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S100 calcium-binding protein A12 knockdown ameliorates hypoxia-reoxygenation-induced inflammation and apoptosis in human cardiomyocytes by regulating caspase-4-mediated non-classical pyroptosis

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Abstract. Ischemic heart disease, especially myocardial infarction, poses a serious risk to human health. S100 calcium-binding protein A12 (S100A12) expression was previously reported to be up-regulated in ST-segment elevation myocardial infarction. Therefore, the present study investigated the role of S100A12 in hypoxia/reoxygenation (H/R)-induced cardiomyocytes injury and the associated mechanism. An *in vitro* H/R-induced cardiomyocyte injury model was first established using AC16 cells. The expression level was found to be hugely upregulated in H/R-induced AC16 cells. Subsequently, cell transfection was conducted to knock down the expression level of S100A12, and the following cellular biological assays revealed that S100A12 knockdown could not only inhibit H/R-induced AC16 cell injury by improving cell viability and decreasing the release of lactate dehydrogenase, as well as reducing apoptotic cells, but also reduce the production of inflammatory cytokines (TNF-α, IL-1β and IL-6), restore the balance of oxidation-antioxidant factors (malondialdehyde, superoxide dismutase and glutathione), and inhibit the activated pyroptosis upon H/R induction. Then, co-immunoprecipitation was used to verify the interaction between S100A12 and caspase-4 (CASP4). CASP4 overexpression reversed the inhibitory effects of S100A12 downregulation on H/R-induced cardiomyocyte injury. In conclusion, these results suggest that S100A12 knockdown can ameliorate H/R-induced cardiomyocyte injury by regulating CASP4 expression. Therefore, S100A12 serves as a potential therapeutic target for the treatment of myocardial ischemia/reperfusion injury.

Key words: S100 calcium-binding protein A12 — Caspase-4 — Cardiomyocyte — Inflammatory — Pyroptosis

Introduction

Ischemic heart disease is a cardiac condition that occurs due to the narrowing of the coronary arteries. Physiologically, it is caused by an imbalance in oxygen supply and demand within the myocardium (Choi et al. 2009). Although reperfusion after ischemia is effective in unblocking the blocked vessels to restore blood perfusion to the myocardial tissues, this process inevitably causes deleterious effects on cardiac function (Fransen et al. 1998). This is because during the delivery of oxygen and blood to the myocardium, reperfusion stimulates the excessive production of reactive oxygen species (ROS), nitrogen and the proinflammatory cytokines (Kalogeris et al. 2016). Additionally, excess ROS can cause damage to the mitochondria in cardiomyocytes, which

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disrupts normal cardiac contractile function, stimulates the release of pro-apoptotic proteins and eventually leads to cardiomyocyte apoptosis (Li et al. 2019). Accordingly, oxidative stress, inflammation and apoptosis are key characteristics of cardiomyocyte injury. Traditional therapies, such as drug application, bypass surgery and stent implantation, have all made significant improvements to the prognosis of ischemic heart disease. However, ischemia/reperfusion (I/R)-induced cardiomyocyte injury frequently leads to heart failure to limit therapeutic efficacy (Yu et al. 2017). Therefore, reparation and regeneration of damaged cardiomyocytes is considered to be a key objective for improving ischemic heart disease.

S100 calcium-binding protein A12 (S100A12) belongs to a member of the S100 family of calcium-binding proteins, which in turn forms a part of the damage-associated molecular pattern protein (DAMPs) family (Marenholz et al. 2006). Previous studies have revealed that S100A12 expression is upregulated during ischemic heart disease. S100A12 expression was found to be highly expressed in patients with ST-segment elevation myocardial infarction (STEMI) (Gobbi et al. 2019). In another previous study, the levels of S100A12 in plasma were documented to be significantly higher in patients with chronic heart failure compared with those in the healthy control group (Allemani et al. 2015). Furthermore, high levels of S100A12 expression were associated with increased risks of cardiovascular disease (Basta et al. 2006). Supporting this, S100A12 knockdown was previously observed to significantly suppress apoptosis and inflammation in primary aortic smooth muscle cells (SMCs) harvested from patients with thoracic aortic aneurysms (Oesterle and Bowman 2015). Lower levels of serum S100A12 were also associated with the anti-inflammatory effects of methotrexate on patients with inflammatory arthritis (Foell et al. 2003).

Pyroptosis is a type of programmed cell death that is dependent on inflammation. It has been identified to be an important signaling pathway leading to the death of cardiomyocytes (Li et al. 2020; Jiang et al. 2021). Pyroptosis is mainly mediated by two main signaling pathways: Caspase (CASP)-1, which represents the classical pathway; and CASP-4/5/11, which is the non-classical pathway (Jia et al. 2019). CASP4 is a protein that has been reported to regulate immunity and inflammation. In addition, it is a key regulator of the non-classical pyroptotic pathway (McIlwain et al. 2013; Shi et al. 2015). CASP4 can cleave gasdermin D (GSDMD) to induce the programmed pyroptosis (Kayagaki et al. 2015; Shi et al. 2015). Based on the HumanBase website (www.flatironinstitute.org), S100A12 can target CASP4 and regulate CASP4 expression in cardiac tissues. Therefore, it is hypothesized that S100A12 can interact with CASP4 to regulate the pyroptosis upstream of cardiomyocyte injury.

Therefore, the present study aims to explore the specific role of S100A2 in I/R-induced cardiomyocyte injury and to explore its regulatory mechanism. It is speculated that S100A2 can regulate inflammation, oxidative stress and apoptosis in I/R-induced cardiomyocytes, which may be associated with CASP4-mediated pyroptosis. It is hoped that results from the present study will provide novel insights into identifying effective therapeutic strategies for ischemic heart disease.

