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Xinnaotongluo liquid protects H9c2 cells against hypoxic damage through IRF2/HIF-1α-mediated oxidation, inflammation, and apoptosis

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Summary. Myocardial ischemia is the primary reason for ischemic heart disease. Xinnaotongluo liquid has been reported to have an improving effect on cardiovascular diseases. In our study, we detected the effects of Xinnaotongluo liquid on H9c2 cell oxidation, inflammation, and apoptosis induced by hypoxia stimulation. H9c2 cells were exposed to hypoxia and/or Xinnaotongluo liquid stimulation. Cell viability, oxidation, inflammation, and apoptosis, along with HIF-1α expression were measured. Subsequently, si-HIF-1α was transfected into H9c2 cells to detect whether HIF-1α depletion was involved in the effect of Xinnaotongluo liquid on H9c2 cells stimulated by hypoxia. Then, the regulatory effect of IRF2 on HIF-1α was detected. Hypoxia exposure induced H9c2 cell viability reduction, oxidation, inflammation, and apoptosis. Xinnaotongluo liquid alleviated the H9c2 cell viability reduction, oxidation, inflammation, and apoptosis induced by hypoxia. HIF-1α was activated in hypoxia-exposed H9c2 cells, and the knockdown of HIF-1α strengthened the effects of Xinnaotongluo liquid on hypoxia-exposed H9c2 cells. Additionally, HIF-1α was transcriptionally regulated by IRF2, and IRF2 was associated with the upregulation of HIF-1α in hypoxia-exposed H9c2 cells. Xinnaotongluo liquid alleviated H9c2 cell apoptosis and inflammation induced by hypoxia, which might be achieved by regulating IRF2/HIF-1α expression.

Key words: HIF-1α, H9c2 cells, Inflammation, IRF2, Xinnaotongluo liquid

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Introduction

Myocardial ischemia is one of the major causes of death and disability in the world, seriously endangering human health (Pagliaro et al., 2020). At present, reperfusion therapy is the most effective method to treat myocardial ischemia (Bhaskar et al., 2018). However, although reperfusion therapy can save the ischemic myocardium, the restoration of ischemia tissue perfusion can lead to changes in the ultrastructure, function, metabolism, and other aspects of myocardial cells, aggravate the existing damage of myocardial ischemia, or cause new damage to myocardial cells, leading to myocardial ischemia-reperfusion injury (Ibáñez et al., 2015). Although the understanding of the pathological mechanism of myocardial ischemia is deepening, and relevant drugs have been developed for this disease both domestically and internationally, an ideal drug or method has not yet been found to achieve recognized, breakthrough therapeutic effects in the treatment of myocardial ischemia. To break through this "bottleneck" in treatment and research, some researchers have turned their attention to traditional Chinese medicine (TCM), which has a history of thousands of years, and began to seek its help (Sun et al., 2015).

With the internationalization and modernization of TCM, in-depth research and exploration of the mechanism of TCM in treating this disease through modern medicine has become a hot topic of concern and research for scholars. TCM formulas, such as salvia miltiorrhiza, panax notoginseng, and borneol, can produce compound salvia miltiorrhiza dripping pills with good therapeutic effects in coronary heart disease, palpitations, shortness of breath, and angina pectoris through certain manufacturing processes (Chen et al., 2018b). Xinnaotongluo liquid is an empirical prescription originally summarized by Professor Li Yan, a famous TCM doctor in Heilongjiang Province, based on the characteristics of ischemic cerebrovascular

disease, which is characterized by deficiency of origin and excess of essence. It is mainly composed of astragalus, ginseng, angelica, chuanxiong, salvia miltiorrhiza, corydalis yanhusuo, safflower, trichosanthes, pinellia ternata, red peony root, fructus aurantii, earthworm, and licorice root. The clinical effect is excellent but the specific targets and molecular mechanisms still need further exploration.

Hypoxia-inducible factor-α (HIF-α) is an important factor in mediating the adaptive response to hypoxia. The increased expression of HIF-1α is one of the first adaptations of human myocardium to ischemia (Lee et al., 2000; Semenza, 2000). Published articles reported that HIF-1α overexpression could promote angiogenesis, decrease infarct size, and improve heart function (Tang et al., 2011; Peng et al., 2015). Our preliminary experiments demonstrated that high expression of HIF-1α was inhibited by Xinnaotongluo liquid under hypoxia conditions. However, whether HIF-1α can be used as a target of Xinnaotongluo liquid still needs further exploration.

Interferon regulatory factors (IRFs) are multifunctional transcription factors that play an important regulatory role in the interferon signaling pathway and are involved in host immune response, cell differentiation, and immune regulation (Yanai et al., 2012). IRF2, as a member of the IRF family of transcription factors, could transcriptionally induce direct target genes (Tamura et al., 2008). By searching the website, IRF2 was identified as a transcription regulator of HIF-1α, which should be further explored in this study.

Materials and methods

Cell culture and treatment

H9c2 cardiomyocytes cultured in Dulbecco's Modified Eagle Medium (DMEM) including 10% fetal bovine serum (FBS) in an incubator with 5% CO_2 at 37°C. The hypoxia/reoxygenation (H/R) model was performed as previously described (Lu et al., 2022). H9c2 cells were exposed to hypoxic conditions supplemented with 1% O_2, 5% CO_2, and 94% N_2 at 37°C for 6h and then subjected to reoxygenation under normoxic conditions equilibrated with 95% air and 5% CO2 at 37°C for 6h.

Xinnaotongluo liquid was purchased from the Xianyang Buchang Pharmaceutical Co., LTD., (Xi'an, China) and dissolved in saline solution to a concentration of 3, 6, 9, 12, and 15 μmol/L. Si-HIF-1α and si-IRF2 were obtained from Sangon (Shanghai, China) and transfected into H9c2 cells by using Lipofectamine 2000 (Invitrogen, USA). PcDNA-3.1-IRF2 was used to upregulate IRF2 expression.

Oxidative stress indicator detection

H9c2 cells were seeded in 60 mm dishes. After hypoxia treatment as described above, 50 μL of culture supernatant from each dish was collected, and lactate dehydrogenase (LDH) activity in the medium was detected using commercial assay kits and presented as U/mL. The malondialdehyde (MDA) content and antioxidant enzyme activity were detected by using a commercial assay kit. The content of MDA is expressed as nmol/mg protein. The activities of superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) are expressed as U/mg protein.

Cell counting kit-8 (CCK-8) assay

Cell viability was determined by using a CCK-8 assay kit based on the supplier's instructions. In brief, H9c2 cells were seeded in 96-well plates at a density of 2×10^3 cells/well and pretreated with different concentrations of Xinnaotongluo liquid. Then, cells were cultivated under H/R conditions for 6h, the CCK-8 solution was added to each well, and cells were cultured for another 2h at 37°C. The absorbance at 450 nm was read by using a microplate reader.

RNA extraction and quantitative PCR

H9c2 cells were collected by centrifugation and lysed with TRIzol reagent (Invitrogen, USA). Then, total RNA was extracted and reverse transcribed to cDNA using the PrimeScript RT reagent kit (TaKaRa, Japan). qPCR for target genes was carried out using the SYBR Green kit (TaKaRa, Japan) run on the Bio-Rad CFX Connect Real-Time PCR Detection System (Bio-Rad). The housekeeping gene, *GAPDH*, was used as a loading control.

Western blot assay

RIPA lysis buffer including phenylmethylsulfonylfluoride (PMSF) and a protease inhibitor cocktail was applied to extract the protein from the treated cells. The bicinchoninic acid (BCA) method was used to determine the protein content. Then, the equivalent quantities of protein were separated by 12.5% sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. The protein blots were sealed with non-fat milk for 1h, followed by treatment with primary antibodies overnight. After incubating with a secondary antibody for 2h, the protein blots were developed by a chemiluminescence kit. The following antibodies were used: HIF-1α, IL-1β, IL-6, TNF-α, Bcl-2, Bax, cleaved caspase 3, IRF2, and Tubulin.

Dual luciferase assay

The cloned human *IRF2* gene was first inserted into the vector pcDNA3.1 (pcDNA3.1-IRF2). Then, two mutant promoter sequences of human HIF-1α were inserted into the vector pGL3-Basic (pGL3-HIF-1α-

mutant1, pGL3-HIF-1α-mutant). The recombinant luciferase reporter plasmid including the HIF-1α promoter (pGL3-HIF-1α, pGL3-HIF-1α-mutant1, pGL3-HIF-1α-mutant) and pcDNA3.1-IRF2 or negative control were co-transfected into cells by Lipofectamine 3000 transfection reagent for 48h at 37°C. After 48h transfection, the luciferase activity was determined using a dual-luciferase reporter gene assay kit based on the manufacturer's instructions. The firefly luciferase activity was normalized to Renilla luciferase activity for each sample.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed with the ChIP assay kit based on the supplier's instructions. In brief, H9c2 cells were fixed with 1% formaldehyde for 10 min at 37°C to crosslink the protein and DNA. Then, glycine was applied to terminate the crosslinking. H9c2 cells were centrifuged and lysed at 4°C for 10 min. The fragments were gathered by shearing crosslinked complex DNA with ultrasound, followed by IP with the antibody directed against IRF2 overnight at 4°C, with IgG as the negative control. The fragments were detected by qPCR.

Statistical analysis

The experimental data from three independent experiments are presented as mean ± standard deviation (SD). Comparisons between two groups were made with the Student's t-test, whereas differences among multiple groups were compared by one-way analysis of variance (ANOVA) followed by Bonferroni's *post-hoc* test. A *p*-value less than 0.05 was deemed statistically significant.

