally used therapeutically for threating chronic venous insufficiency (CVI), varicosity, and capillary fragility. Both in vivo and in vitro experiments indicated that troxerutin exhibited significant free radical scavenging properties (*16*−*18*). It has been demonstrated that troxerutin has hepatoprotective properties and thus protects the liver against a possible lipid peroxidation, which was caused by a high coumarin concentration (*19*). Troxerutin also exhibited a high ability to protect the liver against radiation-induced lipid peroxidation (*20*).

It has been demonstrated that D-gal could cause oxidative stress and mitochondrial dysfunction in murine liver (*7*, *8*, *11*, *12*), and inflammation is one of a variety of biological phenomena caused by oxidative stress (*21*). As previously described, troxerutin has hepatoprotective effects (*19*, *20*). Nevertheless, little work has been done to clarify the detailed mechanism of hepatoprotective effects of troxerutin. These facts formed the basis for a study of whether the antioxidant and anti-inflammatory mechanisms are involved in troxerutin-mediated protection against

*To whom correspondence should be addressed. Tel: +86 516 83500348. Fax: +86 516 83500348. E-mail: ylzheng@xznu.edu.cn or yzheng170@yahoo.com.cn.

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D-gal-induced liver injury. The purpose of this study was to explore whether troxerutin protects mouse liver from D-gal-induced injury by attenuating oxidative stress and suppressing the inflammatory response.

MATERIALS AND METHODS

Reagents. Troxerutin, used in the present study, is 3′,4′,7-Tris [O-(2-hydroxyethyl)] rutin (CAS #7085-55-4 and EINECS #230-389-4; formula, $C_{33}H_{42}O_{19}$; mol wt, 742.68). Troxerutin was obtained from Baoji Fangsheng Biotechnology Co., Ltd. (Baoji, China), and its purity was 99%. Other reagents were obtained from the following sources: D-Gal was from Sigma-Aldrich (St. Louis, MO); alanine aminotransferase (ALT) and aspartate aminotransferase (AST) assay kits were from the Jiancheng Institute of Biotechnology (Nanjing, China); chemicals used in the assays of thiobarbituric acid-reactive substances (TBARS) content and antioxidant enzymes activities were from Sigma Chemical Co. (St. Louis, MO); Optimal Cutting Temperature (OCT) was from Leica (CA, Germany); a BCA assay kit was from Pierce Biotechnology, Inc. (Rockford, IL); the polyvinylidene fluoride (PVDF) membrane was from Roche Diagnostics Corp. (Indianapolis, IN); mouse antihuman nuclear factor-κB (NF-κB) p65 antibody and goat antimouse IgG-HRP were from Santa Cruz Biotechnology (CA); rabbit antihuman cyclooxygenase-2 (COX-2) antibody and goat antirabbit IgG-HRP were from Cell Signaling Technology (Beverly, MA); mouse antimouse inducible NO synthase (iNOS) antibody was from BD Transduction Laboratories (San Jose, CA); and mouse antichicken β-actin antibody was from Chemicon International Inc. (Temecula, CA).

Animals and Treatments. Eight-week-old male Kunming strain mice (30.5 ± 3.8 g; the Branch of National Breeder Center of Rodents, Shanghai, China) were used in the following experiments. The mice were maintained under constant conditions (23 ± 1 °C and 60% humidity) and had free access to rodent food and tap water. Eight mice were housed per cage on a 12 h light/dark schedule (lights on 08:30−20:30). After acclimatization to the laboratory conditions, two groups of mice received daily subcutaneous injection of D-gal at a dose of 500 mg/(kg day) for 8 weeks, and another group with injection of saline (0.9%) only. Then, one group of D-gal-treated mice received troxerutin 150 mg/(kg day) in distilled water by oral gavage for another 4 weeks. Meanwhile, the other group of D-gal-treated mice and the third group serving as the vehicle control were given dH_2O without troxerutin. During the additional 4 weeks, mice were maintained to receive D-gal or saline (0.9%) injection, which was the same as the treatments in the foregoing 8 weeks, resepectively. At the end of treatment, following an overnight fast, mice were sacrificed, and the livers were immediately collected for experiments. The body weights and liver weights of mice were both recorded, and then, the livers were stored at −70 °C for later use. Concurrently, the whole blood of mice was collected into heparinized test tubes and centrifuged at 2000*g* for 15 min at 4 °C to separate serum, and the serum was stored at −70 °C for ALT and AST assays. All experiments were performed in compliance with the Chinese legislation on the use and care of laboratory animals and were approved by the respective university committees for animal experiments.

