Manuscript title:

Myocardial ischemia-reperfusion injury is attenuated by sodium arsenite which inhibits NLRP3 inflammasome activation and induces a cellular stress response

Author

Min Li[1], Yingwu Mei[1], Jingeng Liu[2], Kaikai Fan[3] , Xu Zhang [1], Jitian Xu[4], Yuebai Li[1], Yang Mi[1*]

1. Department of Biochemistry and molecular biology, Basic Medical College, Zhengzhou University, No.100 Science Avenue, Zhengzhou, China, 450001.

2. Department of Thoracic Surgery, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, 450052.

3. Department of Cardiovascular Surgery, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, 450052.

4. Department of Physiology, Basic Medical College, Zhengzhou University, China, 450001.

***Corresponding author. Tel: 13503860420; email: miyang1980@126.com**

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Abstract

Numerous studies focus on reducing the myocardial ischemia and reperfusion injury which produces a spectrum of clinical syndromes. NLRP3 inflammasome, Heme oxygenase-1 (HO-1) and heat shock proteins are all involved in myocardial ischemia-reperfusion pathophysiology. Through a targeted chemical screen, we identify sodium arsenite as a potent inhibitor of NLRP3 inflammasome. We show that the delivery of arsenite 30 minutes before myocardial ischemia limits infarct size and preserves left ventricular (LV) function in an in vivo model of myocardial ischemia/reperfusion (I/R). Further studies show that arsenite induces eIF2α phosphorylation and downstream gene expression which may be beneficial for cell survival.

Introduction

During the last 30 years, considerable effort has focused on limiting myocardial infarct size and post-ischemic injury [1]. Reperfusion strategies have been widely incorporated into clinical practice to reduce ischemic injury. However, reperfusion itself causes additional cardiac myocyte death which is termed myocardial ischemia-reperfusion (I/R) injury. Great efforts have been devoted to develop new strategies to alleviate I/R injury. The most representative cardio-protection methods are ischemic pre- and post conditioning which refers to brief episodes of coronary occlusion/reperfusion preceding or following sustained myocardial ischemia with reperfusion [2, 3].

Increasing evidence indicates that NLRP3 inflammasome plays an essential role in the pathogenesis of myocardial ischemia-reperfusion injury[4-6]. Inflammasome is a molecular platform activated upon signs of cellular danger to trigger innate immune defenses. NLRP3 inflammasome also mediates the sterile inflammatory response triggered by tissue damage. Inflammasome activation in cardiac fibroblasts, but not

in cardiomyocytes, is crucially involved in the initial inflammatory response after myocardial I/R injury. Blocking inflammasome activation by genetic method markedly diminishes infarct development and myocardial fibrosis and dysfunction [4-6].

In a screening for chemicals that influence inflammasome activation, one hit that drew our attention was the compound sodium arsenite. Arsenic is a metalloid that generates various biological effects on living organism. Cells exposed to arsenite start a cytosolic stress response including attenuation of general translation through phosphorylation of eukaryotic translation initiation factor 2 (eIF2α) which is an important strategy in the cell's armory against stressful insults [7-9]. Sodium arsenite also induces proteins with roles in stress response such as heme oxygenase-1 (HO-1), heat shock proteins, CHOP and GADD34 in various types of tissue and cells [10-15]. It's reported that cardiac-specific expression of heme oxygenase-1 or heat stress proteins protected against ischemia and reperfusion injury in mice [14-17].

Arsenic trioxide (As_2O_3) induces apoptosis and was used in the treatment of acute promyelocytic leukemia (APL) and showed a striking effectiveness in china [18, 19]. Arsenic targets Pin1 and cooperates with retinoic acid to inhibit cancer-driving pathways and tumor-initiating cells [20].

We wonder the effect of arsenite on myocardial ischemia-reperfusion injury. The in vivo myocardial ischemia-reperfusion model in rats showed that arsenite protected rather than further damaged myocardial cells during the first 24 hours.

Results and Discussion

Arsenite blocks NLRP3 inflammasome activation

In a screening for chemicals that acted on inflammasome activation, we found that sodium arsenite strongly inhibited IL-1β secretion. To determine the effects of arsenite on NLRP3 inflammasome activation, bone-marrow-derived macrophages (BMDMs) or THP-1 cells were treated with arsenite before

LPS priming or before agonist challenge. We observed that arsenite blocked IL-1b secretion at both situations. It has been reported that arsenite can inhibit protein synthesis [9] and NF-κB activation [21], we then examined whether arsenite had an impact on LPS-induced priming for inflammasome activation. As shown in Figure 1C, Arsenite inhibited LPS-induced pro-IL-1b, pro-caspase-1 and NLRP3 expression.

