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PPARγ Activation Protects against Hydrogen Peroxide-Induced Oxidative Stress and Apoptosis in Human Liver Cells

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Abstract—The deleterious role of oxidative stress in liver damage is a growing problem, and effective therapeutic interventions are highly warranted. This study evaluated whether peroxisome proliferator-activated receptor gamma (PPARγ) activation protects against H_2O_2-induced oxidative stress and apoptosis in human L02 hepatocytes. Cells pretreated with rosiglitazone, a PPARγ agonist, were incubated with H_2O_2, and cell viability was assessed using CCK8 and LDH release assays 24 h after the treatment. The content of apoptotic cells was determined using Hoechst 33258 staining, and the levels of apoptosis-related proteins were determined by immunoblotting. In addition, several oxidative stress indicators were measured. Possible involvement of the nuclear factor erythroid 2-related factor (Nrf2) pathway was investigated using the Nrf2 inhibitor ML385. Rosiglitazone (20 μM) increased cell viability and improved nuclear morphology in H_2O_2-treated L02 cells, possibly by increasing the Bcl-2/Bax ratio and reducing caspase-3 activation. Rosiglitazone also decreased reactive oxygen species and malonaldehyde levels, as well as increased the activities of catalase, glutathione peroxidase, and superoxide dismutase. Rosiglitazone also promoted nuclear translocation of Nrf2 and increased the antioxidant levels in H_2O_2-treated L02 cells. Inhibition of the Nrf2 pathway by ML385 partially abolished the rosiglitazone-induced amelioration of oxidative stress and apoptosis. We conclude that activation of PPARγ protects liver cells against oxidative stress and apoptosis through the Nrf2 pathway.

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Keywords: rosiglitazone, L02 cells, hepatotoxicity, ML385, Nrf2

INTRODUCTION

Liver damage is common in chronic conditions such as viral infections, metabolic diseases, excessive alcohol and drug consumption, and autoimmune diseases [1, 2]. Both experimental and clinical evidence indicate that oxidative damage, inflammation, dysregulation of signaling pathways, and dysfunction of the innate immune system play critical role in the progression of liver damage [3]. As the largest detoxification organ in the body, the liver metabolizes a wide

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range of compounds, a process that generates reactive oxygen species (ROS) as byproducts [4]. Excessive ROS production disrupts redox balance, ultimately leading to oxidative stress. Oxidative stress promotes lipid peroxidation, DNA damage, irreversible protein modifications, and alterations in signaling pathways involved in the regulation of gene transcription, protein expression, and apoptosis, eventually resulting in hepatic damage [5, 6]. Because of its high metabolic activity, the liver is particularly susceptible to oxidative stress, which is closely associated with the emergence and progression of various liver diseases. Therefore, developing novel therapeutic strategies, in particular, those targeting oxidative stress-induced death of hepatocytes, might contribute significantly to the treatment of liver diseases.

High concentrations of ROS inhibit expressions of the anti-apoptotic Bcl-2 protein [7]. Excessive ROS disrupt mitochondrial function, reduce cellular energy supply, and promote the release of cytochrome *c*, resulting in the activation of caspase-3, a key enzyme of the apoptotic cell pathway [8]. Activated caspase-3 cleaves essential cytoplasmic proteins, leading to DNA fragmentation in the nucleus, characteristic changes in the nuclear morphology [9], and ultimately apoptosis.

Nuclear transcription factor peroxisome proliferator-activated receptor gamma (PPARγ) plays a central role in the regulation of glucose and lipid metabolism. Its synthetic agonists thiazolidinediones (TZDs), such as rosiglitazone and pioglitazone, are a class of oral antihyperglycemic agents used in clinical practice [10]. Although TZDs possess some side effects that limit their use for glycemic control, PPARγ activation has been shown to exert protective effects in a broad range of other diseases [11, 12]. For instance, rosiglitazone protects against traumatic brain injury in rats by attenuating neuronal apoptosis and autophagy [13]. Pioglitazone increases the expression of tight junction proteins, improves intestinal barrier function, and attenuates dextran sodium sulfate-induced colitis [14]. Although some studies have reported that TZDs, particularly troglitazone (first-in-class drug), induce hepatotoxicity by altering the levels of liver enzymes, the incidence for such toxicity with rosiglitazone is substantially lower than that observed with troglitazone [15]. Newer TZD compounds, such as rosiglitazone and pioglitazone, have been reported to exhibit milder and reversible hepatic toxicity profiles [15, 16]. The beneficial effects of rosiglitazone on liver injury have been reported more often that its adverse effects. For example, pretreatment with rosiglitazone inhibited extracellular signal-related kinase/mitogen-activated protein kinase (ERK/MAPK) signaling and activation of nuclear factor kappa B pathway, thereby effectively preventing the progression of acute liver damage [17]. Rosiglitazone caused 80% higher cell survival in mice with acute liver injury by reducing liver necrosis and apoptosis through the modulation of endoplasmic reticulum stress pathways [18]. These findings suggest that contrary to concerns regarding hepatotoxicity, recently developed TDZ, such as rosiglitazone, protect the hepatic functions. Further studies are required to clarify the underlying mechanisms in order to establish confidence in their potential therapeutic application against clinical hepatotoxicity.

Collectively, previous studies suggest that the beneficial effects of PPARγ activation are largely attributable to its anti-inflammatory action across a range of pathological conditions. However, the role of PPARγ activation in the liver, particularly at the cellular level, has not yet been clarified. In the present study, we investigated whether rosiglitazone-induced PPARγ activation protects human L02 hepatocytes against hydrogen peroxide (H_2O_2)-induced oxidative stress and apoptosis and explored the underlying molecular mechanism. Our findings may suggest additional therapeutic options for the treatment of liver diseases.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium (DMEM) and L02 cells from Jiangsu Kaiji Biotechnology (Nanjing, China), fetal bovine serum (FBS) from Gibco Invitrogen (USA), trypsin from Dingguo Changsheng Biotechnology (Beijing, China), lactate dehydrogenase (LDH) assay kit (A020-2-2) from the Jiancheng Bioengineering Institute (Nanjing, China) were used. Cell counting kit-8 (CCK-8) (C0037), nucleoprotein extraction kit (P0027), Hoechst 33,258 solution (C1017), and assay kits for malonaldehyde (MDA) (S0131S), ROS (S0035S), glutathione peroxidase (GPx) (S0056), catalase (CAT) (S0051), superoxide dismutase (SOD) (S0101S), oxidized glutathione (GSSG) (S0053), and reduced glutathione (GSH) (S0053) were obtained from the Beyotime Institute of Biotechnology (Shanghai, China). Rabbit anti-Bax (sc-70408) and anti-Bcl-2 (sc-7382) antibodies from Santa Cruz Biotechnology (USA); rabbit antibodies against caspase-3 (9662), Kelch-like ECH-associated protein 1 (Keap1) (8047), Nrf2 (12721), quinone oxidoreductase 1 (NQO1) (3187S), and heme oxygenase-1 (HO-1) (43966) from Cell Signaling Technology (USA), rabbit anti-β-actin (AP0731), anti-histone H3(BS40053) antibodies, and secondary antibodies from Bioworld Technology (USA) were used. Rosiglitazone and ML385 were from Sigma (USA).

Cell culture. Cells were grown in DMEM supplemented with 10% FBS and 0.4% streptomycin/penicillin in 5% CO_2 at 37°C in a humidified atmosphere

and used in the experiments after six passages after reaching ~80% confluency.

CCK-8 assay. The cytotoxicity of different concentrations of H_2O_2 was determined using the CCK-8 assay. For this, L02 cells were seeded into 96-well plates (1×10^4 cells/well) and incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO_2. Next, the cells were washed twice with PBS and pretreated with various concentrations of H_2O_2 (0, 200, 400, 600, 800, and 1000 μM) for 24 h after two. CCK-8 solution (10 μL) was added to and the cells were incubated for 1 h, The absorbance at 450 nm was then measured to calculate the concentration of H_2O_2 that inhibited cell growth by 50% (IC50) [19].