Results

Xinnaotongluo liquid alleviated the damage to H9c2 cells after H/R treatment

H9c2 cells are a cardiac phenotype of immortalized cells and are widely used in the analysis of cardiac ischemia-reperfusion injury (Pelloux et al., 2006). In our study, H9c2 cells were placed in an H/R environment to simulate ischemia-reperfusion injury. With the extension of reoxygenation time (0-9h), the viability, LDH, MDA, and SOD levels of H9c2 cells were also examined. After 3h of reoxygenation, the viability, LDH, MDA, and SOD levels of H9c2 cells were unchanged (Fig. 1A-D). However, the viability, LDH, MDA, and SOD levels of H9c2 cells were significantly reduced after 6h or 9h of reoxygenation (Fig. 1A-D). With relatively high cell viability, 6 h reoxygenation was selected for further study.

To exclude the possibility that the pharmacological effect of Xinnaotongluo liquid was caused by its cytotoxicity, a CCK-8 assay was performed after incubating with H9c2 cells. As expected, the concentrations of 6 μmol Xinnaotongluo liquid did not affect cell viability in our study. Moreover, the IC_{50} of Xinnaotongluo liquid was 8.567 μmol (Fig. 2A). In the following experiments, 8.567 μmol Xinnaotongluo liquid was used as the high concentration of Xinnaotongluo liquid, and 6 μmol Xinnaotongluo liquid was used as the low concentration. Under H/R conditions, the OD values of H9c2 cells were significantly reduced, the levels of LDH and MDA

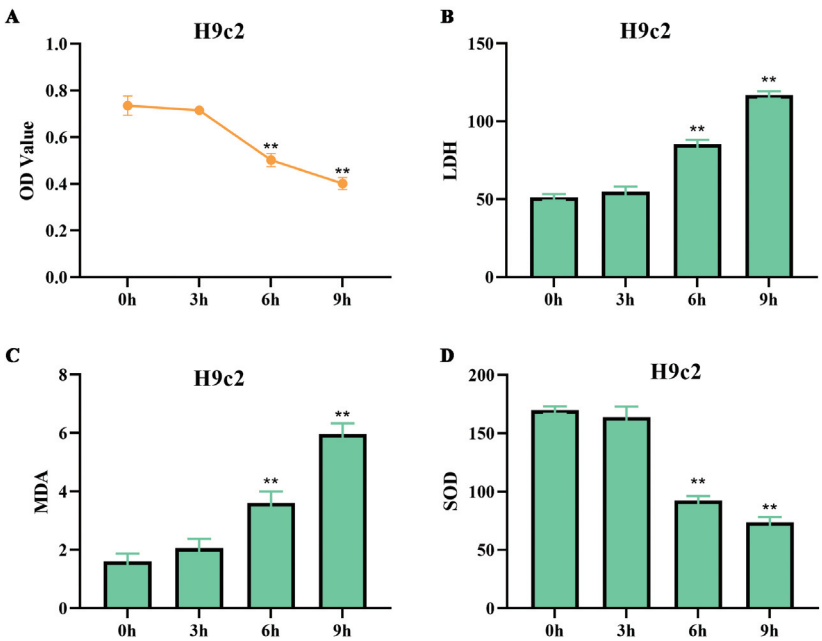

Fig. 1. Hypoxia exposure induced H9c2 cell viability reduction and oxidation. **A.** Under hypoxic conditions, H9c2 cell viability was detected by the CCK-8 assay. **B-D.** The levels of LDH, MDA, and SOD were determined by commercial kits under hypoxic conditions. $^{**}p<0.01$ vs. 0h.

increased, and the levels of SOD, GSH, and CAT of H9c2 cells reduced (Fig. 2B-G). After low-concentration Xinnaotongluo liquid treatment, the OD values of H9c2 cells increased, the levels of LDH and MDA decreased, and the levels of SOD, GSH, and CAT of H9c2 cells increased. After high-concentration Xinnaotongluo liquid treatment, the OD values of H9c2 cells increased, the levels of LDH and MDA decreased, and the levels of SOD, GSH, and CAT of H9c2 cells increased (Fig. 2B-G). Moreover, apoptotic and inflammatory markers were also detected. H/R induced a decrease in Bcl-2 expression, while the expression of Bax and cleaved-caspase 3 increased in an inverse proportion (Fig. 2H,I). Under H/R conditions, high-concentration Xinnaotongluo liquid treatment reversed the changes in Bcl-2, Bax, and cleaved-caspase 3 protein expression (Fig. 2H,I). The addition of low-concentration Xinnaotongluo liquid did not change the expression of Bcl-2, Bax, or cleaved-caspase 3 (Fig. 2H,I). Moreover, the protein levels of IL-1β, IL-6, and TNF-α increased after H/R stimulation, whereas high-concentration Xinnaotongluo liquid treatment reversed the changes in IL-1β, IL-6 and TNF-α protein expression after H/R stimulation (Fig. 2J,K). The addition of low-concentration Xinnaotongluo liquid did not change the expression of IL-1β, IL-6, or TNF-α (Fig. 2 J,K).

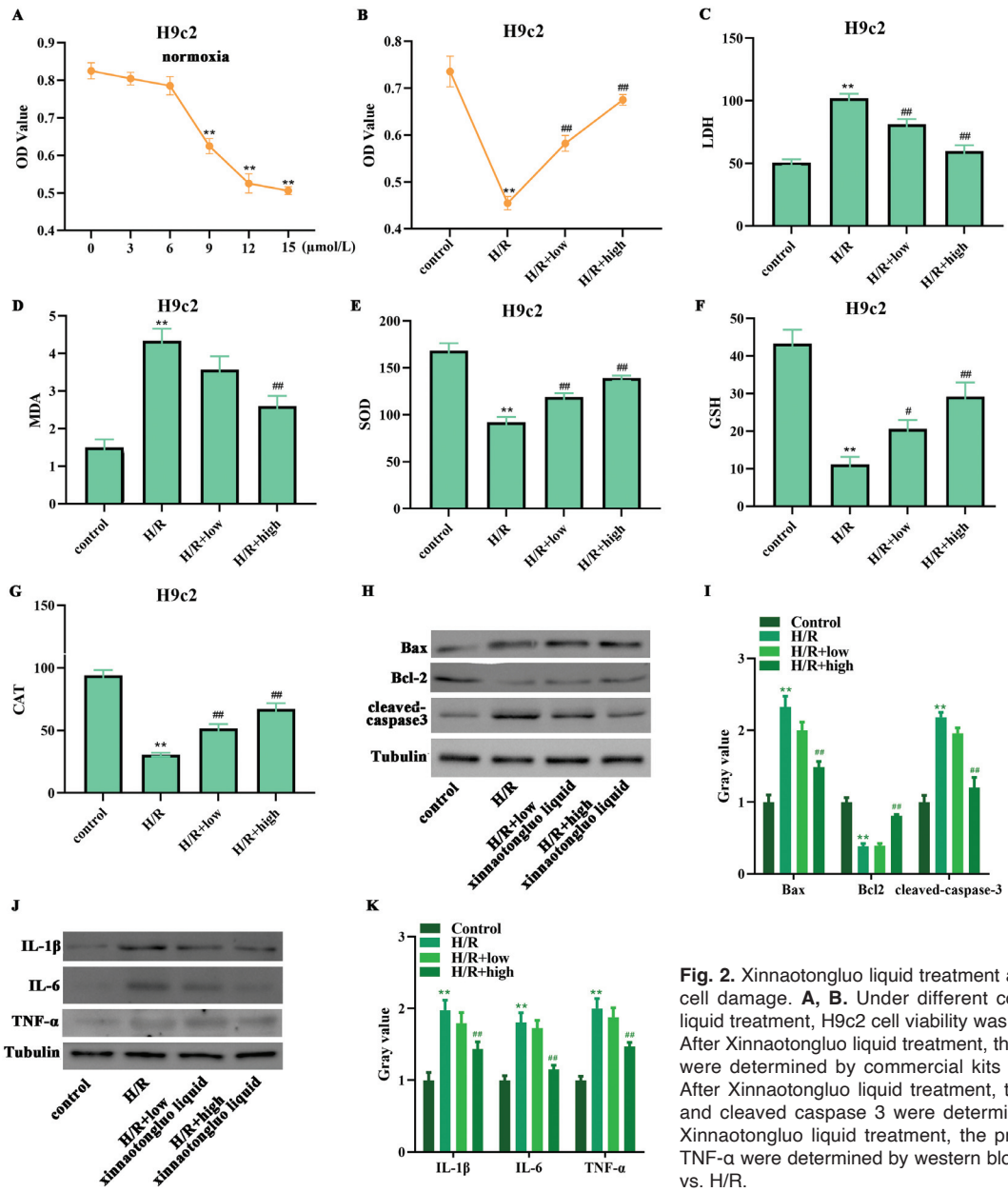

Fig. 2. Xinnaotongluo liquid treatment alleviated the H/R-induced H9c2 cell damage. **A, B.** Under different concentrations of Xinnaotongluo liquid treatment, H9c2 cell viability was detected by CCK-8 assay. **C-G.** After Xinnaotongluo liquid treatment, the levels of LDH, MDA, and SOD were determined by commercial kits under hypoxic conditions. **H, I.** After Xinnaotongluo liquid treatment, the protein levels of Bax, Bcl-2, and cleaved caspase 3 were determined by western blot. J-K. After Xinnaotongluo liquid treatment, the protein levels of IL-1β, IL-6, and TNF-α were determined by western blot. $^{**}p<0.01$ vs. control, $^{\#\#}p<0.01$ vs. H/R.