It's recently reported that recruitment of NLRP3 to dispersed trans-Golgi network (dTGN) is an early and common cellular event that leads to NLRP3 aggregation and activation [22,23]. Different NLRP3 stimuli lead to disassembly of the TGN. To explore whether arsenite has any effect on the recruitment of NLRP3 to dTGN, U2OS and HeLa cells transfected with flag tagged NLRP3 were treated first with arsenite and then with nigericin. Fluorescence microscopy experiments showed that flag_NLRP3 aggregated around nucleus in U2OS or formed small dots in HeLa cells upon nigericin treatment, whereas arsenite decreased the NLRP3 aggregation.

Arsenite attenuates myocardial ischemia-reperfusion injury

It was reported arsenic trioxide induced apoptosis in cancer cells and here we found that arsenite blocks NLRP3 inflammasome activation which is essential for myocardial ischemia/reperfusion injury, thus we wonder what effect arsenite has on myocardial I/R injury. The in vivo model of myocardial ischemia-reperfusion was used to answer this question. Rats were subjected to 40min of LV ischemia and 24h reperfusion. Arsenite was administered by intraperitoneal injection 30min before ligation of the left anterior descending (LAD) coronary artery at dose of 4.5mg/kg. Evaluation of infarct size revealed a significant cyto-protection effect of arsenite as assessed by 2,3,5-triphenyltetrazolium chloride (TTC) staining. Surprisingly, rats receiving 4.5mg/kg arsenite displayed a lower ratio of infarct size to area-at-risk (INF/AAR), as shown in Figure 2 A and B.

To determine the effect of arsenite on cardiac function after I/R, we measured ejection fraction (EF) and

fractional shortening (FS) by echocardiography. The EF and FS were at a relative low level at 1day after myocardial I/R in contrast with the reference, however, treatment with arsenite increased EF and FS after 1 day reperfusion, indicating improved cardiac function. To test whether arsenite affects hemodynamic properties, we measured systolic pressure and diastolic pressure by placing a catheter into the femoral artery of rats at 24 hour after myocardial I/R. The heart rates, systolic pressure, diastolic pressure and pulse pressure were not significantly changed in each group.

Arsenite induces eIF2α phosphorylation and heme oxygenase-1 expression in myocardial tissue

Arsenite was shown to have intricate effect on living organisms. Exposure to arsenite inhibits protein synthesis and activates multiple stress signaling pathways. Phosphorylation of eIF2 at Ser-51 on the alpha subunit was necessary to inhibit protein synthesis initiation in arsenite-treated cells [9]. The eIF2α kinase regulates starvation- and virus-induced autophagy [7] and eIF2α phosphorylation protects cell from ER stress [8]. Sodium arsenite also induces proteins with roles in stress response such as heme oxygenase-1 (HO-1), heat shock proteins, CHOP and GADD34 in various types of tissue [10-17].

To explore whether arsenite has the biological effects as mentioned above on heart, cells or mice were simply treated with arsenite. We first examined the eIF2α phosphorylation and the expression of its downstream genes upon arsenite treatment. As expected, arsenite induced phosphorylation of eIF2α in rodent myocardial tissue and HeLa cells as early as in 1h. The expression of CHOP and GADD34, downstream of eIF2α phosphorylation, was up-regulated in heart tissue as well as in brain at 6-7h after peritoneal injection of arsenite (Fig. 3A, C). We also examined the effect of arsenite on heme oxygenase-1 expression. As shown in Figure 3C, HO-1 mRNA can be induced by arsenite in the heart tissue but not in brain and liver tissue of rats at 7h. The HO-1 protein was not seen increased at 8h after arsenite treatment

111 in cardiac tissue of mice, whereas arsenite indeed induced HO-1 expression in HeLa cells (Fig 3B).