Treatments. Hepatocyte injury model was established by L02 cell exposure to 600 μM H_2O_2. To investigate the protective effects of rosiglitazone, H_2O_2-treated L02 cells were divided into five groups and incubated for 24 h with the following compounds: control (vehicle + vehicle); drug control (20 μM rosiglitazone + vehicle); H_2O_2 (vehicle + 600 μM H_2O_2); low-dose rosiglitazone (10 μM rosiglitazone + 600 μM H_2O_2); and high-dose rosiglitazone (20 μM rosiglitazone + 600 μM H_2O_2). Cells in the low- and high-dose rosiglitazone groups were pretreated with rosiglitazone for 2 h prior to H_2O_2 exposure. After 24 h of incubation, the cells were assessed using the CCK-8 and LDH release assays, Hoechst 33258 staining, and Western blotting.

To investigate the protective mechanism of rosiglitazone against H_2O_2-induced oxidative stress and apoptosis, cells were divided into five groups: (1) vehicle + vehicle; (2) vehicle + 600 μM H_2O_2; (3) 5 μM ML385 + 600 μM H_2O_2; (4) 20 μM rosiglitazone + 600 μM H_2O_2; and (5) 20 μM rosiglitazone + 5 μM ML385 + 600 μM H_2O_2. Cells in the 5 μM ML385 + 600 μM H_2O_2 group were pretreated with ML385 for 2 h, followed by incubation with ML385 and H_2O_2 for 24 h. Cells in the 20 μM rosiglitazone + 5 μM ML385 + 600 μM H_2O_2 group were pretreated with rosiglitazone and ML385 simultaneously for 2 h and then co-incubated with H_2O_2 for 24 h.

LDH release assay. The extent of cell death in H_2O_2-treated cells using the LDH release assay. Cells were seeded in 96-well plates (3×10^5 cells per well) and treated as indicated, after which the activity of LDH released from the cells to the culture medium was determined at 450 nm with a microplate reader. LDH activity reflected membrane damage and H_2O_2-induced cytotoxicity. The same cell density per well was used for subsequent experiments conducted in 6-well plates.

Hoechst 33258 staining was used to investigate whether rosiglitazone protected L02 cells against H_2O_2-induced apoptosis. Cells treated with rosiglitazone and H_2O_2 were washed three times with PBS, incubated in 0.5 mL of fixation buffer at 37°C for 10 min, washed three times with PBS, and stained with Hoechst 33258 at room temperature in the dark for 5 min, and examined under a fluorescence microscope [20].

Intracellular ROS and MDA assessment. Intracellular ROS levels were determined with a ROS assay kit based on the fluorescence probe 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) that is oxidized by ROS to fluorescent dichlorofluorescein (DCF). Thus, DCF fluorescence signal was used to detect the ROS content in the cells. After treatment with H_2O_2 and rosiglitazone, cells were incubated with DCFH-DA (10 μM in PBS) at 37°C for 30 min. Cells were then washed three times with PBS, collected, and analyzed for their fluorescence intensity with a flow cytometer at the excitation wavelength of 488 nm and emission wavelength of 535 nm. MDA levels were measured using a commercial MDA assay kit according to the manufacturer's instructions (S0131S, Beyotime, China).

Assessment of oxidative stress indicators. The content of GSH and GSSG and the activities of CAT, SOD, and GPx were detected using the corresponding commercial kits according to the manufacturers' instructions.

Western blotting. Expression of Bcl-2 and Bax and activation of caspase-3 was evaluated using antibodies against caspase-3 (dilution, 1 : 1000), Bax (1 : 1000), Bcl-2 (1 : 1000), Keap1 (1 : 1000), Nrf2 (1 : 1000), HO-1 (1 : 1000), NQO1 (1 : 1000), β-actin (1 : 3000), and histone H3 (1 : 500). Cells were incubated with lysis buffer (180 μL of RIPA buffer containing phenylmethylsulfonyl fluoride at a 9 : 1 ratio) on ice for 30 min and the lysate was centrifuged at 4°C at 12,000*g* for 10 min to obtain protein-containing supernatant. Nuclear extract was prepared using a nucleoprotein extraction kit. Briefly, the cells were washed twice with PBS, collected by centrifugation at room temperature at 1000*g* for 5 min, and homogenized in an ice-cold hypotonic buffer for 10 min. The lysate was centrifuged at 4°C at 3000*g* for 5 min; the precipitate was washed with the hypotonic buffer and centrifuged at 4°C at 5000*g* for 5 min. Finally, 0.2 mL of lysis buffer was added to the precipitate, and the samples were incubated on ice for 20 min before centrifugation at 4°C at 15,000*g* for 5 min. The obtained supernatant containing nuclear proteins was stored at −40°C.

After determining protein concentration, proteins were separated electrophoretically and transferred to polyvinylidene fluoride membranes. Following blocking overnight with 5% fat-free milk, the membranes were probed with primary antibodies at 4°C and then incubated with an appropriate secondary antibody conjugated with horseradish peroxidase (dilution, 1 : 5000) at room temperature for 2 h.

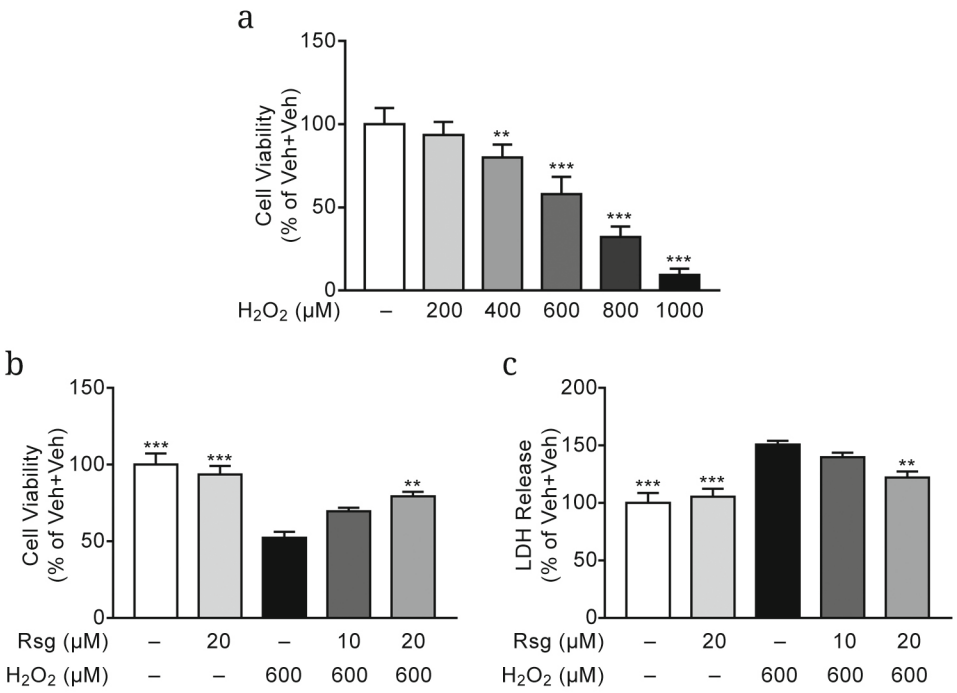

Fig. 1. PPARγ activation ameliorates H_2O_2-induced cytotoxicity in L02 cells. a) Viability of L02 cells after 24-h exposure to different H_2O_2 concentrations (0, 200, 400, 600, 800, and 1000 μM) as assessed by CCK-8 assay. b and c) Viability of L02 cells following indicated treatments assessed by CCK-8 (b) and LDH release (c) assays. Data are shown as mean ± SEM (n = 6); ** $p < 0.01$, *** $p < 0.001$ vs. Veh + H_2O_2 group.

Protein bands were visualized using enhanced chemiluminescence with a gel imaging system (Tanon Science & Technology Co., China). Four biological repeats were performed for each Western blotting experiment.

Statistical analysis was performed with the SPSS software (version 20.0; IBM, USA). The results are expressed as mean ± standard error of mean (SEM). Differences between the groups were evaluated using one-way ANOVA, followed by the Dunnett's *post hoc* test for multiple comparisons; p-value < 0.05 was considered statistically significant.

RESULTS

PPARγ activation ameliorates H_2O_2-induced cytotoxicity in L02 cells. To determine the working concentrations of H_2O_2, L02 cells were exposed to different H_2O_2 concentrations for 24 h, followed by assessment of their viability using CCK-8 assay (Fig. 1a). Treatment with 600 μM H_2O_2 reduced cell viability by ~50%; therefore, this concentration was selected for establishing the cell damage model. Next, we investigated whether rosiglitazone prevented H_2O_2-induced cytotoxicity in L02 cells using CCK-8 detection and LDH release assay. As shown in Fig. 1, the viability of H_2O_2-treated L02 cells was significantly decreased compared to the vehicle treatment group ($p < 0.001$, Fig. 1b). Treatment with 10 μM rosiglitazone slightly increased the viability of H_2O_2-treated cells, but the effect did not reach the level of statistical significance ($p > 0.05$, Fig. 1b). Rosiglitazone at the concentration of 20 μM noticeably mitigated the H_2O_2-induced cytotoxicity, indicating a dose-dependent protective effect ($p < 0.01$, Fig. 1b). H_2O_2 exposure markedly increased the LDH release ($p < 0.001$), whereas pretreatment with 20 μM rosiglitazone reduced it ($p < 0.01$, Fig. 1c). In vehicle-treated cells, rosiglitazone did not exert any cytotoxic effect ($p > 0.05$), as assessed by both CCK-8 and LDH release assays.

PPARγ activation attenuates H_2O_2-induced apoptosis in L02 cell. Hoechst 33258 staining was used to evaluate the protective effect of rosiglitazone against H_2O_2-induced apoptosis in L02 cells. As shown in Fig. 2b, H_2O_2 treatment significantly increased the number of apoptotic cells compared to vehicle-treated controls ($p < 0.01$). L02 cells exposed to 600 μM H_2O_2 for 24 h exhibited characteristic apoptotic features, including chromatin condensation and nuclear shrinkage, manifested as small, bright blue fluorescent dots (Fig. 2a). Pre-incubation with 20 μM rosiglitazone exerted a protective effect on the cells ($p < 0.05$, Fig. 2b). Furthermore, treatment with rosiglitazone alone (20 μM) did not induce apoptosis in vehicle-incubated cells. These results suggest that PPARγ activation protects against H_2O_2-induced cell apoptosis.

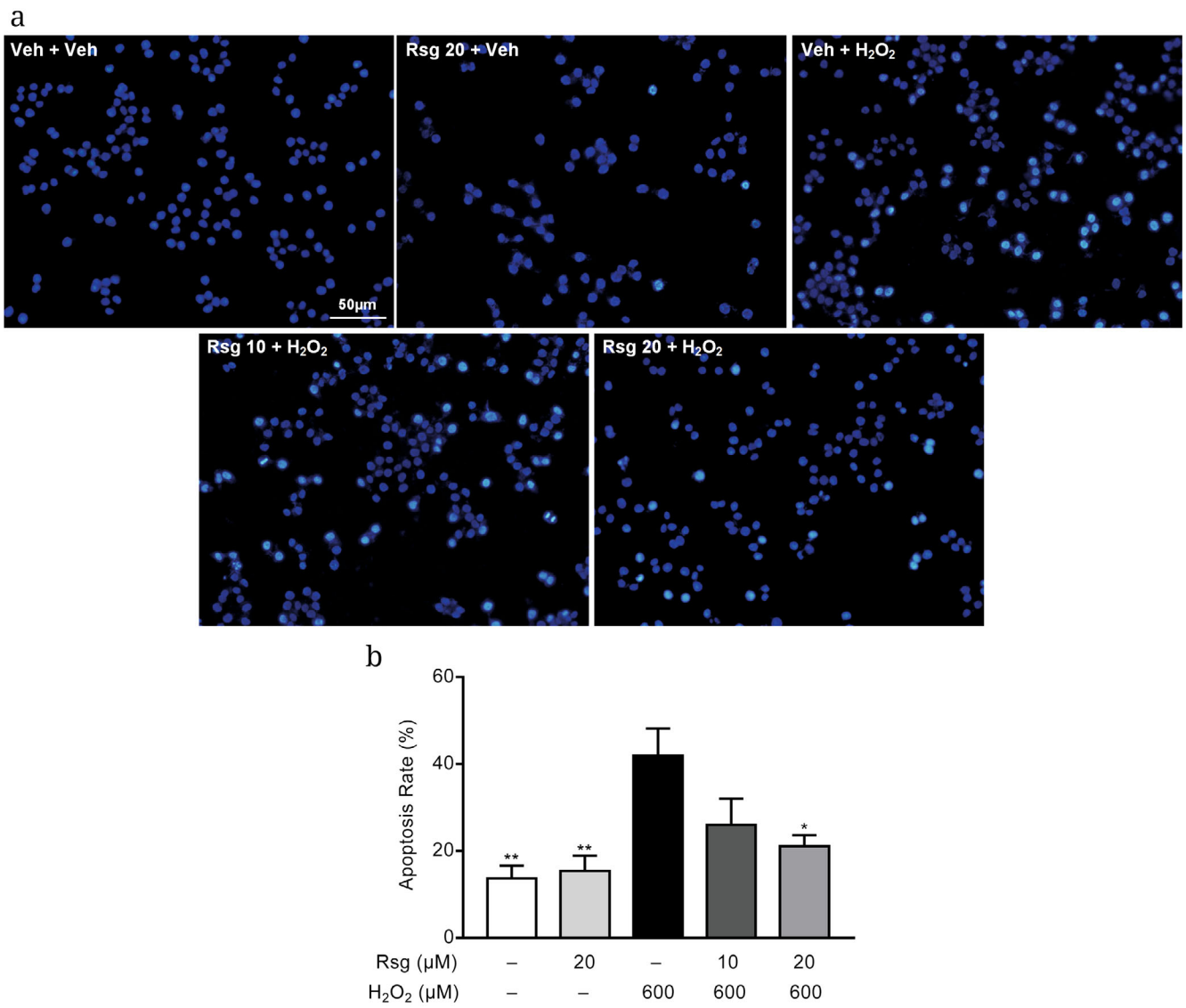

Fig. 2. PPARγ activation inhibits H_2O_2-induced L02 cell apoptosis. a) Representative images of apoptotic L02 cells stained with Hoechst 33258. b) Cell apoptosis rate in treatments groups. Data are shown as mean ± SEM (n = 4); * $p < 0.05$, ** $p < 0.01$, vs. Veh + H_2O_2 group.

PPARγ activation increases the Bcl-2/Bax ratio and inhibits caspase-3 activation in H_2O_2-treated cells. To further investigate whether PPARγ activation mitigates H_2O_2-mediated apoptosis, expression levels of Bax, Bcl-2, and caspase-3 were assessed by Western blotting. The results demonstrated that H_2O_2 significantly reduces the Bcl-2/Bax ratio ($p < 0.001$, Fig. 3, a and c) and elevated the level of caspase-3 ($p < 0.001$, Fig. 3, b and d) in L02 cells. Pretreatment with 20 μM rosiglitazone increased the of Bcl-2/Bax ratio ($p < 0.05$, Fig. 3, a and c). Rosiglitazone at 10 μM and 20 μM significantly inhibited caspase-3 activation ($p < 0.05$ and $p < 0.01$, respectively) (Fig. 3, b and d). Rosiglitazone (20 μM) treatment alone had no significant effect on the expression of these proteins in vehicle-incubated cells.

PPARγ activation alleviates oxidative stress and enhances the antioxidative capacity in H_2O_2-treated cells. To evaluate the effect of PPARγ activation on oxidative stress, we measured the content of ROS as key indicators of oxidative stress based on DCF fluorescence signal intensity. Treatment with H_2O_2 increased ROS levels ($p < 0.01$, Fig. 4a) in L02 cells compared to the vehicle group, while pretreatment with 20 μM rosiglitazone significantly reduced ROS accumulation in H_2O_2 treated-cells ($p < 0.05$, Fig. 4a). Similarly, the content of MDA (lipid peroxidation product) was elevated by oxidative stress ($p < 0.01$, Fig. 4b) in L02 cells, and pretreatment with 20 μM rosiglitazone markedly decreased its content in H_2O_2-incubated cells ($p < 0.05$, Fig. 4b). Moreover, H_2O_2 treatment decreased the activities

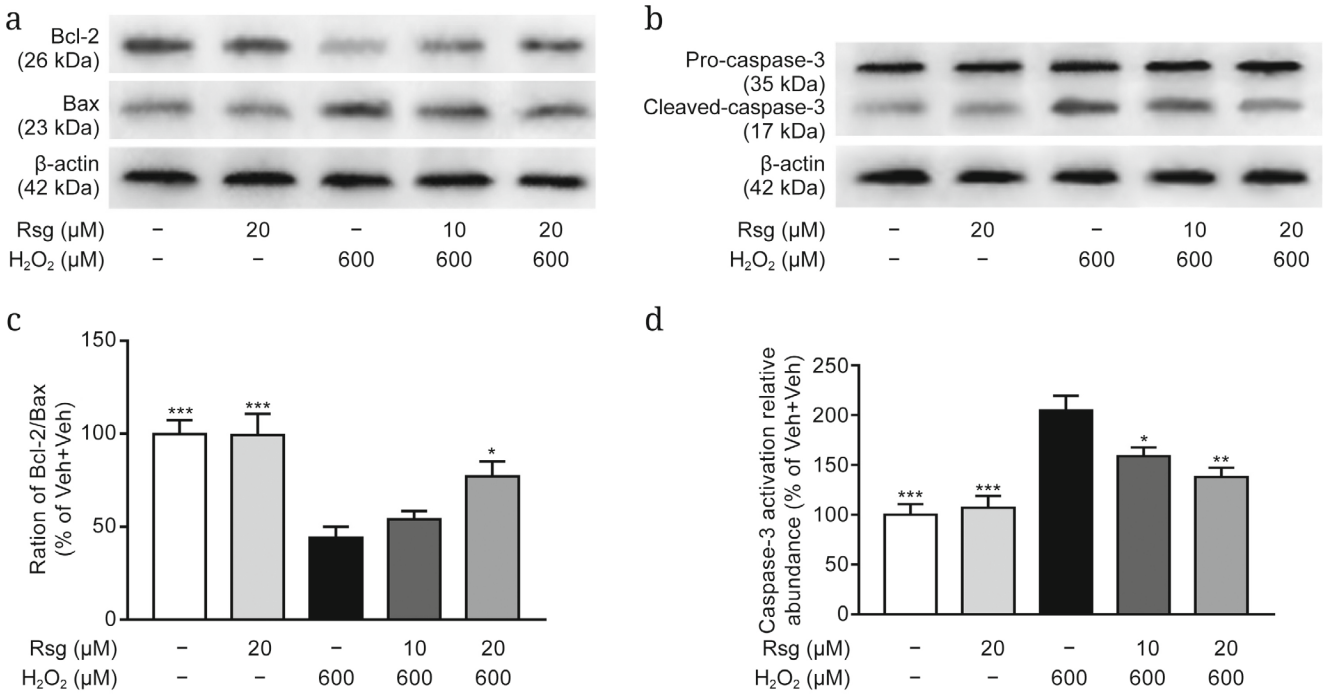

Fig. 3. PPARγ activation increases Bcl-2/Bax ratio and inhibits caspase-3 activation in H_2O_2-treated cells. a and b) Protein expression levels of Bax, Bcl-2, procaspase-3, and cleaved caspase-3 as detected by Western blotting. c) Bcl-2/Bax ratio expressed as percentage of the Bcl-2/Bax in Veh + Veh group. d) Caspase-3 activation evaluated as the ratio of caspase-3 fragment to procaspase-3 and expressed as percentage of this ration in Veh + Veh group. β-actin was used as an internal control. Data are shown as mean ± SEM (n = 4); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Veh + H_2O_2 group.

of antioxidant enzymes, such as SOD ($p < 0.01$, Fig. 4c), CAT ($p < 0.05$, Fig. 4d), and GPx ($p < 0.01$, Fig. 4e), and increased the GSSG/GSH ratio ($p < 0.01$, Fig. 4f). Pretreatment with 20 μM rosiglitazone restored the parameters to near-normal levels (SOD, $p < 0.05$, Fig. 4c; CAT, $p < 0.05$, Fig. 4d; GPx, $p < 0.05$, Fig. 4e; GSSG/GSH ratio, $p < 0.01$, Fig. 4f). At the same time, treatment with 20 μM rosiglitazone alone had no significant effect on ROS levels, MDA content, antioxidant enzyme activities, or GSSG/GSH ratio in vehicle-treated cells.

PPARγ activation stimulates Nrf2 signaling and expression of downstream antioxidant proteins in H_2O_2-treated cells. Western blot was performed to examine the Nrf2 pathway, which plays a crucial role in the regulation of cytoprotective responses against oxidative stress [21]. Under physiological conditions, Nrf2 binds Keap1, forming a complex that remains in the cytoplasm, thereby maintaining the pathway in an inactive state. Our data showed that H_2O_2 exposure increased the levels of cytoplasmic Nrf2 ($p < 0.01$, Fig. 5, a and c) and Keap1 ($p < 0.05$, Fig. 5, e and f), while markedly decreasing the content nuclear Nrf2 ($p < 0.05$, Fig. 5, b and d) compared to the vehicle group. Pretreatment with 20 μM rosiglitazone significantly reduced cytoplasmic Nrf2 ($p < 0.05$, Fig. 5, a and c) and Keap1 ($p < 0.05$, Fig. 5, e and f) and concomitantly increased nuclear Nrf2 levels ($p < 0.05$, Fig. 5, b and d). Also, expression of the Nrf2 pathway target proteins, such as HO-1 and NAD(P)H:quinone oxidoreductase 1 (NQO1), was assessed [22, 23]. Treatment with H_2O_2 decreased expression of both HO-1 ($p < 0.01$, Fig. 5, e and g) and NQO1 ($p < 0.01$, Fig. 5, e and h), but this effect was reversed by pretreatment with 20 μM rosiglitazone ($p < 0.05$ for HO-1 and $p < 0.05$ for NQO1). Therefore, PPARγ activation rescues the Nrf2 pathway suppressed by H_2O_2 and further upregulates expression of downstream target proteins.

PPARγ-mediated protection against oxidative stress and cell apoptosis is partially abolished by Nrf2 inhibitor in H_2O_2-treated cells. To identify the signal transduction pathway through which PPARγ activation protects against H_2O_2-induced oxidative stress and apoptosis, L02 cells were co-incubated with rosiglitazone and Nrf2 inhibitor ML385 (5 μM) [24]. Coadministration of ML385 and rosiglitazone significantly attenuated the rosiglitazone-induced increase in cell viability ($p < 0.05$, Fig. 6a) and reversed rosiglitazone-mediated suppression of LDH release ($p < 0.05$, Fig. 6b) in H_2O_2-treated cells. Consistent with these observations, co-treatment with ML385 and rosiglitazone partially abolished rosiglitazone-mediated reduction of cell apoptosis ($p < 0.05$, Fig. 6, c and d).

PPARγ-mediated protection against oxidative stress and cell apoptosis is partially abolished by Nrf2 inhibitor in H2O2-treated cells. Rosiglitazone-induced reduction in the levels of ROS ($p < 0.05$, Fig. 7a)

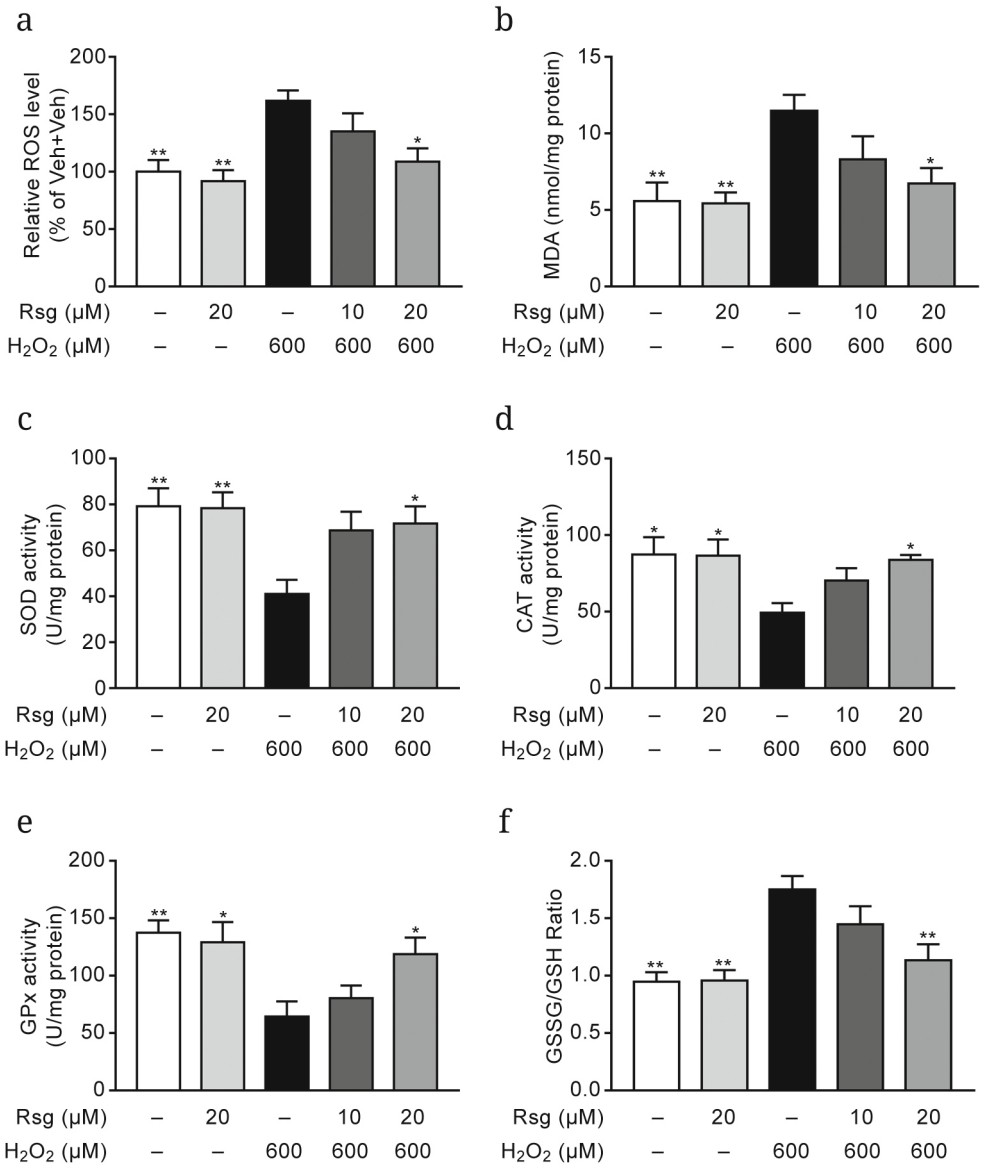

Fig. 4. PPARγ activation alleviates oxidative stress and enhances antioxidant capacity in H_2O_2-treated cells. a) ROS levels in L02 cells expressed as percentage of DCF signal intensity relative to that in the Veh + Veh group. b) MDA levels. c-d) Activities of SOD (c), CAT (d), and GPx (e). f) GSSG/GSH ratio. Data are shown as mean ± SEM (n = 6); * p < 0.05, ** p < 0.01, vs. Veh + H_2O_2 group.

and MDA (p < 0.05, Fig. 7b) was reversed by coadministration of ML385. Moreover, cotreatment with rosiglitazone and ML385 attenuated rosiglitazone-enhanced antioxidant defense in H_2O_2-treated cells, as indicated by the decreased activities of CAT (p < 0.05, Fig. 7d) and GPx (p < 0.05, Fig. 7e) and elevated GSSG/GSH ratio (p < 0.05, Fig. 7f). Although ML385 also reduced rosiglitazone-upregulated SOD activity in H_2O_2-treated cells, the effect was not statistically significant (p > 0.05, Fig. 7c).

Cytoprotective effect of PPARγ activation against H_2O_2-induced injury is associated with the activation of Nrf2 pathway in L02 cells. Inhibition of Nrf2 attenuated rosiglitazone-mediated reduction of cell apoptosis and improvement of antioxidant capacity, prompting us to use Western blotting to examine whether this protective effect was associated with the Nrf2 pathway. Coadministration of ML385 and rosiglitazone elevated cytoplasmic levels of Nrf2 (p < 0.05, Fig. 8, a and c) and Keap1 (p < 0.05, Fig. 8, e and f) downregulated by rosiglitazone, while rosiglitazone-enhanced nuclear Nrf2 level (p < 0.05, Fig. 8, b and d) was diminished. In line with the inhibition of Nrf2 nuclear translocation by ML385, coadministration of ML385 and rosiglitazone downregulated HO-1 expression level (p < 0.05, Fig. 8, e and g) in H_2O_2-treated cells compared to cells pretreated with rosiglitazone alone. Additionally, a trend toward

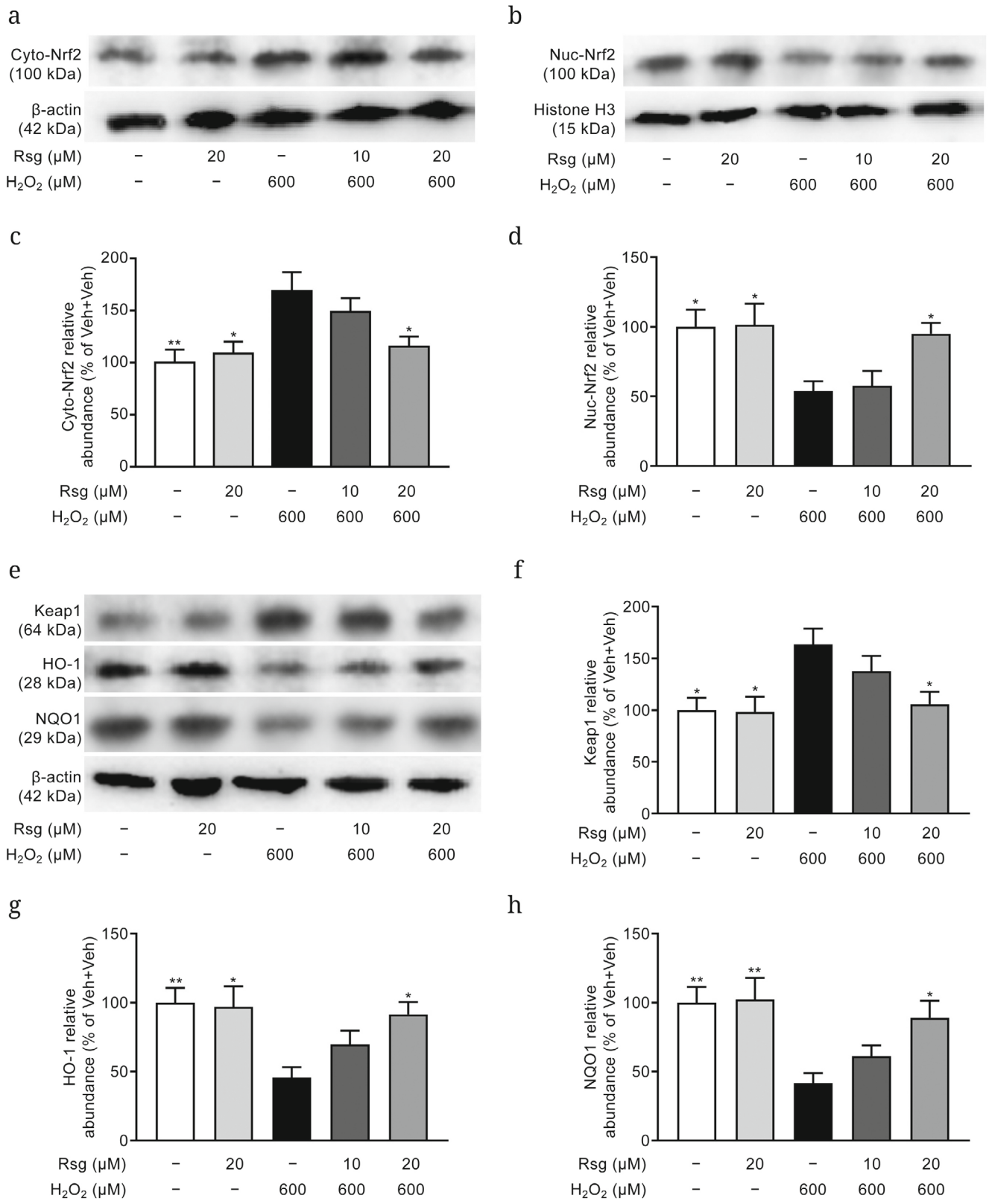

Fig. 5. PPARγ activation stimulates Nrf2 signaling and expression of downstream antioxidant proteins in H_2O_2-treated cells. Protein levels of cytoplasmic Nrf2, nuclear Nrf2, Keap1, HO-1, and NQO1 were detected by Western blotting (a, b, and e). Quantification of cytoplasmic Nrf2 (c), nuclear Nrf2 (d), Keap1 (f), HO-1 (g), and NQO1 (h) is presented as a percentage of the corresponding values in the Veh + Veh group. β-Actin was used as an internal control for cytoplasmic and total protein, and histone H3 was used as an internal control for nuclear protein. Data are shown as mean ± SEM (n = 4); * $p < 0.05$, ** $p < 0.01$, vs. Veh + H_2O_2 group.

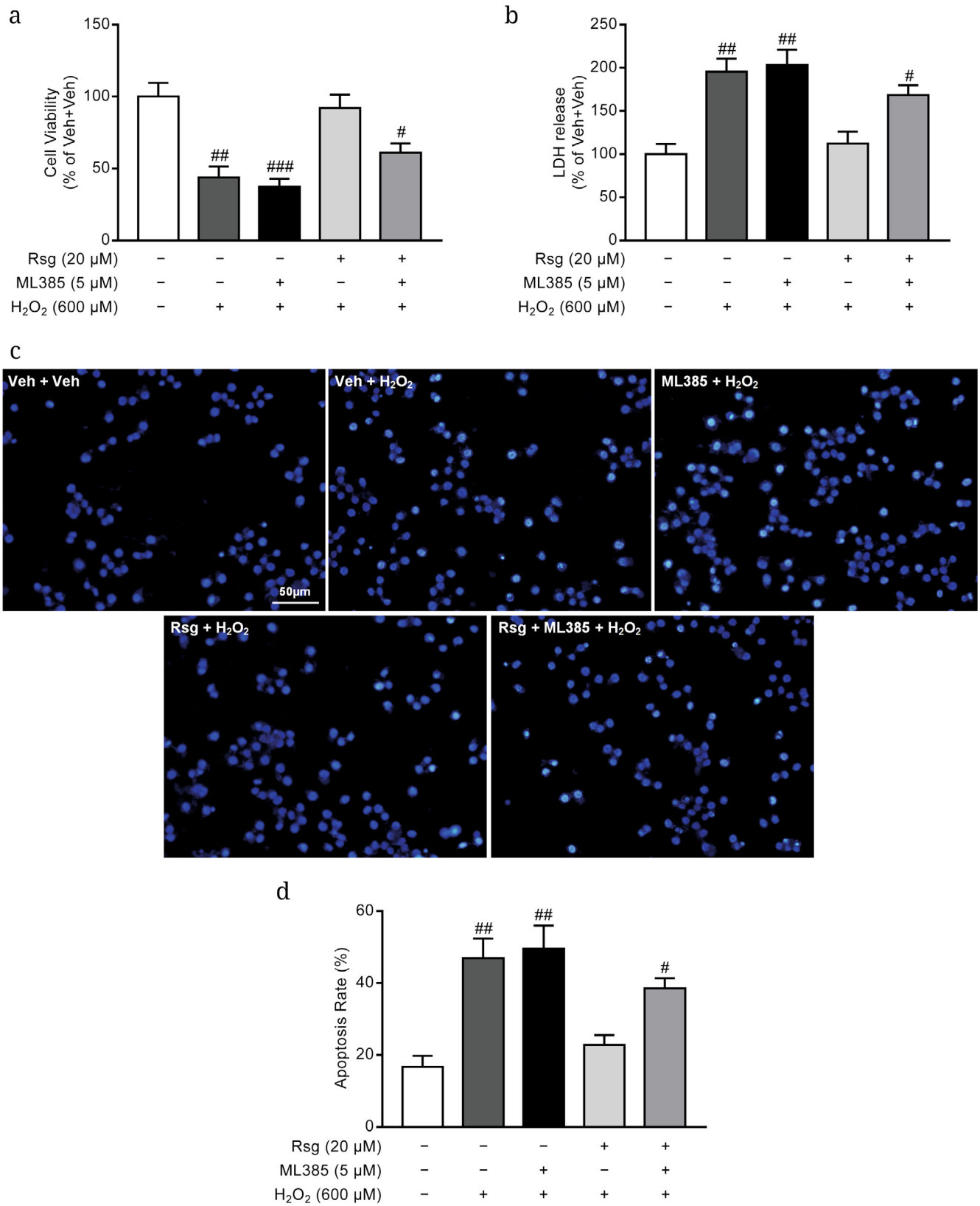

Fig. 6. Nrf2 inhibitor partially reverses the protective effect of PPARγ activation against oxidative stress and cell apoptosis in H_2O_2-treated cells. Viability of L02 cells following indicated treatments assessed by CCK-8 (b) and LDH release (c) assays. c) Representative microphotographs of apoptotic cells stained by Hoechst 33258. d) Cell apoptosis rate in treatment groups. Data are shown as mean ± SEM (for CCK-8 and LDH release assay, $n = 6$; for Hoechst 33258 staining, $n = 4$). # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. Rsg + H_2O_2 group.

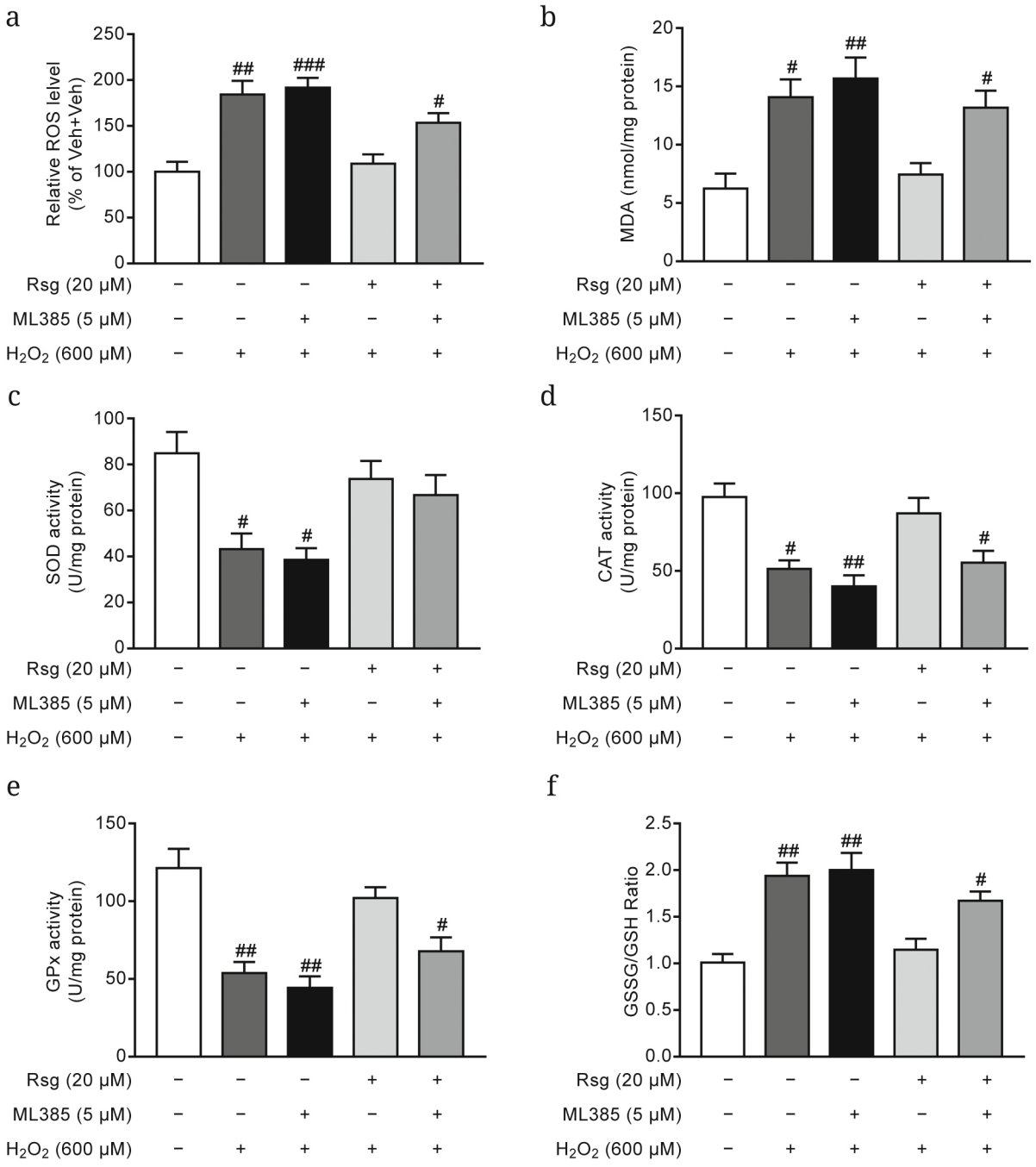

Fig. 7. PPARγ-mediated protection against oxidative stress and cell apoptosis is partially abolished by Nrf2 inhibitor in H_2O_2-treated cells. a) ROS levels in L02 cells expressed as percentage of DCF signal intensity relative to that in the Veh + Veh group. b) MDA levels. c-d) Activities of SOD (c), CAT (d), and GPx (e). f) GSSG/GSH ratio. Data are shown as mean ± SEM (n = 6); # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. Rsg + H_2O_2 group.

reduced NQO1 levels ($p > 0.05$, Fig. 8, e and h) was observed in H_2O_2-induced cells cotreated with ML385 and rosiglitazone, although this effect did not reach statistical significance relative to rosiglitazone administration alone. Collectively, these data indicate that the Nrf2 signaling pathway plays a critical role in the protective effect of PPARγ activation against H_2O_2-induced oxidative stress and cell apoptosis.

DISCUSSION

The present study demonstrated that PPARγ activation attenuates oxidative stress and apoptosis in L02 cells through the Nrf2 signaling pathways. Pretreatment with rosiglitazone at relatively higher concentration significantly enhanced cell viability and suppressed chromatin condensation in H_2O_2-treated

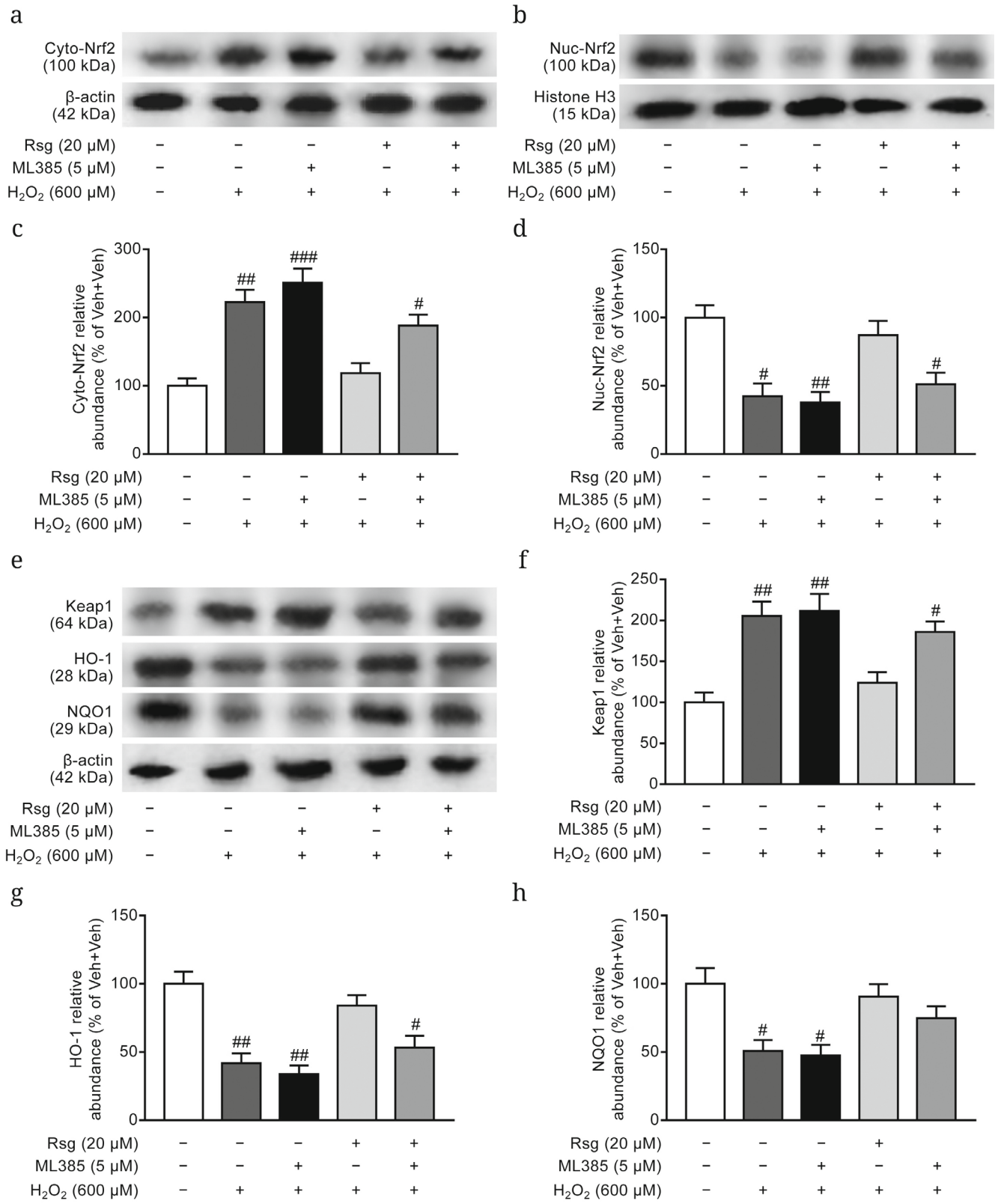

Fig. 8. Cytoprotective effect of PPARγ activation against H_2O_2-induced injury in L02 cells is associated with the activation of Nrf2 pathway. a, b, and e) Protein levels of cytoplasmic Nrf2, nuclear Nrf2, Keap1, HO-1, and NQO1. Quantification of cytoplasmic Nrf2 (c), nuclear Nrf2 (d), Keap1 (f), HO-1 (g), and NQO1 (h) expressed as percentage of the corresponding values in the Veh + Veh group. β-Actin was used as an internal control for cytoplasmic and total protein, and histone H3 was used as an internal control for nuclear protein. Data are shown as mean ± SEM (n = 6); # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. Rsg + H_2O_2 group.

L02 cells. Also, rosiglitazone pretreatment markedly reduced decreased ROS levels and MDA content, while restoring the activity of antioxidant enzymes, such as SOD, CAT, and GPx, indicating a significant mitigation of oxidative stress. Pretreatment with ML385 partially abolished the protective effects of rosiglitazone on cell viability and redox homeostasis in H_2O_2-exposed cells. Collectively, these findings suggest that PPARγ activation protects against oxidative stress and cell apoptosis, an effect that is strongly associated with the Nrf2 pathway activation.

The onset and development of liver diseases may be explained by the development of oxidative stress, a key factor implicated in the pathogenesis of numerous hepatic disorders [25, 26]. ROS play an important role in several cellular functions, such as signal transduction, defense against harmful stimuli, and expression of genes involved in cell proliferation and death. However, excessive ROS accumulation that overwhelms endogenous antioxidant defense capacity disrupts redox homeostasis, leading to oxidative stress and severe cellular damage. H_2O_2 has a long lifetime and could easily convert to the highly reactive and cytotoxic hydroxyl radical [27]. H_2O_2 is widely used to induce the oxidative stress in various cell types [28, 29]. In the present study, exposure of L02 cells to 600 μM H_2O_2 resulted in a reduced cell viability and oxidative stress induction, as evidenced by increased ROS levels, elevated MDA content, and suppression of the antioxidant defense system. Previous studies have reported that rosiglitazone inhibits inflammatory responses and reduces oxidative stress, thereby ameliorating renal tubular injury. Similarly, pioglitazone administration decreased the ROS levels while upregulating nitric oxide synthase phosphorylation and nitric oxide production, protecting aging cerebral arteries against oxidative stress damage [30]. Consistent with previous studies, our data demonstrate that administration of 20 μM rosiglitazone reversed H_2O_2-induced oxidative damage in H_2O_2-treated L02 cells.

ROS are mainly generated in mitochondria; therefore, mitochondria are the primary organelles damaged by excessive ROS exposure. Mitochondrial DNA (mtDNA) is particularly susceptible to oxidative damage due to its unique structural features, lack of histone protection, and close proximity to the mitochondrial electron transport chain, a major site of ROS production [31]. Damage to mtDNA compromises ATP production and further enhances ROS generation. There is a growing body of evidence indicating that excessive mitochondrial ROS accumulation contributes to abnormal mitochondrial fission/fusion and calcium overload, which, in turn, promotes additional ROS production [32]. These pathological processes interact and form a vicious cycle that triggers apoptotic signaling. Alterations in the Bax/Bcl-2 ratio promote the release of cytochrome *c* and trigger a caspase cascade, playing a critical role in mitochondrial dysfunction and oxidative stress-induced apoptosis. As a potent oxidant, H_2O_2 induces the permeability transition pore opening and increases mitochondrial permeability, leading to the caspase-dependent apoptosis in mammalian cells [33]. Here, we observed that H_2O_2-stimulated L02 cells exhibited chromatin condensation (a hallmark of apoptosis), reduced Bcl-2/Bax ratio, and elevated levels of cleaved caspase-3, a key executor of cell apoptosis. Previous studies [19] demonstrated that epigallocatechin-3-gallate alleviates high-fat diet-induced nonalcoholic fatty liver disease by inhibiting apoptosis though the ROS/mitogen-activated protein kinase pathway [19]. Similarly, melatonin attenuates oxidative stress, inhibits apoptosis in hepatocytes, and suppresses autophagy in animal models of acute liver failure [34]. In addition to the antioxidant effect, PPARγ activation significantly reduced H_2O_2-induced cell apoptosis, supporting the idea that antioxidants exhibit protective effects and hold a strong therapeutic potential against hepatic injury.

The transcription factor Nrf2 is a cellular redox sensor expressed in multiple organs. Accumulating evidence indicates that chronic and severe stress can suppress Nrf2 activity and that dysregulation of the Nrf2 pathway is involved in the pathogenesis of several liver diseases [35]. We found that exposure to H_2O_2 elevated the Keap1 level and reduced nuclear translocation of Nrf2, thereby affecting the downstream antioxidant enzymes and reducing the antioxidant capacity of cells. The binding of ligands to PPARγ activates it, leading to the PPARγ translocation to the nucleus and interaction with the retinoid X receptor (RXR) partner protein. The formed complex binds to PPAR response elements (PPREs) located in the promoter regions of PPARγ-regulated genes. Several studies have demonstrated that PPARγ agonists enhance the PPARγ transcriptional activity and upregulate the expression of key antioxidant proteins, such as HO-1, CAT, and GPx, thereby counteracting oxidative stress [36, 37]. Interestingly, a previous study identified several putative PPREs in the promoter region of *Nrf2* gene; however, their function significance remains unclear, suggesting a potential direct regulatory association between PPARγ and Nrf2 [38]. This might partially explain how PPARγ activation promotes Nrf2 translocation to the nucleus, elevates activities of SOD, CAT, and GPx, and upregulates HO-1 and NQO1 expression, enhancing the antioxidant defense. Consequently, rosiglitazone treatment increased cell viability and reduced apoptosis. ML385 inhibited activation of the Nrf2 pathway and attenuated rosiglitazone-induced upregulation of antioxidant enzyme activities and expressions.

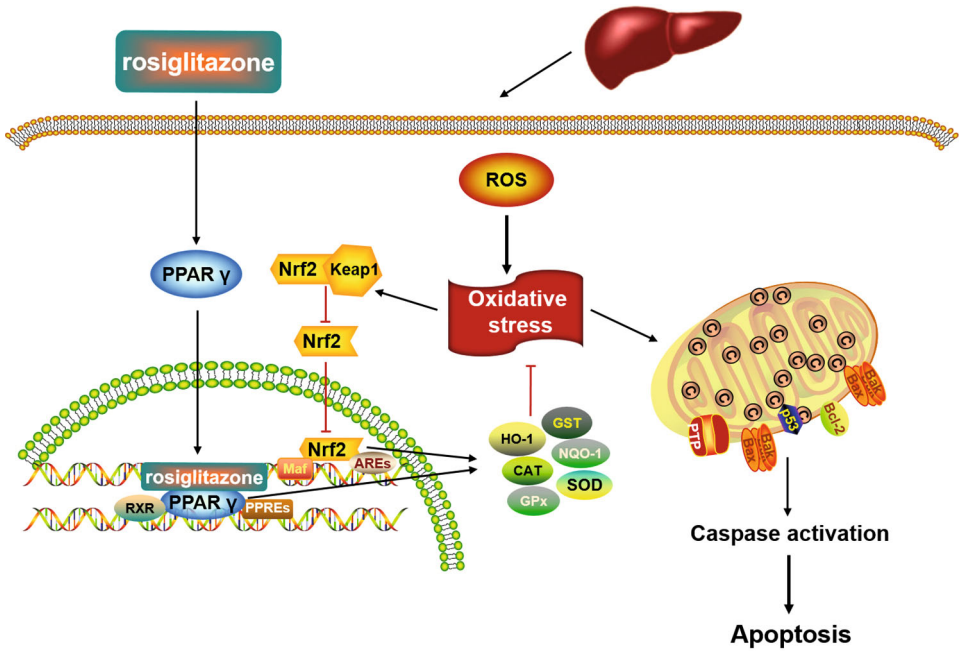

Fig. 9. Proposed mechanism by which rosiglitazone attenuates oxidative stress and apoptosis through activation of the Nrf2 signaling in L02 cells.

Coadministration of rosiglitazone and ML385 weakened the protective effect of rosiglitazone against oxidative stress and cell apoptosis. Intriguingly, several studies have identified antioxidant response elements (AREs) in the PPARγ gene promoter region and demonstrated that increased nuclear translocation of Nrf2 upregulates PPARγ expression [39, 40]. Nrf2 deletion significantly reduced PPARγ expression *in vivo* [41]. We propose an existence of a crosstalk between the Nrf2 and PPARγ pathways that creates a positive feedback loop and promotes expression of downstream antioxidant genes. Further studies are required to confirm the reciprocal regulation between PPARγ and Nrf2 and to clarify the underlying signaling mechanisms.

CONCLUSION

PPARγ activation protects cells against H_2O_2-induced oxidative stress through activation of the Nrf2 pathway, as indicated by increased activities of antioxidant enzymes (CAT, SOD, and GPx), upregulated expression of HO-1 and NQO1, and simultaneous increase in the Bcl-2 level, reduction in the Bax content, and caspase-3 activation (Fig. 9). Collectively, these findings suggest that PPARγ activation holds significant therapeutic potential in prevention and/or treatment of liver disorders.

Abbreviations

CAT	catalase
GPx	glutathione peroxidase
HO-1	heme oxygenase-1
Keap1	Kelch-like ECH-associated protein 1
MDA	malonaldehyde
Nrf2	nuclear factor erythroid 2-related factor 2
NQO1	NAD(P)H:quinone oxidoreductase 1
PPARγ	peroxisome proliferator-activated receptor gamma
PPRE	PPAR response element
ROS	reactive oxygen species
SOD	superoxide dismutase

Contributions

L.Z.W., F.C., Y.G.L., and X.F.W. developed the study concept; L.Z.W., F.C., K.L.Z., and Y.Q.A analyzed the data; F.C., Y.G.L., and X.F.W. acquired the funding; L.Z.W. wrote the article draft; all authors reviewed and edited the manuscript.

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Ethics approval and consent to participate
This work does not contain any studies involving human or animal subjects.

Conflict of interest
The authors of this work declare that they have no conflicts of interest.

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