Materials and Methods

Cell culture

The human cardiomyocyte cell line AC16 was provided by the China Center for Type Culture Collection. AC16 cells were maintained in DMEM supplemented with 10% FBS and 100 U/ml penicillin and 100 mg/ml streptomycin whilst being incubated in a humidified atmosphere at 37°C under 5% CO_2.

Cell transfection

Short-hairpin RNA targeting S100A12 (sh-S100A12-1/2) and its negative control (sh-NC), the overexpression plasmid encoding CASP4 (oe-CASP4) and its corresponding empty vector (oe-NC) were constructed by Shanghai GenePharma Co., Ltd. These vectors were transfected into AC16 cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. In total, 48 h after transfection, the interference or overexpression efficiency were assessed using Western blotting and reverse transcription-quantitative PCR (RT-qPCR).

Cell treatment

Hypoxia/reoxygenation (H/R) injury in the cardiomyocytes was performed to mimic I/R injury *in vivo* (Ge et al. 2019). Briefly, AC16 cells were first cultured in glucose-free/serum-free DMEM at 37°C for 5 h under hypoxic conditions (95% N_2 and 5% CO_2). The cells were then transferred into normal medium after hypoxia and cultured for 1 h at 37°C under reoxygenation conditions (5% CO_2 and 95% O_2). AC16 cells in Control group were kept incubation with the normal condition without transfection, and the untransfected AC16 cells undergoing H/R injury were regarded as H/R group. Meanwhile, AC16 cells transfected with sh-NC, sh-S100A12-1, sh-S100A12-2 and subsequently undergoing H/R injury were regarded as H/R+sh-NC, H/R+sh-S100A12-1, H/R+sh-S100A12-2 group, respectively.

Western blotting

Total proteins were extracted from AC16 cells using the RIPA lysis buffer (Elabscience Biotechnology Inc.) on ice for 30

min. Protein concentration was then determined using the bicinchoninic acid protein assay kit (Beijing Solarbio Science & Technology Co., Ltd.). A total of 30 μg protein sample *per* lane was separated by electrophoresis using 10% SDS-PAGE and transferred onto PVDF membranes (MilliporeSigma). The membranes were subsequently blocked in 5% skimmed milk for 1 h, followed by an incubation with primary antibodies against S100A12, cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS), NADPH oxidase (NOX)2, NOX4, CASP4, GSDMD-N and GAPDH overnight at 4°C overnight. After washing with PBS, the membranes were incubated with HRP-labelled secondary antibodies for another 2 h at room temperature. An enhanced chemiluminescence detection kit (Elabscience Biotechnology Inc.) was used to visualize the protein bands. Finally, densities of the bands were quantified using the ImageJ software (National Institutes of Health).

Quantitative real-time polymerase chain reaction (RT-qPCR)

For the detection of S100A12 mRNA expression, extraction of total RNA from AC16 cells was performed using the TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's protocol. cDNA synthesis was conducted using the Titan™ One Tube RT-PCR kit (Sigma-Aldrich; Merck KGaA). The subsequent qPCR reaction was performed using SYBR Green I (10,000X; Beijing Solarbio Science & Technology Co., Ltd.) in the ABI PRISM 7000 Sequence Detection System (Thermo Fisher Scientific, Inc.). GAPDH served as the internal reference. The relative mRNA expression of S100A12 was evaluated using the $2^{-\Delta\Delta Cq}$ method.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The viability of AC16 cells after H/R conditioning was measured using MTT assay. AC16 cells were seeded into 96-well plates and cultured for 48 h. The media was carefully aspirated before 50 μl serum-free media and 50 μl MTT reagent (Abcam) was added into each well for 3 h incubation at 37°C. Subsequently, 150 μl DMSO (Abcam) was added into each well. The plates were wrapped with foil and then shaken on an orbital shaker for 15 min. Finally, absorbance at 590 nm was read in each well using a microplate reader.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL)

Assessment of AC16 cell apoptosis was performed using TUNEL assay (He et al. 2017). AC16 cells were washed with PBS and fixed with 4% paraformaldehyde for 30 min. After washing, cells were incubated with PBS containing 0.3% Triton X-100 at room temperature for 5 min. Appropriate TUNEL detection solution (Beyotime Institute of Biotechnology) was prepared by following the protocols of the manufacturer. After being washed twice with PBS, 50 μl TUNEL assay solution was added into the sample and incubated for 1 h at 37°C in the dark. After three times of washing, observation of apoptotic cells with green fluorescence was performed by fluorescent microscopy.

Lactate dehydrogenase (LDH) kit assay

The level of LDH was detected to measure the degree of AC16 cell impairment. AC16 cells were first grown in 96-well plates. After various treatments, 120 μl supernatant from each well was transferred into another 96-well plate, which were added with 60 μl reagent from the LDH assay kit (Beyotime Institute of Biotechnology) and incubated at room temperature in the dark for 30 min. Absorbance at 490 nm was measured in each well using a microplate reader.

Enzyme-linked immunosorbent assay (ELISA)

The levels of TNF-α, IL-1β and IL-6 were measured in AC16 cells in each group using corresponding ELISA kits (Beyotime Institute of Biotechnology) according to the manufacturer's protocols. A FlexStation 3 multi-mode microplate reader (Molecular Devices, LLC) was used to measure the absorbance at 450 nm. These experiments were run in triplicate.

Malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione (GSH) assays

The levels of MDA, SOD and GSH in AC16 cells were assessed using their corresponding MDA, SOD and GSH commercial kits (Nanjing Jiancheng Bioengineering Institute). Manufacturer's operating protocols were strictly followed. The absorbance at 532 nm (MDA), 560 nm (SOD) and 412 nm (GSH) were measured using a microplate reader.

Co-immunoprecipitation assay (Co-IP)

The potential interaction between S100A12 and CASP4 was measured in AC16 cells using Co-IP assay. Briefly, cells were harvested and washed carefully with cold PBS twice before being lysed with 1 ml cold RIPA lysis buffer. The lysates were then centrifuged at 14,000 × *g* at 4°C for 15 min before the supernatant was collected. A part of the untreated sample was regarded as the input control, and the rest were added with anti-CASP4 antibody or anti-rabbit IgG antibody for incubation at 4°C overnight. Then, the protein A/G agarose beads (Santa Cruz Biotechnology, USA) was added

to samples, followed by shaking for 30 min at 4°C, so as to precipitate the bound protein. Subsequently, the immunoprecipitated protein complex was boiled and denatured. Following centrifugation, the supernatant was taken for SDS-PAGE electrophoresis and Western blot analysis.

Statistically analysis

The experimental data were presented as the mean ± standard deviation and analyzed using the SPSS 20.0 software (IBM Corp.). Significant differences among multiple groups were analyzed using one-way analysis of variance followed by Tukey's *post hoc* test. $p < 0.05$ was considered to indicate a statistically significant difference. Experiments were performed independently more as three times.

Results

S100A12 knockdown inhibits H/R-induced injury in AC16 cells

First, to mimic myocardial I/R injury, cardiomyocytes AC16 cells were received H/R induction, which has been widely applied in multiple researches (Ge et al. 2019). Following H/R treatment, we not only found a significantly upregulated expression of S100A12, but also observed that H/R induction greatly decreased cell viability, increased the release of LDH and the apoptotic cells, inflecting a successful *in vitro* model of myocardial I/R injury, in which S100A12 was aberrantly high-expressed (Fig. 1).

Subsequently, to examine the impact of S100A12 on the human cardiomyocytes injuries, cell transfection was carried

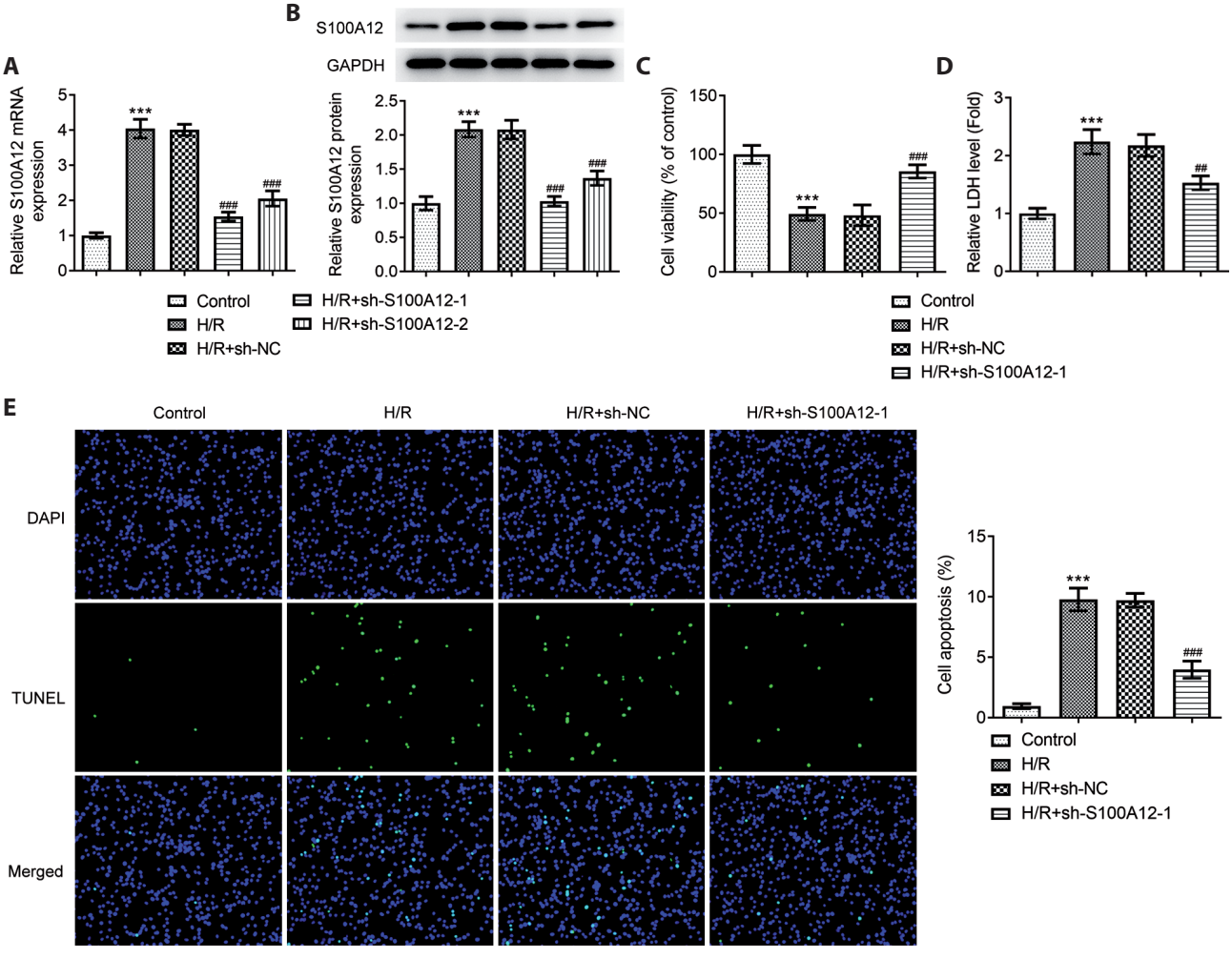

Figure 1. S100A12 knockdown inhibits H/R-induced injury in AC16 cells. S100A12 expression in AC16 cells was measured by reverse transcription-quantitative PCR (**A**) and Western blotting (**B**). **C.** Cell viability in AC16 cells was determined using the MTT assay. **D**. The levels of lactate dehydrogenase (LDH) in AC16 cells were measured using the LDH kit. **E.** Apoptosis of AC16 cells were assayed by TUNEL. *** $p < 0.001$ *vs.* Control; ## $p < 0.01$, ### $p < 0.001$ *vs.* H/R+sh-NC. H/R, hypoxia-reoxygenation group; sh, short-hairpin; NC, negative control; S100A2, S100 calcium-binding protein A2.

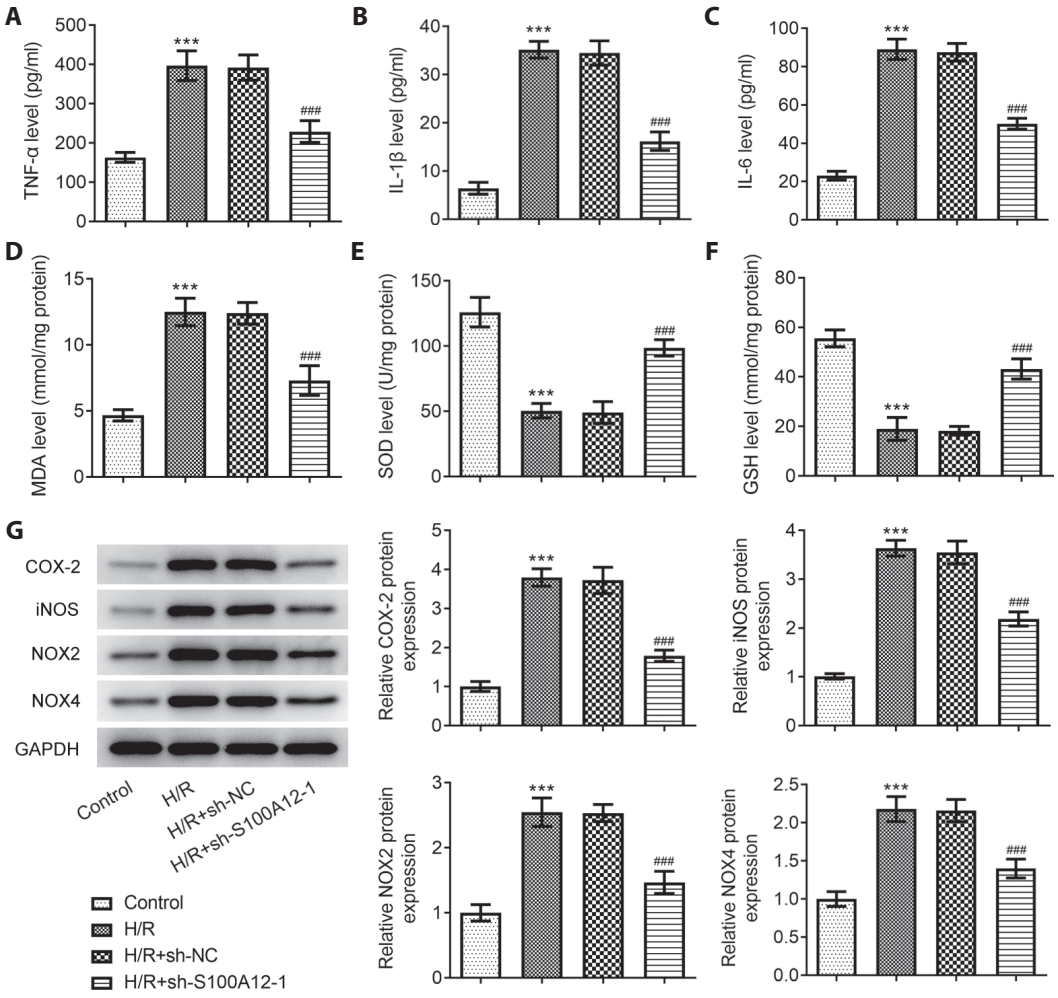

Figure 2. S100A12 knockdown suppresses H/R-induced inflammation and oxidative stress in AC16 cells. The levels of inflammatory cytokines TNF-α (**A**), IL-1β (**B**) and IL-6 (**C**) in AC16 cells were examined using ELISA. The content of MDA (**D**), SOD (**E**) and GSH (**F**) in AC16 cells was assessed using the corresponding commercial kits. **G.** The protein expression levels of COX-2, iNOS, NOX2 and NOX4 in AC16 cells were measured by Western blotting. *** $p < 0.001$ *vs.* Control; ### $p < 0.001$ *vs.* H/R+sh-NC. MDA, malondialdehyde; SOD, superoxide dismutase; GSH, glutathione; COX, cyclooxygenase; NOX, NAPDH oxidase; iNOS, inducible nitric oxide synthase. For more abbreviations, see Figure 1.

out in H/R-induced AC-16 cells. As shown in Figure 1A and B, transfection with either sh-S100A12-1 or sh-S100A12-2 in H/R-induced AC16 cells reduced the expression of S100A12 compared with that in cells in the sh-NC group. In addition, sh-S100A12-1 transfection reduced the expression of S100A12 to the greater magnitude compared with that in the sh-S100A12-2 group. Accordingly, sh-S100A12-1 was selected for subsequent experiments. Then, MTT assay showed that sh-S100A12-1 transfection increased cell viability compared with that in the sh-NC group. This effect on cell viability was determined further by measuring LDH. Elevated levels of LDH were observed in the H/R group compared with those in the control group, whilst decreased levels of LDH were found in the H/R+sh-S100A12-1 group compared with those in the sh-NC group (Fig. 1D). In addition, the percentage of apoptotic cells in the H/R group was markedly higher compared with that in the control group, which was reversed by sh-S100A12-1 transfection (Fig. 1E). These results suggest that H/R can induce AC16 cell injury, whereas S100A12 knockdown protects AC16 cells against H/R-induced damage.

S100A12 knockdown suppresses H/R-induced inflammation and oxidative stress in AC16 cells

To examine the influence of S100A12 on inflammation and oxidative stress in H/R-induced AC16 cells, the levels of inflammatory cytokines TNF-α, IL-1β, and IL-6 were first measured by ELISA. H/R was found to induce a marked rise in the levels of TNF-α, IL-1β and IL-6 compared with those in the control group, which were in turn reversed by sh-

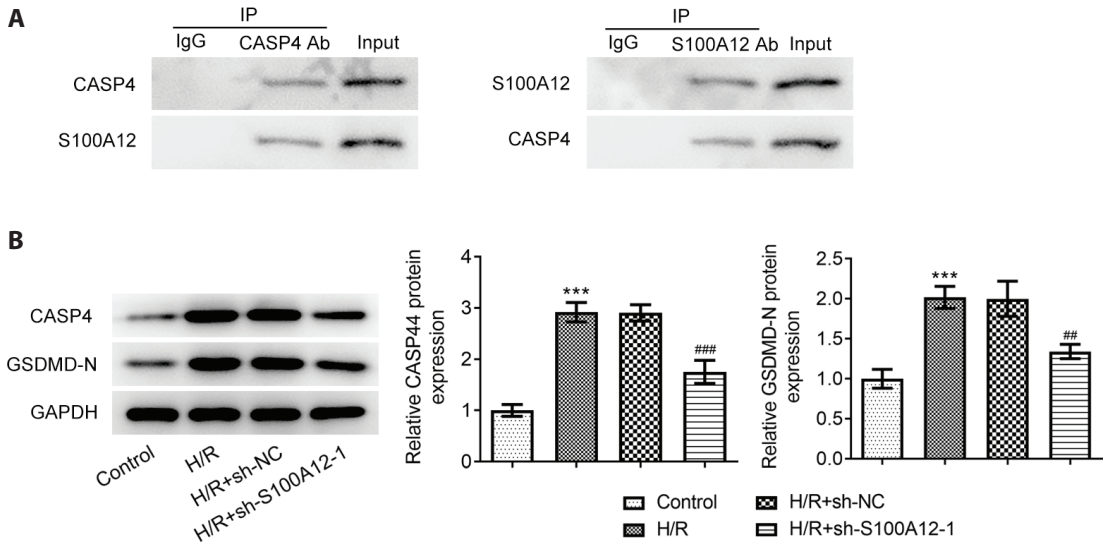

Figure 3. S100A12 interacts with CASP4 and to regulate CASP4-mediated pyroptosis. **A.** The interaction between S100A12 and CASP4 was verified in AC16 cells by Co-immunoprecipitation assay. **B.** The protein expression levels of CASP4 and GSDMD-N in AC16 cells were measured in the Control, H/R, H/R+sh-NC and H/R+sh-S100A12-1 groups by Western blotting. *** $p < 0.001$ *vs.* Control; ## $p < 0.01$ and ### $p < 0.001$ *vs.* H/R+sh-NC. CASP4, caspase 4; GSDMD-N, gasdermin-N. For more abbreviations, see Figure 1.

S100A12-1 transfection (Fig. 2A–C). Further measurements of oxidative stress indicators (Fig. 2D) revealed that the levels of MDA were rapidly increased after H/R induction in AC16 cells compared with those in the control group but were reversed sharply after transfection with sh-S100A12-1 compared with those in the sh-NC group. By contrast, the levels of SOD and GSH were markedly lower in the H/R group compared with those the control group but were higher in the H/R+sh-S100A12-1 group compared with those in the H/R+sh-NC group (Fig. 2E and F). Furthermore, H/R was observed to increase the protein expression levels of COX-2 and iNOS, markers associated with inflammation, in AC16 cells compared with those in the control group (Fig. 2G). By contrast, these effects on COX-2 an iNOS expression were reversed by sh-S100A12 transfection compared with those in the sh-NC group (Fig. 2G). The expression of oxidative stress markers NOX2 and NOX4 were also significantly elevated in H/R-induced AC16 cells compared with that in the control group, but was subsequently decreased after AC16 cells were transfected with sh-S100A12-1 compared with that in the H/R+sh-NC group. Taken together, these findings suggest that S100A12 knockdown can inhibit H/R-induced inflammation and oxidative stress in AC16 cells.

S100A12 interacts with CASP4 and regulates CASP4-mediated pyroptosis

To explore the potential mechanism underlying the regulatory role of S100A12 in H/R-induced AC16 cells, we used HumanBase to find a potential connection between S100A12 and CASP4 in cardiac tissues as aforementioned. Subsequently, the results obtained from Co-IP assay verified that S100A12 and CASP4 interacted with each other in AC16 cells (Fig. 3A). In addition, CASP4 was found to be elevated in H/R-induced AC16 cells, which was partly revered by S100A12 knockdown. Meanwhile, GSDMD-N, the executor of pyroptosis, was also found to be remarkably increased in the H/R group compared with the control group, but dropped by about half in the H/R+sh-S100A12 group by contrast to the H/R+sh-NC group (Fig. 3B). Considering the importance of CASP4 during pyroptosis, these results uncover that S100A12 knockdown effectively suppresses H/R-induced AC16 cell pyroptosis through interacting and regulating CASP4 expression.

CASP4 overexpression abolishes the inhibitory effects of S100A12 knockdown on H/R-induced AC16 cell injury

To test if CASP4 is responsible for the regulatory role of S100A12 in H/R-induced AC16 cells, AC16 cells were transfected with oe-CASP4 to overexpress CASP4, which was successful (Fig. 4A). Subsequently, after the AC16 cells were co-transfected with sh-S100A12 and oe-CASP4, the viability of H/R-induced cells was reduced compared with that in H/R+sh-S100A12-1+oe-NC group (Fig. 4B). In addition, oe-CASP4 transfection increased the levels of LDH compared with those in the H/R+sh-S100A12-1+oe-NC group (Fig. 4C). Increased percentages of apoptotic AC16 cells were also

found after CASP4 overexpression compared with those in the H/R+sh-S100A12-1+oe-NC group (Fig. 4D).

CASP4 overexpression abolishes the inhibitory effects of S100A12 knockdown on H/R-induced inflammation, oxidative stress and pyroptosis in AC16 cells

Next, the expression of inflammatory cytokines TNF-α, IL-1β and IL-6 were measured to investigate the changes after the H/R-induced AC16 cells were co-transfected with sh-S100A12-1 and oe-CASP4. oe-CASP4 was found to restore the levels of TNF-α, IL-1β and IL-6 compared with those in the H/R+sh-S100A12-1+oe-NC group (Fig. 5A–C). Elevated levels of MDA but reduced levels of SOD and GSH were observed in the H/R+sh-S100A12-1+oe-CASP4 group compared with those in the H/R+sh-S100A12-1+oe-NC group. Co-transfection with sh-S100A12 and oe-CASP4 also increased the expression of COX-2, iNOS, NOX2 and

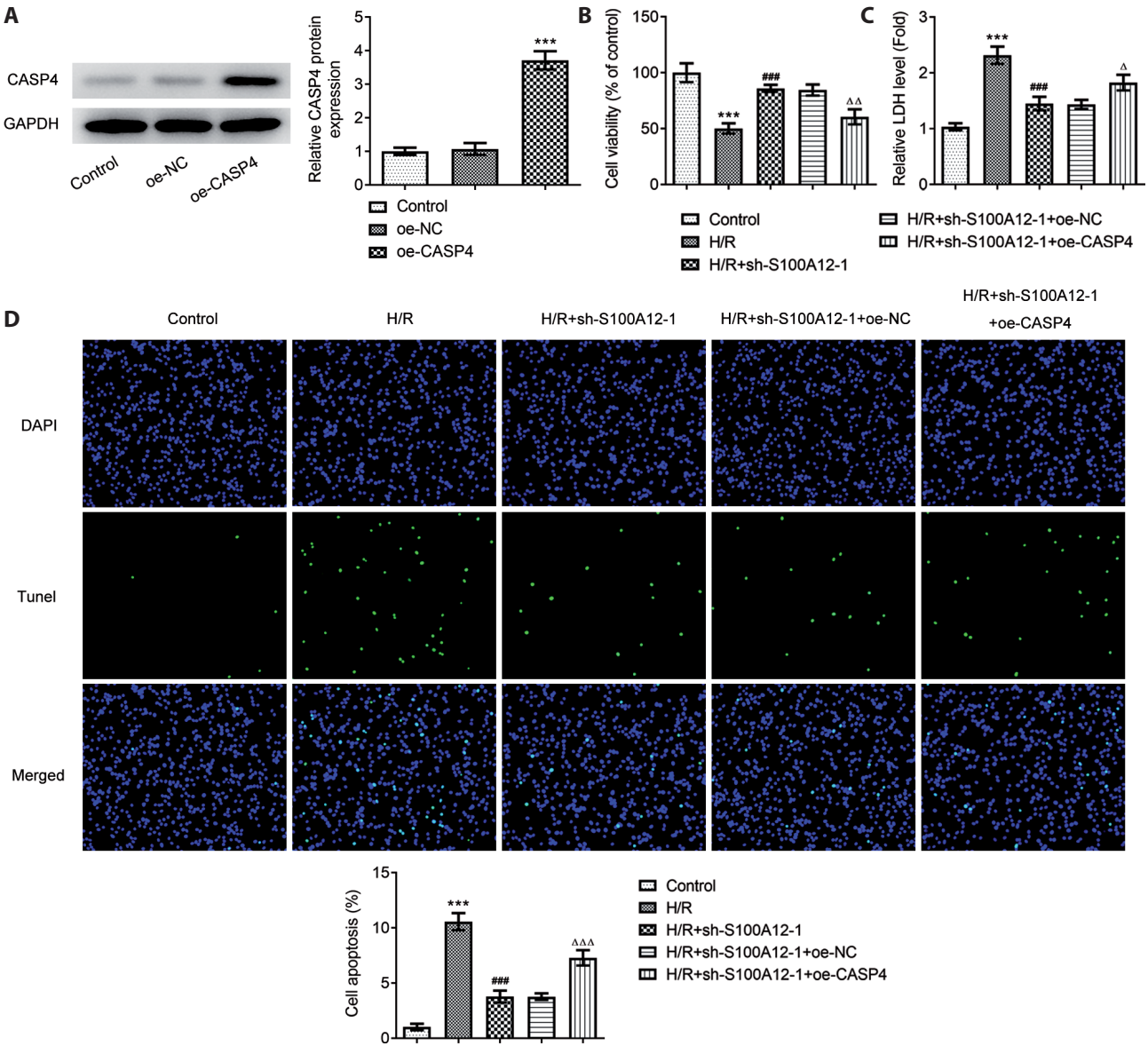

Figure 4. CASP4 overexpression abolishes the protective effects of S100A12 knockdown against H/R-induced AC16 cell injury. **A.** CASP4 expression was examined by Western blotting. *** $p < 0.001$ *vs.* oe-NC. **B.** Cell viability in AC16 cells was measured using MTT assay. **C.** The levels of LDH in AC16 cells were measured using the LDH kit. **D.** Apoptosis of AC16 cells were measured using TUNEL assay. *** $p < 0.001$ *vs.* Control; ### $p < 0.001$ *vs.* H/R; $^{\Delta}$ $p < 0.05$ and $^{\Delta\Delta}$ $p < 0.01$ *vs.* H/R+sh-S100A12-1+oe-NC. CASP4, caspase 4; LDH, lactate dehydrogenase. For more abbreviations, see Figure 1.

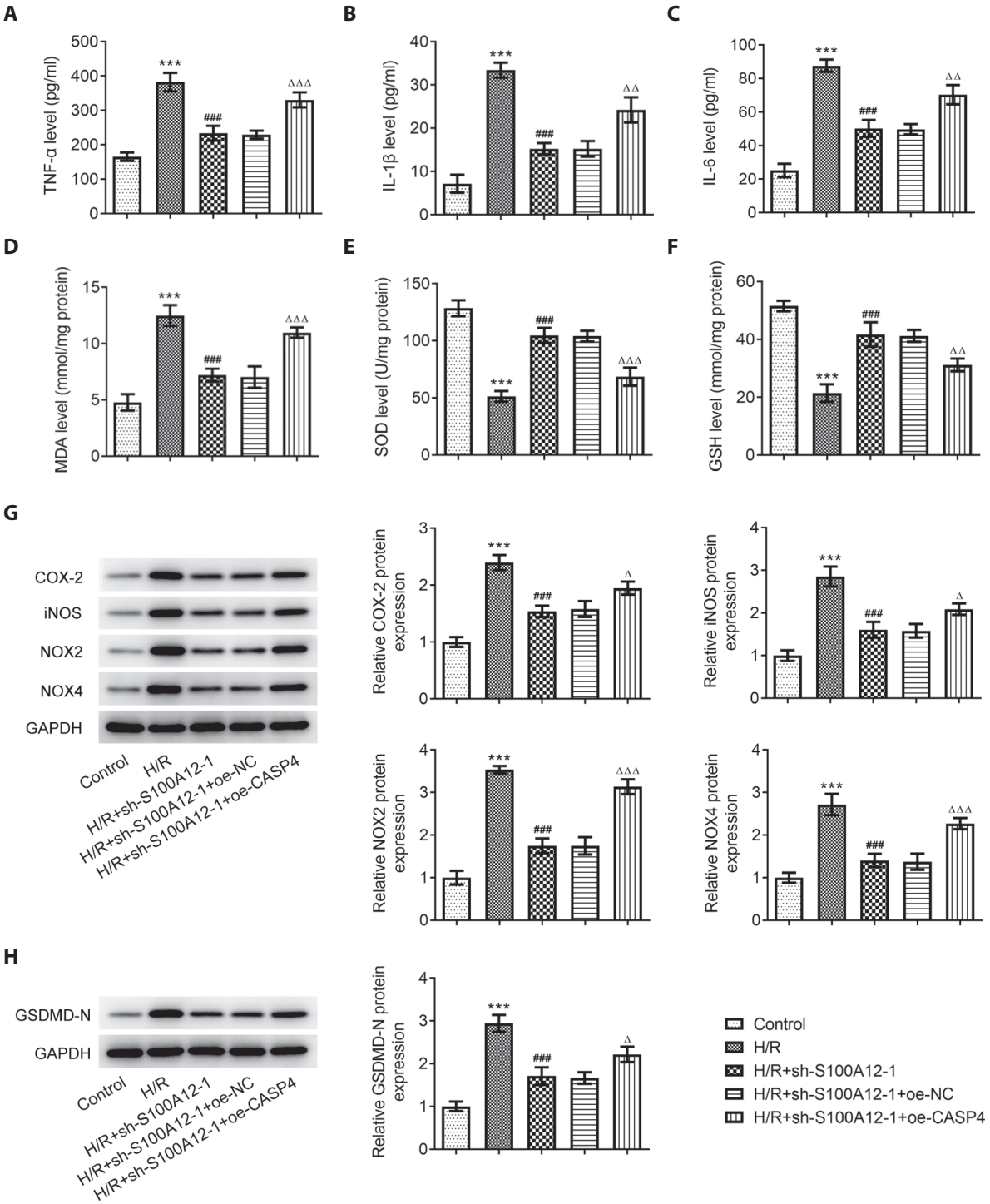

Figure 5. CASP4 overexpression abolishes the protective effects of S100A12 knockdown against H/R-induced inflammation, oxidative stress and pyroptosis in AC16 cells. The levels of inflammatory cytokines TNF-α (**A**), IL-1β (**B**), IL-6 (**C**) in AC16 cells were examined by ELISA. The content of MDA (**D**), SOD (**E**) and GSH (**F**) in AC16 cells was assessed using corresponding commercial kits. **G.** The protein expression levels of COX-2, iNOS, NOX2 and NOX4 in AC16 cells were measured using Western blotting. **H.** The protein expression levels of GSDMD-N in AC16 cells were measured using Western blotting. *** $p < 0.001$ *vs.* Control; $^{###}$ $p < 0.001$ *vs.* H/R; $^{\Delta}$ $p < 0.05$, $^{\Delta\Delta}$ $p < 0.01$ and $^{\Delta\Delta\Delta}$ $p < 0.001$ *vs.* H/R+sh-S100A12-1+oe-NC. Oe, overexpression; MDA, malondialdehyde; SOD, superoxide dismutase; GSH, glutathione; COX, cyclooxygenase; NOX, NAPDH oxidase; iNOS, inducible nitric oxide synthase; GSDMD-N, gasdermin-N. For more abbreviations, see Figure 1.

NOX4 compared with that in the H/R+sh-S100A12-1+oe-NC group (Fig. 5G). Similarly, the inhibitory effects of S100A12 knockdown on GSDMD-N expression were partially abolished by CASP4 overexpression in H/R-induced AC16 cells (Fig. 5H).

Discussion

I/R-induced cardiomyocyte injury in patients following myocardial infarction induces cardiac inflammation, which results in infarct expansion and ultimately heart failure (Reitz et al. 2019). Previously, it has been shown that oxidative stress, inflammation and pyroptosis are key factors in mediating cardiomyocyte injury (Liu et al. 2019; Li et al. 2020). It has also been shown that S100A12 is a viable specific diagnostic marker of inflammation, since its expression is frequently increased in settings of cardiac diseases (Pietzsch and Hoppmann 2009). According to these previous observations, the present study investigated the specific role of S100A12 in cardiomyocyte injury and its associated mechanism. It was found that S100A12 knockdown inhibited the H/R-induced reductions in AC16 cell viability whilst also suppressing inflammation, oxidative stress and pyroptosis induced by H/R. After CASP4 overexpression, these protective effects of S100A12 knockdown against the loss of viability, inflammation, oxidative stress and pyroptosis were reversed in H/R-induced AC16 cells.

S100A12 has been previously reported to promote inflammation, apoptosis and oxidative stress. S100A12 can promote inflammation and apoptosis in sepsis-induced acute respiratory distress syndrome (Zhang et al. 2020b). In addition, S100A12 has been found to promote I/R-induced inflammation, oxidative stress and apoptosis in PC12 cells through the activation of ERK signaling (Zhang et al. 2020a). By contrast, S100A12 knockdown was observed to significantly reverse the inhibitory effects of H_2O_2 on the proliferation, apoptosis and the release of IL-6 and TNF-α from human vascular smooth muscle cells (Jiang et al. 2017). However, the role of S100A12 in cardiomyocytes remains unexplored. Abundant quantities mitochondria are required in cardiomyocytes to produce sufficient ATP to maintain normal cardiac contractile function. However, myocardial ischemia results in the loss of mitochondrial cristae by enhancing the opening of the mitochondrial permeability transition pore, which mediates mitochondrial damage to promote apoptosis (Shi et al. 2019). In the present study, the increased cell viability, decreased levels of apoptosis and LDH release from H/R-induced AC16 cells transfected with sh-S100A12 suggest that S100A12 knockdown can protect AC16 cells from H/R-induced cell viability loss and apoptosis. In addition, I/R can activate the toll-like receptor-dependent induction of proinflammatory cytokines, including TNF-α, IL-1β, and IL-6 (Eltzschig and Eckle 2011). By contrast, since lipid peroxidation leads to the production of MDA following attack by ROS, MDA content can be applied to indirectly reflect the degree of myocardial peroxidative damage (Laumbach et al. 2014). SOD is the key enzyme for destroying ROS in cells, whereby increased SOD activity would indicate restoration and enhancement of the antioxidant defense system (Liu et al. 2019). In the present study, the reduced levels of inflammatory cytokines TNF-α, IL-1β and IL-6, coupled with the elevated levels of oxidative stress markers SOD and GSH but reduced levels of MDA, in addition to the reduced protein expression levels of COX-2, iNOS, NOX2 and NOX4, all indicated that S100A12 knockdown can exert inhibitory effect on inflammation and oxidative stress in H/R-induced AC16 cells.

To further study the mechanism of S100A12 in AC16 cells, the Humanbase website predicted that S100A12 can interact with CASP4 to regulate CASP4 expression in cardiac tissues, which was subsequently verified using Co-IP assays. Following activation, CASP4 specifically cleaves the linker between the amino-terminal gasdermin-N and the carboxy-terminal gasdermin-C structural domains in GSDMD upstream of activating pyroptosis (Shi et al. 2015). This is considered to be a key stage of the non-classical pathway of pyroptosis (Hu et al. 2020). GSDMD has been reported to serve an important role in cardiomyocyte pyroptosis. The NF-κB/GSDMD axis functions as a mechanistic link between oxidative stress and nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain-containing inflammasome-mediated cardiomyocyte pyroptosis (Lei et al. 2018). Suppression of GSDMD was previously found to reduce cardiomyocyte pyroptosis and I/R-induced myocardial injury (Shi et al. 2021). Accordingly, CASP4 may regulate GSDMD in the pyroptotic pathway in cardiomyocytes. Based on the results from the present study, the protective effects of S100A12 knockdown against cell viability loss, apoptosis, inflammation, oxidative stress and pyroptosis in H/R-induced AC-16 cells were partially abolished by CASP4 overexpression. This suggests that S100A12 knockdown may protect AC16 cells against H/R-induced injuries at least partially by regulating CASP4-mediated pyroptosis.

However, limitations still exist in this study. As we know, S100A12 is a calcium-binding protein and it is known that calcium level changes during excitation-contraction coupling. The calcium ions can interact with S100A12 by binding on two EF-hand structures, which can induce some changes in the protein structure. Thus, even though we used H/R-induced AC16 cells to mimic myocardial I/R injury, the *in vivo* conditions were complicated. An *in vivo* validation may improve our findings. In addition, we only used co-IP to preliminarily confirm the interaction between S100A12 and CASP4, but how did they bind to each other and the more precise molecular mechanisms required an in-depth research.

To the best of our knowledge, the present study is the first to investigate the protective effects of S100A12 on H/R myocardial injury *in vitro*. These findings suggest that S100A12 knockdown can alleviate H/R-induced cardiomyocyte injury by regulating the non-classical CASP4-dependent pyroptotic pathway. Therefore, S100A12 may serve an important role in ischemic heart disease and is a promising target for clinical treatment.

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Conflict of interest. The authors declare no conflicts of interest.

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