Determination of ALT and AST Levels. The serum levels of ALT and AST were measured spectrophotometrically using kits according to manufacturer's instructions. The activities of ALT and AST were expressed as an international unit (U/L).

Preparation of Tissue Homogenates. Tissues were homogenized in 3 mL of ice cold RIPA lysis buffer (1 × Tris-buffered saline, 1% Nonidet P 40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.004% sodium azide) combining 30 μL of 10 mg/mL phenylmethanesulfonyl fluoride solution (PMSF), 30 μL of sodium orthovanadate (Na_3VO_4), and 30 μL of protease inhibitor cocktail per gram of tissue. Homogenates were sonicated four times for 30 s with 20 s intervals using a VWR Bronson Scientific sonicator and centrifuged at 5000*g* for 10 min at 4 °C, and then, the supernatants were collected and centrifuged again. The supernatants were collected. Protein levels in the supernatants were determined using the BCA assay kit. The supernatants were stored at −70 °C until used for TBARS assay, antioxidant status, and Western blot analysis.

Measurement of Lipid Peroxidation Level. The level of TBARS in liver tissue homogenates was determined using the method of Uchiyama and Mihara (*22*). Half a milliliter of each homogenate was mixed with 3 mL of H_3PO_4 solution (1%, v/v) followed by the addition of 1 mL of thiobarbituric acid solution (0.67%, w/v). The mixture was incubated at 95 °C in a water bath for 45 min. The colored complex was extracted into *n*-butanol, and the absorption at 532 nm was measured using tetramethoxypropane as the standard. The TBARS levels were expressed as nmol per milligram of protein.

Measurement of Antioxidant Enzymes Activities. Cu, Zn-superoxide dismutase (SOD) activity was measured using the method of McCord and Fridowich (*23*). Solution A was prepared by mixing 100 mL of 50 mM phosphate-buffered saline (pH 7.4) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 2 μmol of cytochrome *c* with 10 mL of 0.001 N NaOH solution containing 5 μmol of xanthine. Solution B contained 0.2 U xanthine oxidase/mL and 0.1 mM EDTA. Fifty microliters of a tissue supernatant was mixed with 2.9 mL of solution A, and the reaction was started by adding 50 μL of solution B. The change in absorbance at 550 nm was monitored in a spectrophotometer (Shimadzu UV-2501PC, Shimadzu Corp., Japan). A blank was run by replacing the supernatant with 50 μL of ultrapure water. Cu, Zn-SOD levels were expressed as units per mg protein with reference to the activity of a standard curve of bovine Cu, Zn-SOD under the same conditions.

The catalase (CAT) activity was assayed by the method of Aebi (*24*). In brief, to a quartz cuvette, 0.65 mL of the phosphate buffer (50 mmol/L; pH 7.0) and 50 μL of sample were added, and the reaction was started by addition of 0.3 mL of 30 mM hydrogen peroxide (H_2O_2). The decomposition of H_2O_2 was monitored at 240 nm at 25 °C. The CAT activity was calculated as nanomolar H_2O_2 consumed/min/mg of tissue protein.

The glutathione peroxidase (GPx) activity assay was based on the method of Paglia and Valentine (*25*). *tert*-Butylhydroperoxide was used as the substrate. The assay measured the enzymatic reduction of H_2O_2 by GPx through consumption of reduced glutathione (GSH) that is restored from oxidized glutathione GSSG in a coupled enzymatic reaction by GR. GR reduced GSSG to GSH using NADPH as a reducing agent. The decrease in absorbance at 340 nm due to NADPH consumption was measured in a Molecular Devices M2 plate reader (Molecular Devices Corp., Menlo. Park, CA). The GPx activity was computed using the molar extinction coefficient of 6.22 mM^{-1} cm^{-1}. One unit of GPx was defined as the amount of enzyme that catalyzed the oxidation of 1.0 μmol of NADPH to $NADP^+$ per minute at 25 °C.