HIF-1α was regarded as a potential target of Xinnaotongluo liquid

Under H/R conditions, we observed an increase in the expression of HIF-1α (Fig. 3A-C). However, the increased HIF-1α can be diminished by Xinnaotongluo liquid treatment under H/R conditions (Fig. 3A-C). To further explore the relationship between HIF-1α and Xinnaotongluo liquid, si-HIF-1α was transfected into H9c2 cells (Fig. 3D,E). The data from Fig. 3F shows that the reduced OD values induced by H/R were suppressed by si-HIF-1α transfection. Moreover, si-HIF-1α transfection accelerated the improvement in cell viability of Xinnaotongluo liquid (Fig. 3F). Additionally, the increased levels of LDH and MDA caused by H/R were diminished after si-HIF-1α transfection, and the reduced levels of SOD, GSH, and CAT caused by H/R were suppressed by si-HIF-1α transfection (Fig. 3G-K). Meanwhile, si-HIF-1α treatment accelerated the improvement of Xinnaotongluo liquid on the levels of LDH, MDA and SOD, GSH and CAT (Fig. 3G-K). The addition of si-HIF-1α induced an increase in Bcl-2 expression, while the expression of Bax and cleaved-caspase 3 decreased in an inverse proportion compared with the H/R group. The addition of si-HIF-1α strengthened the effects of Xinnaotongluo liquid on the levels of Bcl-2, Bax, and cleaved-caspase 3. The addition of si-HIF-1α reduced the protein level of IL-1β, IL-6, and TNF-α compared with the H/R group, and enhanced the effects of Xinnaotongluo liquid on the

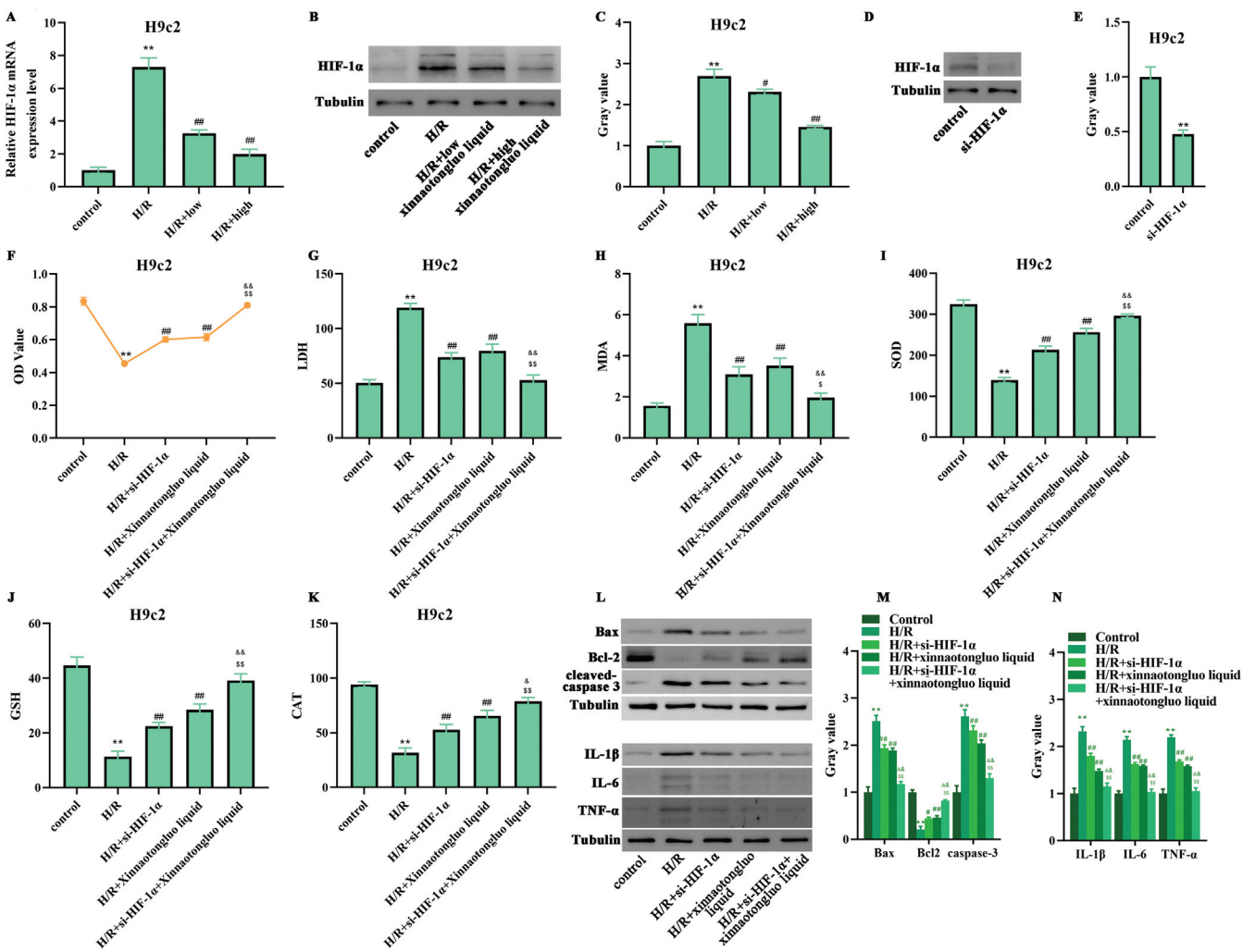

Fig. 3. Knockdown of HIF-1α strengthened the beneficial effects of Xinnaotongluo liquid in H/R-induced H9c2 cells. **A-C.** After Xinnaotongluo liquid treatment, qPCR and western blot were used to detect HIF-1α expression in H/R-induced H9c2 cells. **D, E.** HIF-1α expression reduced after si-HIF-1α treatment. **F.** After si-HIF-1α and Xinnaotongluo liquid treatment, H9c2 cell viability was detected by CCK-8 assay under H/R conditions. **G-K.** After si-HIF-1α and Xinnaotongluo liquid treatment, the levels of LDH, MDA, and SOD were determined by commercial kits under hypoxic conditions. **L-N.** After si-HIF-1α and Xinnaotongluo liquid treatment, the protein levels of Bax, Bcl-2, cleaved caspase 3, IL-1β, IL-6, and TNF-α were determined by western blot. $^{**}p<0.01$ vs. control, $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs. H/R, $^{\$}p<0.05$, $^{\$\$}p<0.01$ vs. H/R+si-HIF-1α, $^{\&}p<0.05$, $^{\&\&}p<0.01$ vs. H/R+Xinnaotongluo liquid.

levels of IL-1β, IL-6, and TNF-α (Fig. 3L-N).

IRF2 was identified as an upstream regulator of HIF-1α

By searching the PROMO (https://alggen.lsi.upc.es/) and UCSC (https://genome.ucsc.edu/) websites, IRF2 was predicted as a target of HIF-1α. To further explore the relationship between HIF-1α and IRF2, dual luciferase and ChIP assays were performed. As presented in Fig. 4A,B, the luciferase activity of the MUT-1 or MUT-2 group was significantly diminished compared with the WT group. Moreover, the relationship between HIF-1α and IRF2 was verified by the ChIP assay (Fig. 4C). Then, we detected the mRNA and protein levels of IRF2 in H9c2 cells under H/R conditions. The data from Fig. 4D-F show that IRF2 was upregulated in H9c2 cells under H/R stimulation, whereas Xinnaotongluo liquid treatment reversed this phenomenon. Subsequently, we detected the protein levels of HIF-1α after IRF2 up- or downregulation. The data from Fig. 4G,H show that HIF-1α expression was significantly upregulated after IRF2 overexpression, and Xinnaotongluo liquid treatment inhibited the promoting effect of IRF2 overexpression on HIF-1α expression. The data from Fig. 4I,J show that HIF-1α expression was downregulated after IRF2 depletion, and Xinnaotongluo liquid treatment suppressed the inhibitory effect of IRF2 depletion on HIF-1α expression.

Discussion

It is well known that myocardial ischemia-reperfusion is still a terrible complication of reperfusion therapy (Boag et al., 2017), which is implicated in diverse pathophysiological events, including cardiomyocyte inflammation and apoptosis (Wang et al.,

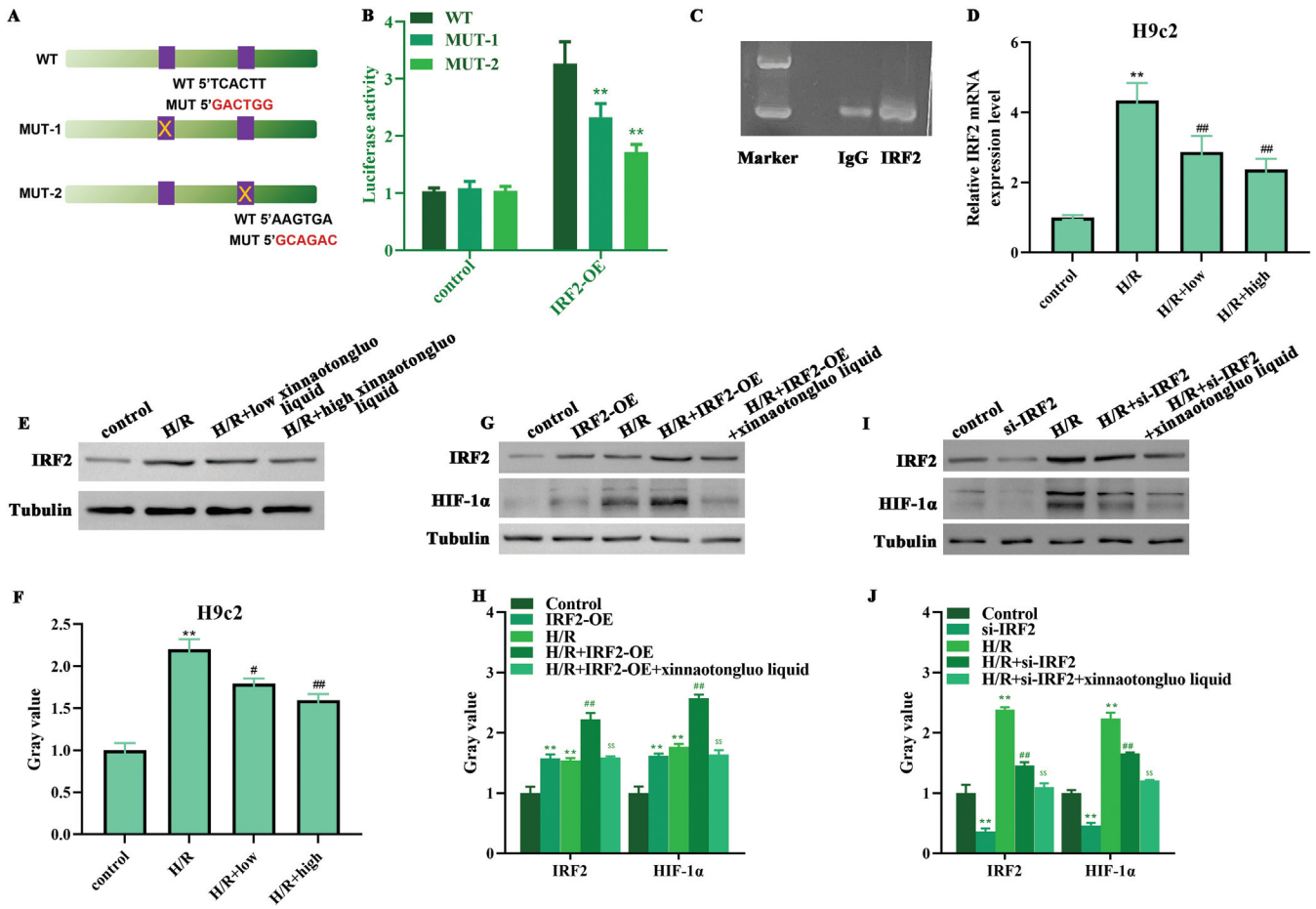

Fig. 4. IRF2 transcriptionally regulated HIF-1α expression in H/R-induced H9c2 cells. **A.** The target sites between HIF-1α and IRF2 are presented. **B.** Dual luciferase assay was used to detect the relationship between HIF-1α and IRF2, $^{**}p<0.01$ vs. WT group. **C.** ChIP assay was used to detect the relationship between HIF-1α and IRF2. **D-F.** After Xinnaotongluo liquid treatment, qPCR and western blot were used to detect IRF2 expression in H/R-induced H9c2 cells, $^{**}p<0.01$ vs. control, $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs. H/R. **G, H.** Under H/R conditions, the protein levels of IRF2 and HIF-1α were examined by western blot after IRF2-OE and/or Xinnaotongluo liquid treatment, $^{**}p<0.01$ vs. control, $^{\#\#}p<0.01$ vs. H/R, $^{\$\$}p<0.01$ vs. H/R+IRF2-OE. **I, J.** Under H/R conditions, the protein levels of IRF2 and HIF-1α were examined by western blot after si-IRF2 and/or Xinnaotongluo liquid treatment, $^{**}p<0.01$ vs. control, $^{\#\#}p<0.01$ vs. H/R, $^{\$\$}p<0.01$ vs. H/R+si-IRF2.

2018). Our study demonstrated that Xinnaotongluo liquid functioned as a beneficial agent for alleviating H/R injury (Cui et al., 2024). In our study, we illustrated that Xinnaotongluo liquid inhibited apoptosis and inflammation of H/R-induced H9c2 cells by suppressing IRF2/HIF-1α, thereby alleviating H/R-induced H9c2 cell damage.

HIF-1α is an important regulator that controls the cellular response to hypoxia, and the increased expression of HIF-1α is one of the first adaptations of the human myocardium to ischemia (Du et al., 2018). For example, inducible miR-145 expression by HIF-1α protects cardiomyocytes against apoptosis in a simulated myocardial infarction hypoxic microenvironment (Sun et al., 2018). However, whether HIF-1α can play a role as a Xinnaotongluo liquid target in myocardial ischemia remains unclear. In our study, we observed that HIF-1α expression significantly increased in H9c2 cells after H/R stimulation, whereas the addition of Xinnaotongluo liquid reduced HIF-1α expression. Moreover, si-HIF-1α transfection significantly reduced H/R-induced damage to H9c2 cells and strengthened the efficacy of Xinnaotongluo liquid.

After consulting the literature, we found that the pathophysiological process of myocardial ischemia-reperfusion injury may be related to oxidative stress, inflammation, and apoptosis (Ai et al., 2021; Xia et al., 2022). Cardiomyocyte apoptosis is involved in the occurrence and development of many cardiovascular diseases. The energy metabolism disorder caused by mitochondrial oxidative stress after cardiomyocyte damage is the cause of many cardiovascular diseases, and myocardial injury will cause a strong inflammatory reaction, which will further aggravate cardiomyocyte apoptosis (Zhang and Dhalla, 2024). Therefore, we focused on indicators related to oxidation, apoptosis, and inflammation in our study. Increasing evidence has demonstrated that the myocardial ischemia-reperfusion process can produce a large number of free radicals, destroying the body's oxidation and antioxidant processes (Zhai et al., 2017). MDA is the end product of lipid peroxidation and is cytotoxic (Qiu et al., 2021). SOD can restore the dynamic balance of oxidation and antioxidant, and prevent reactive oxygen species from causing damage to cells (Yang et al., 2018). Additionally, oxidative stress in cultured cells has also been assessed by markers such as LDH release and the production of MDA, as well as the inhibition of SOD, GSH, and CAT (Todd et al., 2016). In our study, the levels of LDH and MDA were significantly increased in H/R-induced H9c2 cells, whereas, after treatment with Xinnaotongluo liquid, the levels of LDH and MDA in H/R-induced H9c2 cells were decreased. Moreover, the knockdown of HIF-1α not only suppressed the levels of LDH and MDA in H/R-induced H9c2 cells but also strengthened the inhibitory effects of Xinnaotongluo liquid on LDH and MDA levels. Similarly, the levels of SOD, GSH, and CAT experienced opposite changes.

As typical pro-inflammatory cytokines, IL-1β, IL-6, and TNF-α are the main indicators of cellular inflammation (Chen et al., 2018a). In our study, the protein levels of IL-1β, IL-6, and TNF-α were significantly increased in H/R-induced H9c2 cells whereas, after treatment with Xinnaotongluo liquid, their levels decreased. Moreover, the knockdown of HIF-1α not only suppressed the protein levels of IL-1β, IL-6, and TNF-α in H/R-induced H9c2 cells but also strengthened the inhibitory effects of Xinnaotongluo liquid on IL-1β, IL-6, and TNF-α expression.

Cardiomyocyte apoptosis is an irreversible event induced by myocardial ischemia-reperfusion injury, and reducing apoptosis has proven to be an effective strategy for protection against myocardial ischemia-reperfusion injury (Ai et al., 2021). The opening of mitochondrial permeability transition pores leads to suppression of Bcl-2 activity; Bax is recruited from the cytoplasm to the mitochondria, followed by the release of various apoptotic factors, including Cyt-c, from the mitochondria into the cytoplasm, thereby activating the downstream effector caspase (including caspase 3) (Yao et al., 2022). In our study, the protein levels of Bax and cleaved-caspase 3 were significantly increased in H/R-induced H9c2 cells whereas, after treatment with Xinnaotongluo liquid, their levels decreased. Moreover, the knockdown of HIF-1α not only suppressed the protein levels of Bax and cleaved-caspase 3 in H/R-induced H9c2 cells but also strengthened the inhibitory effects of Xinnaotongluo liquid on Bax and cleaved-caspase 3 expression. Bcl-2 protein showed the opposite trend.

The IRF family is best known for its role in the regulation of gene expression. As a member of the IRF family, IRF2 is implicated in diverse biological processes, including cell proliferation and apoptosis, inflammation, and tumorigenesis (Yanai et al., 2012). A recent study suggested that IRF2 could contribute to myocardial infraction by regulating gasdermin D-induced pyroptosis (Li et al., 2022). Similar to the previous study, our study discovered that IRF2 expression was upregulated in H/R-induced H9c2 cells. Moreover, high expression of IRF2 in H/R-induced H9c2 cells was suppressed after Xinnaotongluo liquid treatment. Additionally, IRF2 was identified as a transcription factor of HIF-1α and regulated HIF-1α expression.

In summary, we demonstrated that Xinnaotongluo liquid ameliorated H/R-induced damage in H9c2 cells by regulating oxidation, inflammation, and apoptosis through the IRF2/HIF-1α pathway.

Ethics Approval. Not applicable.

Competing interests. We declare that we have no conflict of interest.

Authors' contributions. Jiankun Cui wrote the manuscript, performed the experiments, and analyzed the experimental data. Hui Liu and Yanmei Feng supported the study and edited the whole manuscript.

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Availability of data and materials. The datasets used during the study are available from the corresponding author upon reasonable request.

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