Tanshinone IIA alleviates hypoxia/reoxygenation induced cardiomyocytes injury via lncRNA AK003290/miR-124-5p signaling

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Abstract

Objective

Acute myocardial infarction (AMI) is the leading cause of death and one of the heaviest healthy burden globally nowadays. Tanshinone IIA (TSA) is the major active compound extracted from *Salvia miltiorrhiza* Bunge possessing various pharmacology activities. The present study aimed at investigating the role of TSA in AMI and underlying mechanism.

Methods

Quantitative real-time PCR (qRT-PCR) and western blot assay were performed to detect the expression of genes and proteins. Cell apoptosis was detected by Annexin/V PI staining and flow cytometry. The amount of LDH, MDA and ROS were detected using commercial kits. FISH experiment was used to detect the expression of AK003290 in cardiomyocytes. Luciferase activity assay was performed to verify the interaction between AK003290 and miR-124-5p.

Results

We have shown that TSA decreased the apoptosis rate, the amount of LDH, MDA as well as ROS of cardiomyocytes. Meantime, it elevated mitochondrial membrane potential (MMP) which was decreased by H/R treatment. It was also determined that miR-124-5p targets AK003290 directly. TSA up-regulated the expression of AK003290 and its function can be reversed by knock down of AK003290 as well as miR-124-5p overexpression.

Conclusion

TSA exerts the protective role against H/R induced apoptosis, oxidative and MMP loss of cardiomyocytes via regulating AK003290 and miR-124-5p signaling.

Background

Acute myocardial infarction (AMI) is characterized by the block of blood supply to the heart. It is the leading cause of death and one of the heaviest healthy burden globally nowadays [1, 2]. Restoration of blood flow has been demonstrated as one of the most effective therapeutic methods to prevent the hearts from dysfunction or damage caused by imbalance of oxygen demand and supply. However, the recovery of blood supply termed as reperfusion together with reoxygenation will result in exacerbation of tissue injury as well as inflammatory responses which is called ischemia reperfusion (I/R) injury.

Tanshinone IIA (TSA) is the major active compound from the root extracts of *Salvia miltiorrhiza* Bunge, which is prescribed for treating cardiovascular disease in the pharmacopoeia of China as a traditional Chinese medicine (TCM) "Danshen". TSA has been shown to exert anti-angiogenic, antioxidant, anti-inflammatory and antitumor activities. Increasing evidences have demonstrated the anti-tumor function

of TSA in human cancers such as liver, cervical, gastric, colorectal, prostate, bladder, and breast cancers [3–10]. Moreover, due to its anti-oxidative and anti-inflammatory effects, TSA was indicated to possess cardio-protective effects. For instance, TSA has an anti-oxidative effect and prevents atherosclerosis by reducing vascular oxidative stress, inhibiting platelet aggregation, and protecting from endothelium damage [11–14]. Tanshinone IIA pretreatment protects myocardium against ischaemia/reperfusion injury through the phosphatidylinositol 3-kinase/Akt-dependent pathway in diabetic rats [15].

In the present study, we focused on the function of TSA on myocardial ischemic reperfusion injury. In vitro studies revealed that TSA alleviated the apoptosis, oxidative stress and MMP loss of cardiomyocytes subjected to hypoxia/reoxygenation (H/R) treatment. Mechanically, we found that TSA participates in the regulation of lncRNA AK003290/miR-124-5p signaling. These findings offer important insights into fundamental mechanisms underlying functions of TSA and lncRNAs, meantime, provided novel therapeutic targets for cardiac ischemic injury.

Methods

Cell culture

H9c2 cell were obtained from Cell bank of Chinese Academy of Sciences and cultured in DMEM (Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, CA, USA), 100 U/mL penicillin and 100 µg/mL streptomycin under 5% CO₂ at 37 °C in a humidified atmosphere.

Drug treatment

TSA were purchased from National Institute for the Control of Pharmaceutical and Biological Products (> 99% purity, Beijing, China). Cells were treated with TSA (0.5, 1, 5 µM) respectively 2 hours before inducing the hypoxia and during the hypoxia period.

Plasmids construction

MiR-124-5p mimic and mimic control as well as siRNA for AK003290 and the negative control are designed and synthesized by Genepharma (Shanghai, China). The wild and mutant region of AK003290 targeted by miR-124-5p were synthesized by Genepharma (Shanghai, China) and cloned into pGL3 luciferase reporter vectors (Promega, CA, USA).

Flow cytometry

The cultured H9c2 cells were digested with trypsin, washed with cold PBS and dualstained with AnnexinV FITC/propidium iodide according to the manufacturer's instructions. Cell apoptosis was detected by flow cytometry on a BD FACSCalibur (Becton Dickinson, NJ, USA).

Reverse transcription and quantitative realtime PCR

RNA extraction were performed with Trizol reagent (Invitrogen, CA, USA), precipitated with isopropanol, washed with 75% ethanol and dissolved in RNase free water. Reverse transcription were performed with 1 µg RNA using the cDNA transcription kit (Transgen, USA). 20 ng cDNA was used for qPCR to validate the expression of relative mRNA. It was determined by using SYBR green mix (Yisheng, Shanghai, China).

Western blotting

Cells were lysed on ice by lysis buffer (Beyotime, Shanghai, China), and the protein were extracted by centrifugation at 12000 g, 10 min, 4 °C. Protein lysates were loaded with 5 × loading buffer on the SDS-PAGE. After electrophoresis, the gel was transferred to PVDF membrane. Followed with blocking with skim milk for 1 hour, protein bands on the PVDF membrane were incubated with relative primary antibodies at 4 °C overnight and the corresponding secondary HRP antibody at room temperature for 2 hour. The blots were visualized by ECL chemiluminescence.

RNA Pull-down assay

Biotinylated miR-124-4p probe and the control probe were synthesized by Sangon Biotech (Shanghai, China). Probe-coated beads were generated by coincubating the probe with streptavidin-coated beads (Invitrogen, CA, USA) at 25°C for 2 h. H9c2 cells were collected, lysed and incubated with AK003290 or miR-124-5p probes overnight at 4°C respectively. Thereafter, the beads were eluted and the complex was purified with TRIzol (Takara, Dalian, China). Then the abundance of AK003290 and miR-124-5p was analyzed by qRT-PCR.

LDH and MDA detection

After reperfusion, the hearts were resected and homogenized in the cold phosphate buffer. After centrifuge at 3000 rpm for 15 min the supernatant were collected for measurement at -20°C. The level of LDH as well as MDA were evaluated using commercial kits following their manufacturer's protocols (Jiancheng, Nanjing, China)

ROS detection

After I/R treatment, myocardial tissue or cardiomyocytes were collected and washed in PBS followed by co-culturing with 10 µM DCFH-DA (DCF-DA, Shanghai, Beyotime) at 37 °C in an incubator for 20 min with gentle shaking in the dark. The mean fluorescence intensity (MFI) was evaluated using a flow cytometry (BD FACSCalibur, Becton Dickinson, NJ, USA).

Fluorescence in situ hybridization (FISH)

Alexa Fluor 555-labeled AK003290 probes were designed and synthesized by RiboBio (Guangzhou, China). FISH experiment was carried out with a fluorescent in Situ Hybridization Kit (RiboBio, Guangzhou, China). 1×10^5 cells were seeded onto autoclaved glass slides and cultured for 24 h. After fixing with 4% paraformaldehyde for 20 min followed by permeabilization with 0.5% Triton X-100 for 10 min, the cells were cultured at 37°C overnight. Finally, the slides were incubated with DAPI to stain cell nuclei and observed under a fluorescence microscope (Leica, Wetzlar, Germany).

Luciferase activity assay

The wild-type or mutant seed sequence at the predicted region of AK003290 and miR-124-5p were synthesized and cloned into the pGL3 Luciferase Reporter vectors (Promega, CA, USA) at the KpnI and BamHI sites. H9c2 cells were co-transfected with miR-124-5p mimics or mimic control, together with pGL3 vectors, which contained the WT or mutant predicted binding region of AK003290. TRL-SV40 plasmid (Promega, CA, USA) was also transfected as a normalizing control. The cells were harvested for the detection of the activity of luciferase with the use of the Dual-Luciferase Assay (Promega, WI, USA) at 48 h following the transfection.

Statistical analysis

SPSS 20.0 software (SPSS Inc., Chicago, IL, USA) were used to analyze all data for statistical significance. All the data are presented as the means $\pm$ SD. One-way ANOVA was used to assess the difference between multiple groups. Differences between two groups were analyzed by the Student's t-test. $P < 0.05$ was considered as statistical significance.

Results

TSA alleviated H/R induced apoptosis, oxidative stress and loss of mitochondrial membrane potential

TSA has been reported to be capable of attenuating the I/R injury in cardiomyocytes. We first established H/R model of cardiomyocytes to evaluate this function of TSA. As Fig. 1A showed, TSA of 30 and 60 μ M reduced the apoptosis of cardiomyocytes induced by H/R. In addition, MDA, LDH and ROS were detected to evaluate the oxidative stress of cardiomyocytes. We found that H/R significantly elevated the level of MDA, LDH and ROS, meantime, TSA of 30 and 60 μ M notably reduced this elevation (Fig. 1B-D). Moreover, MMP was evaluated via JC-1 staining. As showed in Fig. 1E, TSA of 30 and 60 μ M elevated the MMP which was decreased by H/R treatment. In order to further verify the effect of TSA on cell apoptosis, we detected the expression level of apoptotic proteins. It was indicated that H/R promoted expression

level of apoptotic protein such as bax, cytoplasmic cyt-c, cleaved caspase3 while inhibited that of anti-apoptotic protein bcl-2. 3 dose of TSA reversed these alteration induced by H/R (Fig. 1F).

TSA promoted the expression of AK003290

To explore novel mechanisms underlying the effect of TSA. We focused on the lncRNA which has attracted a lot of attention due to their various functions. We searched several lncRNAs that have been reported to play critical roles in the cardiovascular system diseases and evaluated their expression level after TSA treatment. The results indicated that TSA elevated the level of AK003290, MEG3, CASC7, HOTAIR while decreased that of ROR (Fig. 2A). Further, we selected and evaluated the expression of AK003290 after H/R and TSA treatment using qPCR and FISH assay. As Fig. 2B revealed, AK003290 was notably down-regulated by H/R treatment. TSA significantly reversed the effect of H/R (Fig. 2B, C).

Knock down of AK003290 reversed the effect of TSA on H/R injury

As AK003290 was down-regulated by H/R and up-regulated by TSA treatment, we speculated that AK003290 exerts the protective role against H/R injury in cardiomyocytes. We established the siRNA for AK003290. As expected, knock down of AK003290 reversed the effect of TSA (30 μ M) on apoptosis (Fig. 3A). Moreover, the same trend was found in the oxidative stress (Fig. 3B-D) and loss of MMP (Fig. 3E). Again, we detected the expression of apoptotic proteins. AK003290 knock down increased the expression level of bax, cytoplasmic cyt-c, cleaved caspase3 and reduced that of bcl-2 in cardiomyocytes compare to TSA treatment group (Fig. 3F).

AK003290 sponges miR-124-5p in cardiomyocytes

As we know, lncRNAs are capable of sponging miRs to block their regulatory function on target genes. Here, we predicted and verified the miR targeting AK003290 to further elucidate the mechanism underlying the effect of AK003290. Figure 4A showed the targeting sequences between AK003290 and miR-124-5p. Luciferase activity assay indicated that miR-124-5p directly targets AK003290 in cardiomyocyte (Fig. 4B). Furthermore, the level of miR-124-5p was notably reduced in AK003290 knock down group (Fig. 4C). RNA pull down was carried out to detect whether AK003290 could directly bind to miR-124-5p endogenously. We found that biotin labeled miR-124-5p probe enriched the level of AK003290 which indicating the interaction between AK003290 and miR-124-5p (Fig. 4D). Moreover, the qPCR analyses further confirmed that miR-124-5p overexpression decreased the level of AK003290 while knock down of miR-124-5p increased that of AK003290 (Fig. 4D). Pearson analysis was finally carried out which indicated a negative correlation between miR-124-5p and AK003290 (Fig. 4E).

MiR-124-5p overexpression reversed the effect of TSA on H/R injury

To confirm whether miR-124-5p participates in the function of TSA on H/R injury. Rescue experiment was carried out. It was found that miR-124-5p overexpression reversed the effect of TSA on apoptosis (Fig. 5A). Moreover, the same trend was found in the oxidative stress (Fig. 5B-D) and loss of MMP (Fig. 5E). We further detected the expression of apoptotic proteins and found that miR-124-5p overexpression increased the expression level of bax, cytoplasmic cyt-c and cleaved caspase3 and reduced that of bcl-2 of cardiomyocytes in comparison to TSA treatment group (Fig. 5F).

Discussion

Apoptosis is well known to be the critical form of myocardial cells death induced by dysfunction of mitochondria and increase of lipid peroxides contributes mainly to I/R injury. Deeper understanding of the mechanisms of cardiomyocyte apoptosis is critical to prevent heart injury and treat heart disease. In the present study, we detected apoptosis of cardiomyocytes using flow cytometry. To further evaluate the apoptosis condition of cardiomyocytes, we detected the MMP as well as apoptotic proteins including bax, cytoplasmic cyt-c, bcl-2 and cleaved caspase3. It was found that TSA reduced the apoptosis of cardiomyocytes subjected to H/R and also alleviated the oxidative stress and MMP loss induced by H/R treatment.

TSA has been extensively studied in I/R injury not only in cardiovascular system but also in cerebral I/R injury. These findings proved the protective function of TSA on myocardial I/R injury to a great extent. However, the molecular mechanism underlying the effect of TSA is extremely complicated. It was reported that TSA participate in the regulation of classic signal pathways such as AMPKs/mTOR [16], PI3K/Akt/FOXO3A/Bim [17], MAPK [18] pathways as well as some key proteins including TORC1, pCREB, BDNF [19] and Na⁺/K⁺-ATPase[20]. However, the interaction between TSA and lncRNAs has been rarely studied.

Emerging evidences have shown that non-coding RNAs such as miRNAs and lncRNAs possess crucial roles in many biological processes. Previously, TSA was found to be capable of inhibiting miR-1 expression through p38 MAPK signal pathway in post-infarction rat cardiomyocytes [21]. In the present study, we identified that lncRNA AK003290 was down-regulated in cardiomyocyte subjected to H/R which indicated its protective role against H/R injury. Interestingly, TSA treatment elevated the level of AK003290. We speculated that AK003290 is involved in the effect of TSA on alleviating H/R injury. Further study revealed that knock down of AK020546 notably reversed the effect of TSA reducing cardiomyocyte apoptosis, oxidative stress and loss of MMP. These findings verified that TSA protected cardiomyocytes from H/R induced injury via up-regulating AK003290 expression.

It is well known that lncRNAs always act as sponges of miRNA thus participate in the regulation of their target genes that are involved in a common cellular function. Previous studies have reported that the APF,

MALAT1, PFL mediates I/R injury through sponging miR-188-3p, miR-155/miR-203/miR-133 and let-7d.respectively. Consequently, to further understand the critical role of AK003290 in cardiac I/R injury, we further explored the molecular mechanism by which AK003290 exerts its functions. Bioinformatic tools predicted that AK003290 is a potential target of miR-124-5p, luciferase activity assay verified our speculation indicating AK003290 as a direct target gene of miR-124-5p. Finally, rescue experiment confirmed our speculation indicating miR-124-5p as a downstream gene regulated by TSA. It is the first report demonstrating the function of TSA through lncRNA/miR signaling.

MiR-124-5p is a novel miRNA which has rarely been studied. We can know from the previous studies that miR-124-5p inhibits the growth of high-grade gliomas through posttranscriptional regulation of LAMB1 [22]. And low expression levels of microRNA-124-5p correlated with poor prognosis in colorectal cancer via targeting of SMC4. Here, we confirmed that miR-124-5p directly target AK003290. We performed rescue experiments and found both AK003290 knock down and miR-124-5p overexpression can reverse the effect of TSA. To further confirm the relation between AK003290 and miR-124-4p. Rescue experiment between AK003290 and miR-124-5p should be carried out.

Conclusion

In conclusion, we extended the understanding of the role of lncRNA in cardiac ischemia/reperfusion injury and provided a novel regulatory mechanism underlying the effect of TSA that is AK003290/miR-124-5p signaling. Our findings suggest novel biomarkers or potential therapeutic targets to treat ischemic heart diseases.

Declarations

Ethics approval and consent to participate

Not available.

Consent to publish

Not available.

Availability of data and materials

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

Competing interests

There is no competing interests in the present study.

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Authors' Contributions

study design: George Liu

experimental studies: Liye Chen, Lili Wei, Qiongyang Yu

Statistical analysis: Haozhe Shi,

Manuscript preparation: George Liu

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Not available.

Abbreviations

LncRNA

Long non-coding RNA

AM

Acute myocardial infarction

TSA

Tanshinone IIA

I/R

ischemia reperfusion

H/R

hypoxia/reoxygenation

MMP

mitochondrial membrane potential

LDH

lactate dehydrogenase

MDA

Malondialdehyde

ROS

Reactive oxygen species

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Figures

Figure 1

TSA alleviated the apoptosis, oxidative stress and MMP loss induced by H/R. (A) Apoptosis of cardiomyocytes was evaluated using Annexin/VI-PI staining under different dose of TSA treatment. (B) MDA and LDH amount in cardiomyocytes were analyzed using commercial kit. (C) The ROS of cardiomyocytes was detected using commercial kit and flow cytometry. (D) Western blot was carried out to detect the apoptotic protein expression such as bax, cytoplasmic cyt-c, cleaved caspase3 and bcl-2. *p<0.05.

Figure 2

TSA promoted the expression level of AK003290. (A) qPCR was used to evaluate the expression of several lncRNAs correlated with heart disease. (B) qPCR was used to evaluate the expression of AK003290 in cardiomyocytes under different dose of TSA treatment. (C) FISH experiment was used to evaluate the expression of AK003290 under different dose of TSA treatment. *p<0.05.

Figure 3

Knock down of AK003290 reversed the effect of TSA. (A) Apoptosis of cardiomyocytes was evaluated using Annexin/VI-PI staining under TSA treatment and si-AK003290 transfection. (B) MDA and LDH amount in cardiomyocytes were analyzed using commercial kit. (C) The ROS of cardiomyocytes was detected using commercial kit and flow cytometry. (D) Western blot was carried out to detect the apoptotic protein expression such as bax, cytoplasmic cyt-c, cleaved caspase3 and bcl-2. * $p \leq 0.05$.

Figure 4

MiR-124-5p directly targets AK003290 in cardiomyocytes. (A) targeting region between miR-124-5p and AK003290. (B) Luciferase activity assay was carried out to verify whether miR-124-5p targets AK003290 in cardiomyocytes. (C) qPCR was performed to detect the expression of miR-124-5p. (C) RNA pull down using the probe of miR-124-5p was performed to detect the interaction between miR-124-5p and AK003290. (E) Pearson analysis was performed to investigate the correlation between circCadm1 and Pawr as well as miR-124-5p targets AK003290. * $p \leq 0.05$.

Figure 5

MiR-124-5p overexpression reversed the effect of TSA. (A) Apoptosis of cardiomyocytes was evaluated using Annexin/VI-PI staining under TSA treatment and miR-124-5p mimic transfection. (B) MDA and LDH amount in cardiomyocytes were analyzed using commercial kit. (C) The ROS of cardiomyocytes was detected using commercial kit and flow cytometry. (D) Western blot was carried out to detect the apoptotic protein expression such as bax, cytoplasmic cyt-c, cleaved caspase3 and bcl-2. * $p < 0.05$.