Histological Evaluations. The mice were perfused transcardially with 25 mL of normal saline (0.9%). The liver tissues were fixed in a fresh solution of 4% paraformaldehyde (pH 7.4) at 4 °C for 24 h, incubated overnight at 4 °C in 100 mM sodium phosphate buffer (pH 7.4) containing 30% sucrose, and embedded in OCT. Cryosections were collected on 3-aminopropyl-trimethoxysilane-coated slides (Sigma-Aldrich). The liver slices were stained with hematoxylin and eosin and examined by an expert in liver pathology (S.M.) blinded to the type of treatment received by the animals.

Western Blot Analysis. Samples (60 μg each) were separated by denaturing sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane by electrophoretic transfer (Bio-Rad Laboratories, Inc., Hercules, CA). The membrane was preblocked with 5% nonfat milk and 0.1% Tween-20 in Tris-buffered saline (TBST) and incubated overnight with the primary antibody (in TBST with 5% nonfat dried milk). Each membrane was washed three times for 15 min and incubated with the secondary horseradish peroxidase-linked antibodies. Quantitation of detected bands was performed with the Scion Image analysis software (Scion Corp., Frederick, MD). To prove equal loading, the blots were analyzed for β-actin expression using an anti-β-actin antibody. Each density was normalized using each corresponding β-actin density as an internal control and averaged from three samples, and we standardized the density of vehicle control for relative comparison as 1.0 to compare other groups.

Statistic Analysis. All statistical analyses were performed using the SPSS software, version 11.5. Lipid peroxidation level, antioxidant enzyme activity, and Western blotting data were analyzed with Newman−Keuls or Tukey's HSD posthoc test. Data were expressed as means ± standard errors of the mean (SEM). Statistical significance was set at $P \leq 0.05$.

RESULTS

Effects of Troxerutin on Body Weight and Liver index in D-Gal-Treated Mouse. The body weight of mice was recorded daily, and

Table 1. Effects of Troxerutin on Body Weight and Liver Index in D-Gal-Treated Mouse[a]

group	body weight (g)			liver index (g/100 g body weight)
	before treament	end of 8 weeks	end of 12 weeks	
control	29.98 ± 1.75	37.54 ± 1.86	38.32 ± 2.35	3.98 ± 0.21
D-gal	29.66 ± 0.92	37.51 ± 0.79	37.92 ± 2.47	3.51 ± 0.19[b]
troxerutin + D-gal	30.12 ± 1.11	37.80 ± 1.76	38.34 ± 2.6	3.93 ± 0.25[c]

[a] Data are shown as means ± SEMs ($N = 8$). [b] $P < 0.01$, as compared with the control group. [c] $P < 0.01$, vs D-gal group.

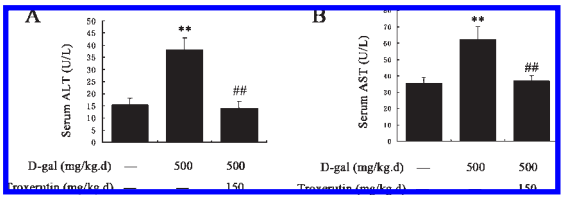

Figure 1. Effects of troxerutin on the levels of serum ALT and AST in D-gal-treated mice. (**A**) Serum ALT levels and (**B**) serum AST levels. All values are expressed as means ± SEMs ($N = 3$). $^{**}P < 0.01$, as compared with the control group; $^{\#\#}P < 0.01$, vs the D-gal group.

the liver index was also determined when the livers were collected (**Table 1**). Neither D-gal injection nor troxerutin oral gavage remarkably affected the body weight of mice during the experimental period. D-Gal-treated mice had a significantly lower liver index than that in vehicle controls ($P < 0.01$). Interestingly, the mice treated with troxerutin after D-gal injection showed a dramatic increase in liver index as compared with D-gal-treated mice ($P < 0.01$). No obvious changes of liver index could be seen in the mice treated with troxerutin after D-gal injection as compared to vehicle controls. Low liver index was an indication of liver injury; therefore, the results suggested that troxerutin could suppress mouse liver injury.

Effects of Troxerutin on Serum ALT and AST Levels in D-Gal-Treated Mouse. The levels of serum ALT and AST were determined to evaluate liver injury (**Figure 1**). Both ALT and AST levels in serum were markedly increased by 147 and 75% in D-gal-treated mice as compared to vehicle controls, respectively ($P < 0.01$ and $P < 0.01$). The serum ALT and AST levels of the mice treated with troxerutin after D-gal injection significantly decreased by 64 and 41% as compared with D-gal-treated mice, respectively ($P < 0.01$ and $P < 0.01$). There was no significant difference in serum ALT and AST levels between the mice treated with troxerutin after D-gal injection and vehicle controls. The increases of serum ALT and AST levels were also indications of liver injury; therefore, the results also suggested that troxerutin could suppress D-gal-induced mouse liver injury.

Effects of Troxerutin on Lipid Peroxidation Level in D-Gal-Treated Mouse Liver. We determined the TBARS level to evaluate lipid peroxidation level of mouse liver (**Figure 2**). A significant increase by 127% in hepatic level of TBARS was observed in D-gal-treated mice as compared with vehicle controls ($P < 0.01$). However, the hepatic TBARS content of the mice treated with troxerutin after D-gal injection was significant reduced by 53% as compared with D-gal-treated mice ($P < 0.01$). No significant difference of TBARS level could be seen in the livers between the mice treated with troxerutin after D-gal injection and vehicle controls. As expected, troxerutin could attenuate oxidative stress through inhibiting D-gal-induced lipid peroxidation levels in mouse liver.

Effects of Troxerutin on Antioxidative Status of D-Gal-Treated Mouse Liver. The activities of antioxidant enzymes including Cu, Zn-SOD, CAT, and GPx were evaluated in mouse liver (**Figure 3**). The activities of hepatic Cu, Zn-SOD, CAT, and GPx were all

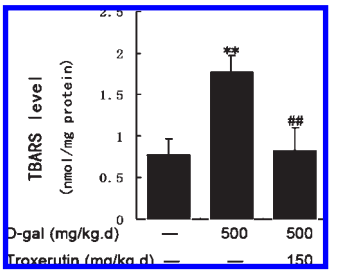

Figure 2. Effects of troxerutin on the levels of lipid peroxidation in D-gal-treated mice livers. Each value is expressed as a mean ± SEM ($N = 3$). $^{**}P < 0.01$, as compared with the control group; $^{\#\#}P < 0.01$, vs the D-gal group.

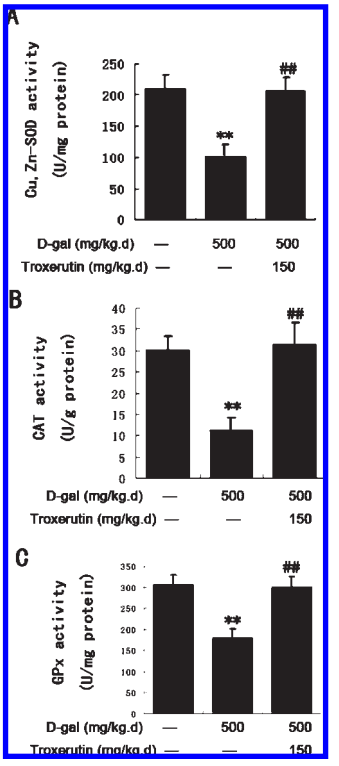

Figure 3. Effects of troxerutin on the activities of antioxidant enzymes in D-gal-treated mice livers. (**A**) Cu and Zn-SOD activities, (**B**) CAT activities, and (**C**) GPx activities. All values are expressed as means ± SEMs ($N = 3$). $^{**}P < 0.01$, as compared with the control group; $^{\#\#}P < 0.01$, vs the D-gal group.

significantly decreased by 52, 62, and 41% in D-gal-treated mice as compared with vehicle controls, respectively ($P < 0.01$, $P < 0.01$, and $P < 0.01$). Interestingly, troxerutin caused dramatic increases by 104, 178, and 67% in hepatic Cu, Zn-SOD, CAT, and GPx activities of D-gal-treated mice, respectively ($P < 0.01$, $P < 0.01$, and $P < 0.01$). There was no significant difference in those antioxidant enzymatic activities between the mice treated with troxerutin after D-gal injection and vehicle controls. Troxerutin

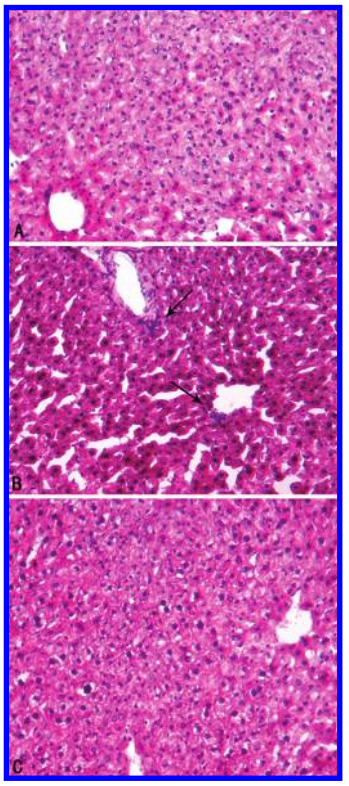

Figure 4. Effects of troxerutin on D-gal-induced histological changes in mouse liver. Representative light micrographs of liver sections from vehicle control mice (**A**); mice treated with D-gal at a dose of 500 mg/(kg day) (**B**); and mice treated with troxerutin at a dose of 150 mg/(kg day) after D-gal injection at dose of 500 mg/(kg day) (**C**). The arrows indicate congregated leucocytes and migratory leucocytes. Magnification, 100×.

could renew the activities of antioxidant enzymes in the livers of D-gal-treated mice.

Effects of Troxerutin on Histopathological Changes of D-Gal-Treated Mouse Liver. The histopathological changes were evaluated by hematoxylin and eosin stain in mouse liver (**Figure 4**). A section of D-gal-treated liver showed complete loss of architecture with the collapse of parenchyma, hepatocyte necrosis, and leucocyte infiltration (**Figure 4B**). Interestingly, we found that troxerutin effectively suppressed those D-gal-induced histopathologic changes in mouse liver. As compared with normal mice, there were no visible histologic changes in the livers of the mice treated with troxerutin after D-gal injection (**Figure 4C**). The results of histopathological evaluation showed that troxerutin exhibited a hepatoprotective effect against D-gal-induced liver injury.

Effects of Troxerutin on NF-κB p65 Activation in the D-Gal-Treated Mouse Liver. The activation of NF-κB p65 was determined by Western blot analysis (**Figure 5**). The activation of NF-κB p65 was markedly increased in the livers of D-gal treated mice as compared with that in the vehicle controls ($P < 0.001$). Interestingly, the D-gal-induced upregulation of NF-κB p65 activation was largely reduced by treatment with troxerutin ($P < 0.001$). No significant changes of NF-κB p65 activation were seen in the mice treated with troxerutin after D-gal injection as compared with the controls. Troxerutin could suppress the D-gal-induced upregulation of the activation of NF-κB p65.

Effects of Troxerutin on Inflammatory Response in the D-Gal-Treated Mouse Liver. The expression levels of iNOS and COX-2 were analyzed by Western blot (**Figure 6**). As compared with the vehicle controls, hepatic iNOS expression was markedly upregulated

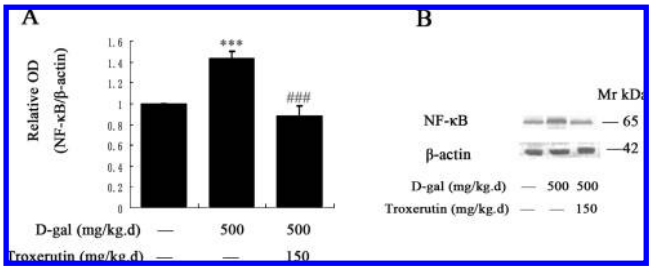

Figure 5. Western blot analysis of NF-κB p65 activation in the D-gal-treated mice livers. (**A**) Relative density analysis of the NF-κB p65 protein bands and (**B**) effect of troxerutin on NF-κB p65 activation in the D-gal-treated mice livers. β-Actin was probed as an internal control. The relative density is expressed as the ratio (NF-κB/β-actin), and the vehicle control is set as 1.0. Values are averages from three independent experiments. Each value is the mean ± SEM ($N = 3$). ***$P < 0.001$, as compared with the control group; ###$P < 0.001$, vs the D-gal group.

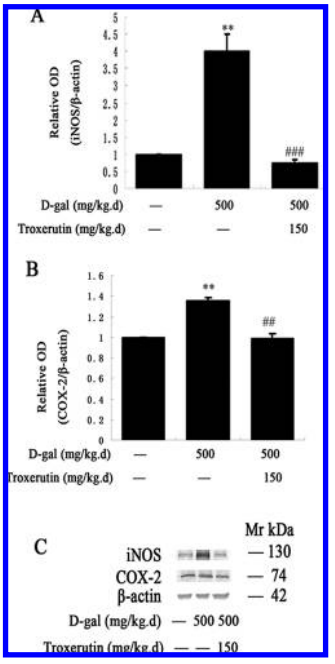

Figure 6. Western blot analysis of the proteins in association with inflammation. (**A**) Relative density analysis of the iNOS protein bands, (**B**) relative density analysis of the COX-2 protein bands, and (**C**) effect of troxerutin on the expression of iNOS and COX-2 in D-gal-treated mice livers. β-Actin was probed as an internal control. The relative density is expressed as the ratio (iNOS/β-actin or COX-2/β-actin), and the vehicle control is set as 1.0. Values are averages from three independent experiments. Each value is the mean ± SEM ($N = 3$). **$P < 0.01$, as compared with the control group; ##$P < 0.01$; and ###$P < 0.001$, vs the D-gal group.

in D-gal-treated mice ($P < 0.01$). However, the upregulation of iNOS expression was largely attenuated in the mice treated with troxerutin after D-gal injection ($P < 0.001$). No marked difference of iNOS expression levels was seen in the livers between the mice treated with troxerutin after D-gal injection and the controls (**Figure 6A**).

D-Gal treatment caused a marked upregulation of COX-2 expression in mice livers ($P < 0.01$), whereas troxerutin largely reduced the upregulation of COX-2 expression in D-gal-treated mice ($P < 0.01$). There were no significant changes of COX-2 expression between the mice treated with troxerutin after D-gal injection and the controls (**Figure 6B**). Troxerutin could suppress

the D-gal-induced upregulation of the expression of iNOS and COX-2.

DISCUSSION

It has been well-demonstrated that oxidative stress played an important role in hepatoxin-mediated liver injury (*1*−*3*). The same mechanism was also involved in D-gal-induced liver injury. The recent studies reported that D-gal provoked severe oxidative stress as indicated by elevated levels of oxidative biomarkers in rodents livers (*7*, *11*, *12*). The level of TBARS, a particular oxidative stress biomarker, indicated the degree of lipid peroxidation. It has been demonstrated that troxerutin protected against lipid peroxidation in rodents livers (*19*, *20*). In this study, the results showed that troxerutin markedly inhibited D-gal-induced increase of TBARS content in mouse liver. Consistent with previous reports (*19*, *20*), the results indicated that troxerutin could attenuate oxidative stress by decreasing the lipid peroxide level in D-gal-treated mouse liver. Cells have several defense mechanisms, such as antioxidant enzyme system, for protection against oxidative damage caused by reactive metabolite. SOD is the first enzyme that can catalyze spontaneous dismutation of superoxide radicals to H_2O_2 (*23*). Then, the enzyme CAT and members of the peroxidase family including GPx can scavenge the end product of the dismutation reaction, $-H_2O_2-$ (*26*). Cellular injury, such as a decrease in antioxidant enzyme activities, occurs when ROS generation exceeds the cellular capacity to remove it.

Many investigators suggested that the rise of antioxidant enzyme activity was a common mechanism in the hepatoprotective effects of flavonoide compounds including rutin (*27*, *28*). Interestingly, the present study showed that troxerutin markedly renewed the activities of those antioxidant enzymes, which have dramatically decreased by the treatment of D-gal in mice livers. Troxerutin exhibited efficient free radical scavenging properties (*16*−*18*). These reports implicated that troxerutin could act as antioxidant, which could directly scavenge free radical. Our findings further indicated that the renewal of the antioxidant enzyme activities should be another mechanism accounting for the hepatoprotective effects of troxerutin.

Furthermore, oxidative stress-mediated liver damage could cause an increase of AST and ALT levels in serum and a decrease of liver index as well as hepatic histological changes, such as structure damage, hepatocellular necrosis, leucocyte infiltration, and massive hemorrhage (*26*, *29*−*31*). In the present study, troxerutin largely reduced AST and ALT levels in serum, markedly attenuated the decrease of liver index, and effectively alleviated tissue architecture changes in D-gal-treated mouse liver. We suggested that troxerutin might improve liver function and structure via production of a more significant elevation of antioxidant enzyme activities to increase the scavenging of ROS, leading to the reduced oxidative stress in D-gal-treated mouse.

Oxidative stress contributed to the pathogenesis of inflammation. In the inflammatory process, there was an increase of microvasculature permeability, leakage of the elements of blood into the interstitial spaces, and migration of leukocytes into the inflamed tissue. It has been reported that rutin could suppress those inflammatory processes (*32*, *33*). In this research, the results of histopathological evaluation showed that troxerutin effectively suppressed the D-gal-induced histopathologic changes, including structure damage and leukocyte infiltration in mouse liver. Once tissue damage occurs, leukocytes rapidly migrate to sites of injury initiating an inflammatory response (*34*). So, the results of histological analysis substantiated that troxerutin could suppress inflammatory response in the livers of D-gal-treated mice.

In addition to inflicting direct damage to vital cell constituents such as lipids, proteins, and DNA, oxidative stress could also modulate the pattern of gene expression through functional alterations of transcription factors such as NF-κB (*35*). NF-κB plays an important role in the process of inflammation. NF-κB regulated the expression of various target genes including those encoding cytokines, nitric oxide, or adhesion molecules implicated in inflammation (*36*). The present study showed that the upregulation of NF-κB p65 activation induced by D-gal was suppressed by the treatment of troxerutin . These results suggested that troxerutin could inhibit the initiation of inflammatory response, at least in part, by suppressing NF-κB p65 activation. iNOS, which is associated with both local and systemic inflammatory response, could consistently release high levels of nitric oxide (NO) (*37*). In the liver, the production of NO by iNOS was implicated in hepatic injury during inflammation. COX-2, which catalyzed the formation of prostaglandins and other eicosanoids from arachidonic acid, was induced at the site of inflammation. The production of prostanoids by COX-2 was often implicated in inflammatory diseases, characterized by edema and tissue injury (*38*, *39*). Some investigators suggested that the NO released from inflammatory cells, which synthesized by iNOS, could increase COX-2 activity (*40*). The present study showed that troxerutin largely attenuated the upregulation of iNOS and COX-2 expression in the livers of D-gal-treated mice. These results implicated that troxerutin could attenuate inflammatory processes by suppressing the expression of iNOS and COX-2. It was well-known that the levels of expression of iNOS and COX-2 were tightly controlled by NF-κB. We suggested that troxerutin might attenuate the upregulation of iNOS and COX-2 expression induced by D-gal due to its inhibition of NF-κB p65 activity. These results suggested that troxerutin could alleviate liver injury caused by D-gal through suppressing inflammatory response.

In conclusion, troxerutin inhibited the increase of TBARS level, renewed activities of antioxidant enzymes, and attenuated the upregulation of NF-κB p65, iNOS, and COX-2 in D-gal-treated mouse liver. Our findings suggested that troxerutin could alleviate liver injury caused by D-gal through antagonizing oxidation stress and inflammatory response.

ABBREVIATIONS USED

D-gal, D-galactose; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBARS, thiobarbituric acid-reactive substances; SOD, superoxide dismutases; CAT, catalase; GPx, glutathione peroxidase; NF-κB, nuclear factor-κB; iNOS, inducible NO synthase; COX-2, cyclooxygenase-2.

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