112

113 The major findings of this study are as follows: (1) Arsenite inhibits NLRP3 inflammasome activation by
114 inhibiting both the priming and activation stage; (2) Arsenite attenuates the myocardial
115 ischemia-reperfusion injury in rats; (3) Arsenite induces eIF2α phosphorylation and HO-1 in myocardial
116 tissue. However, the causal relationship among these activities needs further proof.
117 The relationship between NLRP3 inflammasome and ischemia-reperfusion injury has been studied for
118 several years. Arsenite at least partially acts on myocardial tissue by this way.
119 The most interesting finding is that arsenic trioxide induces apoptosis of tumor cells, whereas arsenite help
120 cells survive during myocardial I/R. The cytosolic stress induced by arsenite may explain its distinct effect
121 on tumor cells and myocardial tissues. The pre-induced cytosolic stress response including eIF2α
122 phosphorylation, heme oxygenase-1 and heat shock protein induction may be beneficial for cell survival
123 during I/R.
124 Pre- and post conditioning with ischemia succeed in significantly reducing infarct size in animal models [2,
125 3]. Both treatments in fact induce a cellular stress response, and arsenite may induce a similar cardiac stress
126 response which is protective for the subsequent ischemia injury.

127 **Materials and Methods**

128 **2.1 In vivo myocardial I/R**

129 SD rats were intraperitoneally anesthetized with Pentobarbital, orally intubated with Teflon tubes
130 (1.2/1.6mm), and connected to a rodent ventilator. A left thoracotomy was then performed. The chest
131 muscle was set aside, and the third and fourth ribs were break off. The area around left auricle and
132 pulmonary arterial cone was exposed. The left anterior descending (LAD) coronary artery was ligated

using 6-0 silk suture around a fine tube with a firm knot. Rats were subjected to 40 minutes of LAD ischemia followed by 24h of reperfusion. The infarct area was determined by 2,3,5-triphenyltetrazolium chloride (TTC) staining. All animal studies were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. All animal experiments performed in this study adhered to the protocols approved by the Institutional Animal Care and Use Committee of Zhengzhou University.

2.2 Infarct area assessment

After 1-day of reperfusion, rats were anesthetized, Evans blue solution (3%, 1ml) were directly injected into the right ventricle, 1 minute later, the heart was quickly excised, immediately frozen, and sliced. Sections were then incubated in a 1% TTC solution which was freshly prepared, and the slices must be covered with a glass sheet during staining. After staining, sections were digitally photographed and the infarct area was determined by computerized planimetry.

2.3 Echocardiography

After 24 hr of reperfusion, echocardiography was performed using a VEVO 2100 high-resolution in vivo imaging system (FUJIFILM Visual Sonics, Toronto, Ontario, Canada). Under anesthesia, the chest of the rat was shaved, and two-dimensional echocardiography was performed. Cardiac function was evaluated by M-mode. Left ventricular (LV) end-diastolic diameter and LV end-systolic diameter were measured on the parasternal LV long-axis or short-axis view. Left ventricular ejection fraction (EF) and fractional shortening (FS) were calculated with computerized algorithms. All measurements represented the mean of five consecutive cardiac cycles.

2.4 Arterial pressure measurements

After 24 hr of reperfusion, before TTC staining method was performed, mice were anesthetized. The

femoral artery was dissected and was cannulated using a catheter pre-filled with heparinized normal saline (0.5 IU/ml). After cannulation, the other end of the catheter was connected to the transducer. The arterial systolic pressure and diastolic pressure was monitored by a biological Signal Acquisition and Analysis System (BL-420F, TECHMAN, Chengdu, China).

2.5 Cell culture and inflammasome stimulation

Human THP-1 cells were grown in RPMI 1640 medium, supplemented with 10% FBS. THP-1 cell were differentiated for 3 hr with 100 nM phorbol-12-myristate-13-acetate (PMA). Bone marrow was flushed from mouse femurs and cultured in bone marrow media (RPMI 1640 containing 20% heat-inactivated fetal bovine serum, 30% L929 cell media, 2 mM L-glutamine) for 7 d at 37℃ in 4 100-mm polystyrene dishes to obtain mature, differentiated macrophages.

For inducing IL-1 , 10^6 macrophages were plated in 12-well plate overnight and the medium was changed to opti-MEM in the following morning, and then the cells were primed with ultrapure LPS (100 ng/ml) for 3 hr. After that, arsenite were added into the culture for 5 minutes, and then the cells were stimulated with ATP (5 mM, pH adjusted to 7.5) or Nigericin (10 M) for 1 hour, or rotenone (10 M) for 3 h.

2.6 Immunostaining

U2OS or HeLa cells were plated on coverslips for 1 day and transfected with pcDNA6-flag-hNLRP3 vector for 36 hours. After that, the cells were treated with nigericin (10 μM) for 30min and stained with antibody.

For immunostaining, cells were fixed with 4% paraformaldehyde for 15 minutes and permeabilized with 0.1% Triton X-100 in PBS, before incubation with anti-FLAG M2 antibody followed by Cy3 secondary antibodies. Nuclei were stained with DAPI.

177 **2.7 Western blotting**

178 The antibodies for phospho-eIF2 Ser51 were purchased from elabscience (E-AB-20864). The antibodies
179 for eIF2 (sc-133132) GADD34 (sc-373815), GADD153 (sc-7351) and Heme Oxygenase 1
180 (sc-136960) were from Santa Cruz biotechnology.

181 Mice intraperitoneally injected with arsenite (4.5ug/g) for various periods were sacrificed and organs were
182 excised quickly. Protein was extracted from frozen tissue by grinding using a pre-chilled mortar. The RIPA
183 lysis buffer (50mM Hepes pH7.5, 150mM NaCl, 2mM EDTA, 2mM EGTA, 1% TritonX-100, 50mM NaF,
184 5mM Sodium Pyrophosphate, 50mM Sodium glycerophosphate, 1mM $NaVO_3$, 1mM DTT, 1mM PMSF,
185 10g/ml Leupeptin, 10g/ml Aprotinin) was used for protein preparation. Following SDS-PAGE,
186 nitrocellulose membranes were blocked with 3% skim milk, and immunoblotting was performed as
187 described elsewhere.

188 **2.8 RT-PCR**

189 Total RNA was extracted from hearts, brain and liver tissue of rats intraperitoneally injected with PBS or
190 arsenite (4.5ug/g) for 7 hours. Semi-quantitive PCR were performed to detect the mRNA expression. The
191 following primers were used: HO-1 (5'-GACCGTGGCAGTGGGAATT-3' and 5'-
192 TGGTCAGTCAACATGGACGC-3'), CHOP (5' -ACTCTTGACCCTGCATCCCT-3' and
193 5'-TCTCATTCTCCTGCTCCTTCTC-3'), XBP1 (5'-TTAGTGTCTAAAGCCACCCACC-3' and
194 5'-GCCAGGCTGAACGATAACTG-3'), GAPDH (5'-TGGAAAGCTGTGGCGTGAT-3' and
195 5'-GGGTGGTCCAGGGTTTCTT

196 **2.9 Statistical analysis**

197 Data are presented as mean± standard deviation. Comparisons between groups were performed by
198 Student's two-tailed unpaired t-test. Statistical significance was set at $P < 0.05$.

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Author contributions

Min Li performed most of the experiments. Yang Mi designed the whole project including every experiment, analyzed all the data, performed some experiments and prepared the manuscript. PhD Yingwu Mei, Professor Jitian Xu, Yuebai Li and Xu Zhang kindly provided some materials. PhD Jingeng Liu and PhD Kaikai Fan help to establish the myocardial I/R model.

Conflict of interest

The authors don't have a conflict of interest.

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Figure Legends

Figure 1. Arsenite blocks NLRP3 inflammasome activation

(A) THP-1 cells were primed with LPS (100 ng/ml) for 3 hr and were stimulated with nigericin (5μM) for 1 hour or Rotenone (10μM) for 3 hours. Sodium arsenite (40μM) was added into medium together with LPS ($Arsenite^{P}$) or with Nigericin ($Arsenite^{N}$). Supernatants were analyzed by ELISA for IL-1β. Experiments were repeated three times.

(B) LPS-primed bone-marrow-derived macrophages (BMDMs) were stimulated for 1 hour with ATP (5 mM) or Nigericin (10μM), Sodium arsenite (10μM) was added into medium together with LPS ($Arsenite^{P}$) or with Nigericin ($Arsenite^{N}$). Supernatants were analyzed by ELISA for IL-1β. Experiments were repeated three times.

(C) BMDMs treated with LPS and arsenite alone or collectively for 4 hours were analyzed by RT-PCR. "As", Sodium arsenite.

Figure 2. NLRP3 aggregation is compromised by arsenite

(A) U2OS and (B) HeLa cells transfected with Flag_NLRP3 vector were treated with nigericin and sodium arsenite alone or collectively for 30 minutes, cells were fixed and stained with FLAG antibody. Scale bars: 10μM

Figure 3. Role of arsenite treatment in myocardial I/R

(A) Quantification of infarct size of myocardial tissues 1 day after reperfusion (n=14 and 15) by TTC staining method. Rats were intraperitoneally administrated with PBS or 4.5mg/kg arsenite 30min before LCA was occluded. The data were analyzed using t-test, $P<0.0001$. AAR, the area at risk.

(B) Representative images of heart slices from different groups at 1 day after reperfusion. The non-ischemic area is indicated in blue, the area at risk in red, and the infarct area in white. