Tranilast reduces cardiomyocyte injury induced by ischemia-reperfusion via Nrf2/HO-1/NF-κB signaling

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Abstract. Tranilast, a synthetic derivative of a tryptophan metabolite, can be used to treat heart diseases. However, the specific mechanism underlying the effect of tranilast on ischemia-reperfusion (I/R) injury-induced cardiomyocyte apoptosis remains unclear. Therefore, the present study aimed to determine if tranilast could attenuate I/R-induced cardiomyocyte injury. A hypoxia/reoxygenation (H/R) model of H9c2 cardiomyocytes was established to simulate I/R-induced cardiomyocyte injury. The viability, apoptosis, inflammation and oxidative stress in H/R-induced H9c2 cells following treatment with tranilast were evaluated by Cell Counting Kit-8 and TUNEL assay. Commercially available kits were used to detect the levels of inflammatory markers and oxidative stress indicators. In addition, the expression levels of the apoptosis- and nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1)/NF-κB signalling pathway-associated proteins were detected by western blotting. The levels of reactive oxygen species were determined using 2',7'-dichlorofluorescin diacetate assay kit. The viability of H9c2 cells was decreased following induction with H/R. However, treatment with tranilast increased viability while decreasing apoptosis, oxidative stress and inflammatory response in H/R-induced H9c2 cells by activating Nrf2/HO-1/NF-κB signalling. Furthermore, treatment with ML-385, an Nrf2 inhibitor, reversed the effects of tranilast on H/R-induced H9c2 cells. In conclusion, the results of the present study suggested that tranilast could attenuate I/R-induced cardiomyocyte injury via the Nrf2/HO-1/NF-κB signalling pathway.

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Abbreviations: I/R, ischemia-reperfusion; H/R, hypoxia/reoxygenation; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; MIRI, myocardial ischemia-reperfusion injury; TRPV2, transient receptor potential vanilloid type 2; PARP, poly(ADP-ribose) polymerase; ROS, reactive oxygen species; MDA, malondialdehyde; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase

Key words: tranilast, ischemia-reperfusion, cardiomyocyte injury, apoptosis, nuclear factor erythroid 2-related factor 2/heme oxygenase-1/NF-κB signaling

Introduction

Ischemic heart disease is the most common cardiovascular disease (1). With the ageing of the population, the incidence of ischemic heart disease is increasing every year in China (2). During 2007-2008 in an urban community of Romania, it was estimated that the incidence of ischemic heart disease was considerably higher in elderly women than in men, with an increase from 55.7 to 65.1% in women and from 47.4 to 60.8% in men (3). Intravenous thrombolysis, percutaneous coronary intervention and coronary artery bypass grafting have improved the long-term prognosis of patients with acute myocardial infarction. However, myocardial ischemia-reperfusion (I/R) injury (MIRI) caused by the aforementioned treatments is a serious complication (4). MIRI refers to the pathological process of progressive aggravation of myocardial tissue damage following reperfusion of interrupted coronary blood flow, which can lead to severe arrhythmia and sudden death (5). However, to the best of our knowledge, there is no effective MIRI prevention strategy in clinical practice.

Tranilast, with the chemical formula N-(3',4'-dimethoxycinnamoyl) anthranilic acid, is an analogue of a metabolite of tryptophan (Fig. 1A), which is used to treat allergic disease, such as bronchial asthma, allergic rhinitis, dermatitis and conjunctivitis (6). A previous study demonstrated that long-term use of tranilast could inhibit allergic reactions in patients with bronchial asthma (7). The anticancer effects of tranilast via different mechanisms have been recently demonstrated (8). Additionally, a study suggested that tranilast could exert a significant therapeutic effect on heart disease (9). Moreover, recent studies showed that tranilast prevents doxorubicin-induced myocardial hypertrophy (10) and decreases myocardial fibrosis in diabetic rats via the TGF-β/SMAD signalling pathway (11). Furthermore, tranilast attenuates cerebral IR injury in rats via NF-κB and peroxisome proliferator-activated receptor-mediated inflammatory responses (12) and improves myocardial infarction in mice by inhibiting nucleotide binding oligomerization domain-like

receptor family pyrin domain containing-3 inflammasome and affecting the phenotype of macrophages (13). However, to the best of our knowledge, the effect of tranilast on MIRI remains unclear.

Tranilast is a selective transient receptor potential vanilloid type 2 (TRPV2) inhibitor. A study demonstrated that TRPV2 downregulation protects against MIRI (14), while TRPV2 knockdown partially reverses the effect of H_2O_2 on nuclear factor erythroid 2-related factor 2 (Nrf2) inhibition in hepatocellular carcinoma (15). In addition, activation of the Nrf2/heme oxygenase-1 (HO-1)/NF-κB signalling pathway protects the heart during MIRI (16).

It was hypothesized that tranilast decreases I/R-induced cardiomyocyte injury via Nrf2/HO-1/NF-κB signalling. Therefore, the present study aimed to investigate if tranilast attenuates I/R-induced cardiomyocyte injury by using a hypoxia/reoxygenation (H/R) model in H9c2 cardiomyocytes to trigger I/R-stimulated cardiomyocyte injury.

Materials and methods

Cell culture and establishment of the in vitro H/R model. H9c2 cardiomyocytes were obtained from the American Type Culture Collection (cat. no. CRL-1446) and cultured in DMEM supplemented with 10% FBS (Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin solution at 37˚C in an incubator with 5% CO_2. Cells were treated with of tranilast (25, 50 and 100 μM; cat. no. S1439; Selleck Chemicals) for 24 h, as previously described (9,17). To establish the H/R *in vitro* model, 3 ml H9c2 cell suspension in 10% FBS-DMEM at a density of $1x10^6$ cells/ml was plated onto a 60-mm dish and cells were cultured in an incubator with 5% CO_2 at 37˚C. When 90% confluency was reached, cells were exposed to H/R. Briefly, H9c2 cardiomyocytes were incubated in serum- and glucose-free DMEM (Thermo Fisher Scientific, Inc.) at 37˚C in a hypoxic incubator with 5% CO_2 and 95% N_2 for 2 h to induce hypoxia. For reoxygenation, cells were transferred to normal culture conditions with routine culture medium in a normal oxygen environment (74% N_2, 5% CO_2 and 21% O_2) at 37˚C for 24 h (15). H9c2 cells cultured in complete culture medium were referred to the Control group. H9c2 cells were pre-treated with 25, 50 or 100 μM tranilast for 24 h at 37˚C prior to being subjected to hypoxia for 2 h and reoxygenation for 4 h. Furthermore, to verify if tranilast exerts its effect on I/R injury via the Nrf2/HO-1/NF-κB signalling pathway, H9c2 cells were pre-treated with 20 μM ML-385, an Nrf2 inhibitor, for 1 h at 37˚C prior to treatment with tranilast for 24 h and exposure to hypoxic conditions.

Cell Counting Kit-8 (CCK-8) assay. H9c2 cells were seeded into a 96-well plate at a density of $5x10^3$ cells/well and incubated for 12 h at 37˚C. Cells were treated with 25, 50 or 100 μM tranilast with the absence or presence of H/R exposure and a CCK-8 assay (Beyotime Institute of Biotechnology) was then used to assess cell viability. Briefly, each well of the 96-well plate was supplemented with 10 μl CCK-8 reagent, followed by incubation in a humidified incubator with 5% CO_2 at 37˚C for 2 h. Finally, absorbance of each well was detected at a wavelength of 450 nm using the Model 680 Microplate Reader (Bio-Rad Laboratories, Inc.).

TUNEL assay. H9c2 cardiomyocytes were fixed in xylene for 15 min at room temperature, blocked with 5% goat serum (Thermo Fisher Scientific, Inc.) for 30 min at room temperature and incubated with antibody provided with the TUNEL kit (cat no. KA4159; Abnova GmbH) at 4˚C overnight. Slides were incubated with a TUNEL reaction mixture for 1 h at 37˚C in the dark followed by treatment with 0.05% DAPI for 10 min in the dark at room temperature. Apoptotic cells were observed under a fluorescence microscope (magnification, x200; Olympus Corporation) from five random fields of views and were then quantified using the Image-Pro Plus 6.0 software (Media Cybernetics).

Western blot analysis. H9c2 cardiomyocytes from each group were collected and total protein was extracted after cell lysis in RIPA buffer (Protech Technology Enterprise Co., Ltd.) at 4˚C for 20 min, followed by centrifugation at 8,798 x g for 10 min at 4˚C. Protein concentration was measured using a BCA kit (cat. no. A045-4-2; Nanjing Jiancheng Bioengineering Institute). Total protein extract from each group (20 μg/lane) was separated by SDS-PAGE on a 10% gel and transferred onto PVDF membranes (EMD Millipore). Following blocking with 5% skimmed milk for 2 h at room temperature, membranes were incubated with the following primary antibodies at 4˚C overnight: Anti-B-cell lymphoma 2 (Bcl-2; cat. no. ab196495; 1:1,000); anti-Bcl-2-associated X protein (Bax; cat. no. ab32503; 1:1,000); anti-poly(ADP-ribose) polymerase (PARP; cat. no. ab191217; 1:1,000); anti-cleaved PARP (cat. no. ab32064; 1:1,000); anti-Nrf2 (cat. no. ab92946; 1:1,000) and anti-HO-1 (cat. no. ab189491; 1:2,000; all Abcam); anti-phosphorylated (p)-NF-κB (cat. no. 3033; 1:1,000) and anti-NF-κB (cat. no. 8242; 1:1,000; both Cell Signaling Technology, Inc.) and β-actin (cat. no. ab8227; 1:1,000; Abcam). The membranes were incubated with corresponding horseradish peroxidase-conjugated secondary antibody (cat. no. ab6721; 1:2,000; Abcam) for 1 h at room temperature. The protein bands were visualized using ECL Western Blotting Substrate (Pierce; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Densitometry analysis was performed using ImageJ 1.52a software (National Institutes of Health) with β-actin as the loading control.

Detection of oxidative stress indicators. Following treatment with 25, 50 or 100 μM tranilast prior to H/R exposure, H9c2 cardiomyocytes were cultured in the presence of 10 μM dichloro-dihydro-fluorescein diacetate solution at 37˚C for 20 min in the dark, according to the manufacturer's instructions [reactive oxygen species (ROS) assay kit; cat. no. S0033S; Beyotime Institute of Biotechnology]. The production of intracellular ROS was detected using a fluorescence microscope (magnification, x400; Olympus Corporation) and results were analyzed using ImageJ 1.8.0 software (National Institutes of Health). Lysates were obtained following centrifugation at 10,000 x g at 4˚C for 12 min. The levels of malondialdehyde (MDA) and activity of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were detected using corresponding kits according to the manufacturer's instructions (cat. nos. A003-1-2, A001-3-2 and A006-2-1, respectively; Nanjing Jiancheng Bioengineering Institute) at wavelengths of 532, 450 and 405 nm, respectively.

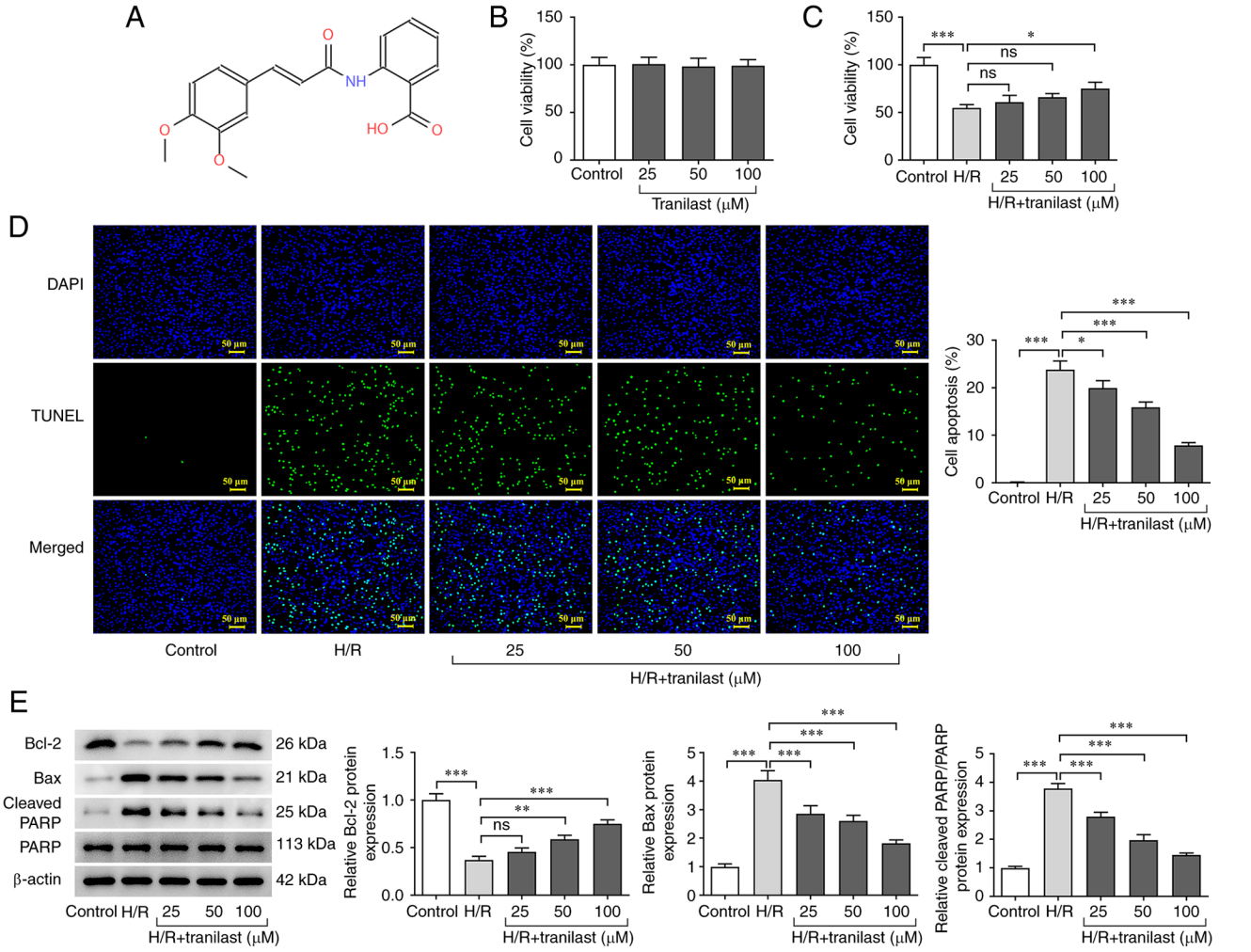

Figure 1. Tranilast promotes H/R-induced H9c2 cell viability. (A) Chemical structure of tranilast. Viability of (B) H9c2 and (C) H/R-induced H9c2 cells treated with tranilast was detected by Cell Counting Kit-8 assay. (D) Apoptosis of H/R-induced H9c2 cells treated with tranilast was analyzed by TUNEL assay (scale bar, 50 μm). (E) Expression of apoptosis-associated proteins in H/R-induced H9c2 cells treated with tranilast was detected by western blotting. *P<0.05, **P<0.01 and ***P<0.001. ns, non-significant; H/R, hypoxia/reoxygenation; PARP, poly(ADP-ribose) polymerase.

ELISA. The cell culture supernatant from each group of H9c2 cardiomyocytes was collected via centrifugation at 1,000 x g for 5 min at 4˚C. The secretion of TNF-α, IL-6 and IL-8 was detected using the TNF-α (cat. no. PT516; Beyotime Institute of Biotechnology), IL-6 (cat. no. PI328; Beyotime Institute of Biotechnology) and IL-8 (cat. no. SEKR-0071; Beijing Solarbio Science & Technology Co., Ltd.) ELISA kits according to the manufacturer's instructions, respectively.

Statistical analysis. All statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, Inc.; Dotmatics). The differences between multiple groups were assessed using one-way ANOVA followed by Tukey's post hoc test. All data are expressed as the mean ± standard deviation of three independent experimental repeats and exhibited normal distribution. P<0.05 was considered to indicate a statistically significant difference.

Results

Tranilast promotes H/R-induced H9c2 cell viability. To investigate if different concentrations of tranilast affect viability of H9c2 cells or H/R-induced H9c2 cells, a CCK-8 assay was performed. In addition, the apoptosis rate of H/R-induced H9c2 cells in the presence or absence of tranilast was determined using TUNEL assay and western blot analysis. Following exposure of H9c2 cells to tranilast, viability was not significantly affected (Fig. 1B). However, cell viability (Fig. 1C) was decreased and apoptosis (Fig. 1D) was increased in H9c2 cells subjected to H/R. The aforementioned effects were reversed following treatment with tranilast and this effect was significant only at 100 μM of tranilast. The expression levels of apoptosis-related proteins Bcl-2 were decreased and Bax/cleaved PARP were increased in H/R-induced H9c2 cells; these effects were also reversed by tranilast treatment (Fig. 1E). These findings indicated that tranilast increased cell viability and decreased the apoptotic rate in H/R-induced H9c2 cells.

Tranilast attenuates oxidative stress and inflammatory response in H/R-induced H9c2 cells. Oxidative stress and inflammatory response in H/R-induced H9c2 cells in the presence or absence of tranilast were assessed. The results showed that the levels of ROS and MDA were increased, while activity of GSH-Px and SOD decreased following H/R injury.

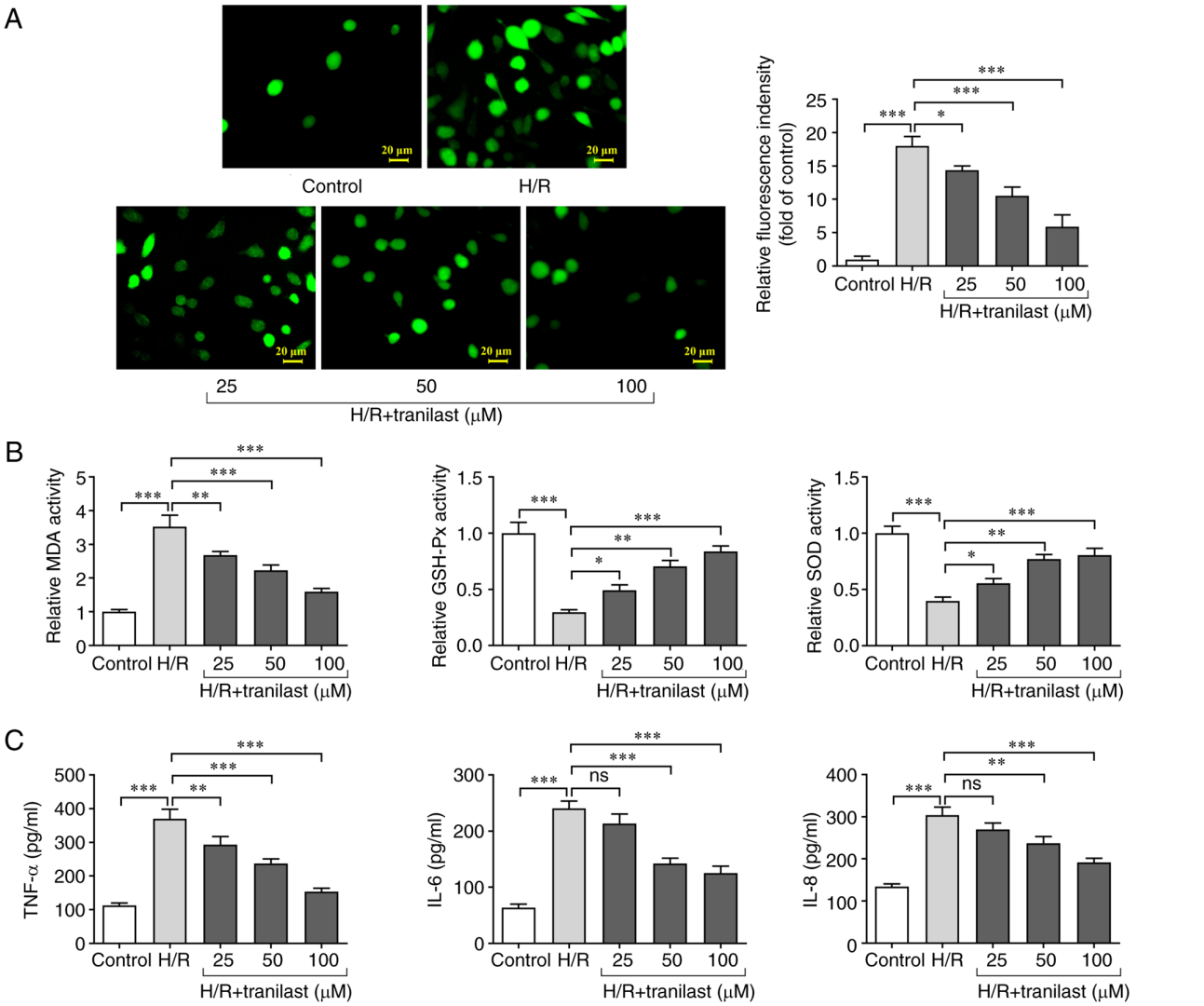

Figure 2. Tranilast decreases oxidative stress and inflammatory response in H/R-induced H9c2 cells. (A) Reactive oxygen species in H/R-induced H9c2 cells treated with tranilast were detected by dichloro-dihydro-fluorescein diacetate assay (scale bar, 20 μm). (B) MDA levels and SOD and GSH-Px activity in H/R-induced H9c2 cells treated with tranilast were evaluated by commercial kits. (C) Secretion levels of TNF-α, IL-6 and IL-8 inflammatory factors in H/R-induced H9c2 cells treated with tranilast were detected by ELISA. *P<0.05, **P<0.01 and ***P<0.001. ns, non-significant; H/R, hypoxia/reoxygenation; MDA, malondialdehyde; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase.

However, treatment of H/R-induced H9c2 cells with tranilast decreased ROS and MDA levels (Fig. 2A) whereas it increased activity of GSH-Px and SOD (Fig. 2B). Furthermore, ELISA revealed that the secretion of TNF-α, IL-6 and IL-8 increased in H/R-induced H9c2 cells while these effects were reversed by tranilast pre-treatment and no significant changes were observed in IL-6 and IL-8 levels at 25 μM of tranilast (Fig. 2C). These findings demonstrated that the H/R-mediated increase in oxidative stress and inflammatory response in H9c2 cells was suppressed by tranilast.

Tranilast activates Nrf2/HO-1/NF-κB signalling in H/R-induced H9c2 cells. Western blot analysis was performed to investigate the role of Nrf2/HO-1/NF-κB signalling in the protective effects of tranilast on H/R-induced H9c2 cells. Nrf2 and HO-1 were upregulated while p-NF-κB was downregulated in H/R-induced H9c2 cells. However, the effect of H/R on Nrf2/HO-1/NF-κB signalling was reversed in a dose-dependent manner following pre-treatment with 25-100 μM tranilast (Fig. 3). These results indicated that tranilast treatment reversed the effects of H/R exposure on Nrf2/HO-1/NF-κB signalling in H9c2 cells.

Tranilast enhances viability of H/R-induced H9c2 cells via Nrf2/HO-1/NF-κB signalling. To investigate the role of Nrf2/HO-1/NF-κB signalling in the promoting effect of tranilast on H/R-induced H9c2 cell viability, cells were co-treated with ML-385, an Nrf2 inhibitor. Viability and apoptosis of H/R-induced H9c2 cells co-treated with tranilast and ML-385 were assessed by CCK-8 and TUNEL assay and western blot analysis. To investigate the effect of tranilast on H/R-induced H9c2 cells, a concentration of 100 μM tranilast was selected for subsequent experiments as 100 μM tranilast exhibited the greatest effect. Pre-treatment with ML-385 of tranilast-treated H/R-induced H9c2 cells decreased viability (Fig. 4A) and increased apoptosis (Fig. 4B and C). Pre-treatment with ML-385 downregulated Bcl-2 and upregulated both Bax and cleaved PARP in tranilast-treated H/R-exposed H9c2

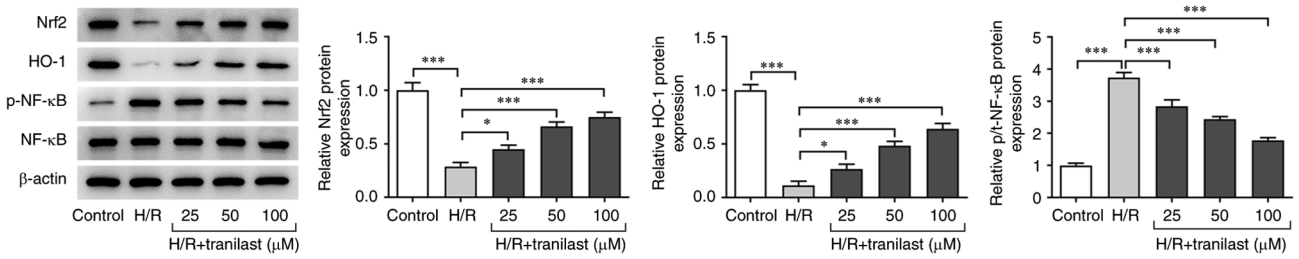

Figure 3. Tranilast activates Nrf2/HO-1/NF-κB signalling in H/R-induced H9c2 cells. Expression of Nrf2/HO-1/NF-κB signalling-associated proteins in H/R-induced H9c2 cells was detected by western blotting. *P<0.05 and ***P<0.001. Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; H/R, hypoxia/reoxygenation; p, phosphorylated.

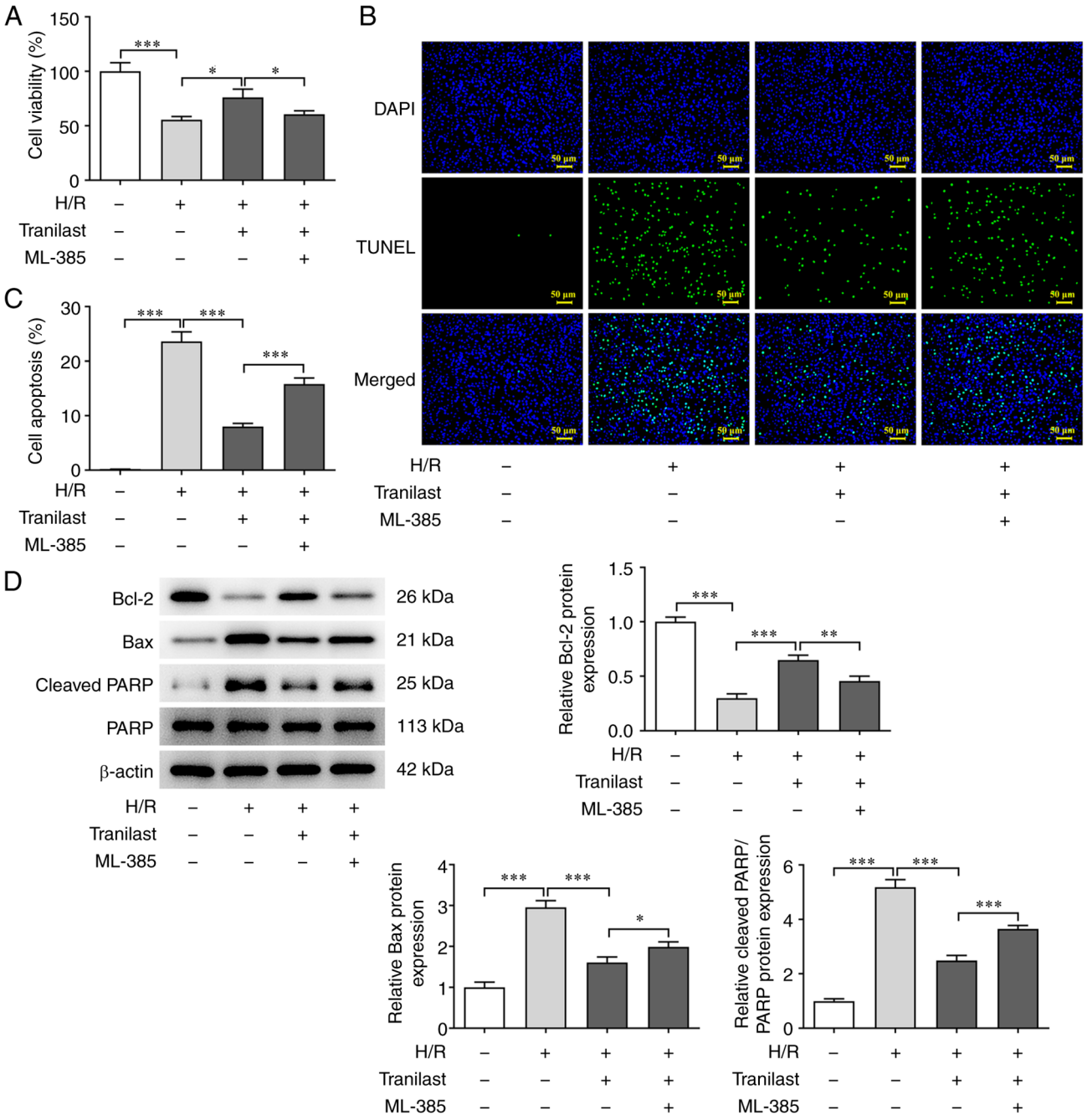

Figure 4. Tranilast enhances H/R-induced H9c2 cell viability via nuclear factor erythroid 2-related factor 2/heme oxygenase-1/NF-κB signalling. (A) Viability of H/R-induced H9c2 cells pre-treated with tranilast and ML-385 was detected by Cell Counting Kit-8 assay. (B) Apoptosis of H/R-induced H9c2 cells pre-treated with tranilast and ML-385 was analyzed by TUNEL assay (scale bar, 50 μm) and (C) quantification. (D) Expression levels of apoptosis-associated proteins in H/R-induced H9c2 cells pre-treated with tranilast and ML-385 were measured by western blotting. *P<0.05, **P<0.01 and ***P<0.001. H/R, hypoxia/reoxygenation; PARP, poly(ADP-ribose) polymerase.

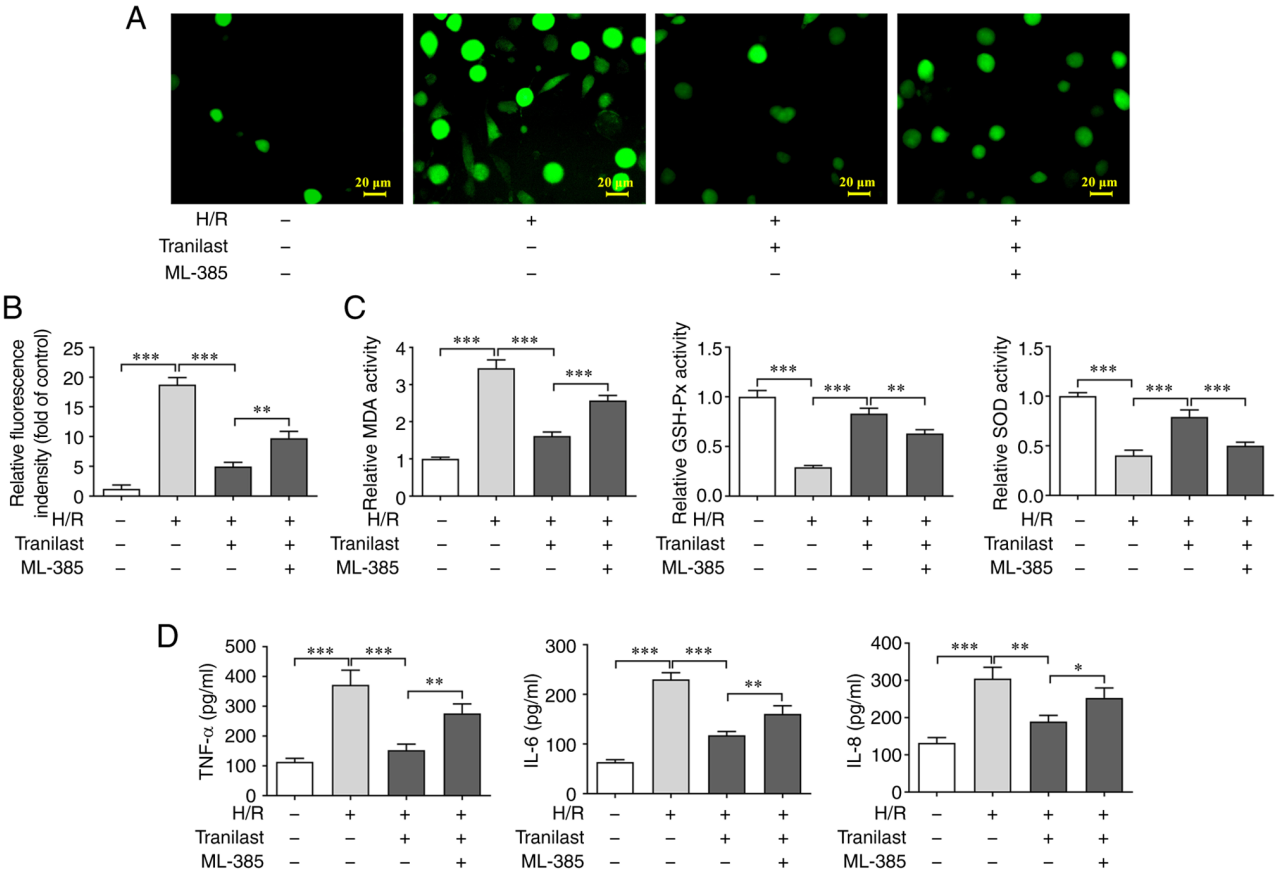

Figure 5. Tranilast decreases H/R-induced oxidative stress and inflammatory response in H9c2 cells via nuclear factor erythroid 2-related factor 2/heme oxygenase-1 signalling. (A) Reactive oxygen species levels in H/R-induced H9c2 cells pre-treated with tranilast and ML-385 were detected by dichloro-dihydro-fluorescein diacetate assay (scale bar, 20 μm) and (B) quantification. (C) MDA levels and SOD and GSH-Px activity in H/R-induced H9c2 cells treated with tranilast and ML-385 were evaluated using commercial kits. (D) Secretion of TNF-α, IL-6 and IL-8 in H/R-induced H9c2 cells treated with tranilast and ML-385 were detected by ELISA. *P<0.05, **P<0.01 and ***P<0.001. H/R, hypoxia/reoxygenation; MDA, malondialdehyde; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase.

cells (Fig. 4D). Overall, ML-385 suppressed Nrf2 expression, thus suggesting that Nrf2 interference inhibited the promotive effect of tranilast on H/R-induced H9c2 cell viability.

Tranilast decreases H/R-induced oxidative stress and inflammatory response in H9c2 cells via Nrf2/HO-1 signalling. To investigate the role of Nrf2/HO-1/NF-κB signalling in the inhibitory effect of tranilast on oxidative stress and inflammatory response, H/R-induced H9c2 cells were pre-treated with ML-385. Oxidative stress and inflammatory response were evaluated using assay kits. The levels of ROS and MDA (Fig. 5A and B) were increased, while activity of GSH-Px and SOD (Fig. 5C) was decreased in H/R-induced H9c2 cells pre-treated with ML-385 prior to tranilast treatment. Additionally, the secretion of TNF-α, IL-6 and IL-8 were increased in H/R-induced H9c2 cells pre-treated with ML-385 prior to tranilast treatment (Fig. 5D). These findings demonstrated that tranilast exerted the inhibitory effect on oxidative stress and inflammatory response in H/R-induced H9c2 cells via activating Nrf2/HO-1 signalling.

Discussion

Tranilast is a fatty cell membrane stabilizer, commonly used in clinical trials and practice as an antiallergic agent (6,7). Tranilast exhibits antifibrotic effects in the myocardium (18) and inhibits inflammatory responses (19-21) and oxidative stress (22). The results of the present study indicated that tranilast decreased secretion levels of IL-6, TNF-α and IL-8 in H/R-induced H9c2 cells. Inflammatory response serve a critical role in MIRI (23). In addition to the release of cytokines, such as TNF-α and IL-6, inflammation is associated with neutrophilic granulocyte-activating cytokines and adhesion molecules, as well as vascular endothelial cell injury (24). Here, treatment of H/R-induced H9c2 cells with tranilast increased activity of GSH-Px and SOD while decreasing levels of MDA. Oxidative stress is also associated with MIRI by promoting ROS production and exacerbating myocardial injury (24). Excessive ROS react with lipids to produce MDA (25). In addition, ROS overproduction is eliminated by endogenous antioxidants, leading to depletion of cell storage and cell structure damage (26). SOD is an important oxygen-free scavenger in the human body based on superoxide anion-free radical (O_2^-). SOD catalyzes its disproportionation into H_2O_2, which is reduced to water through a reaction with glutathione under the catalysis of GSH-Px (27). When cells are exposed to high levels of ROS, they trigger inflammatory cascades and expression of adhesion molecules, thus promoting pro-inflammatory responses characterized by increased levels of TNF-α, IL-1β, IL-6 and IL-8 (28). Additionally, adhesion molecules,

such as vascular cell adhesion molecule-1 and intercellular adhesion molecule-1, are upregulated, thus inducing oxidative stress (29). The present study demonstrated that inflammation and oxidative stress were aggravated in H/R-induced H9c2 cells, accompanied by increased levels of inflammatory factors TNF-α, IL-6 and IL-8, enhanced levels of ROS and MDA, and decreased activities of GSH-Px and SOD.

Nrf2/HO-1 signalling is involved in antioxidant and anti-inflammatory processes, decreased mitochondrial damage and regulation of Ca^{2+} influx and cell death, ultimately affecting the outcome of several diseases, such as lung diseases, cardiovascular disease and neurological disorders and so on (30). A previous study demonstrated that the Nrf2/HO-1 axis is involved in resveratrol-induced inflammation and oxidative stress in rats with MIRI via downregulating the inflammatory factor myeloperoxidase, decreasing MDA content and enhancing SOD and GSH-Px levels (31). Additionally, Konrad *et al* (32) revealed that HO-1 activation attenuates cytoskeletal actin remodelling and the migration of chemokine C-X-C motif ligand 1-related polymorphonuclear leukocytes into the alveolar septa and decreases microvascular endothelial permeability in a conditional HO-1 knockout [flanked by loxP (FLOX/FLOX)] pneumonia mouse model compared with wild-type mice. The same study also showed that HO-1 knockout stabilizes lung barrier function, thus exerting anti-inflammatory effects. In terms of oxidative stress, a study showed that the levels of TNF-α and IL-1β in hippocampus tissues are significantly lower in the rats that received Nrf2 activator sulforaphane compared with the rats that received treatment with advanced glycation end products, while activity of SOD, GSH-Px and catalase was enhanced (33). The aforementioned studies demonstrated that the Nrf2/HO-1 axis exhibits both anti-inflammatory and antioxidant activity. A previous study also demonstrated that MIRI activates NF-κB to promote its translocation into the nucleus, where it serves as a specific DNA target sequence and transcriptionally regulates release of inflammatory factors, such as IL-1, IL-6, TNF-α and IL-8, thus promoting inflammatory responses and aggravating myocardial tissue injury (34). In the present study, Nrf2/HO-1 signalling was suppressed and NF-κB was activated in H/R-induced H9c2 cells. Furthermore, Nrf2/HO-1/NF-κB signalling inactivation decreased viability and promoted apoptosis, inflammation and oxidative stress of H/R-induced H9c2 cells treated with tranilast.

The present study has limitations. Firstly, experiments were only performed on a single cell line. Therefore, further experiments on more cell lines and animal models should be performed in future. Secondly, the effect of tranilast on treating I/R-induced cardiomyocyte injury in clinical practice was not investigated.

In conclusion, the present study demonstrated that tranilast improved viability and alleviated apoptosis, inflammation and oxidative stress of H/R-induced H9c2 cells by activating Nrf2/HO-1/NF-κB signaling. The aforementioned effects were suppressed following treatment with the Nrf2 inhibitor ML-385.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WW and QS designed the study, performed the experiments and revised the manuscript. WW analyzed the data. QS interpreted the data and drafted the manuscript. All authors have read and approved the final manuscript. WW and QS confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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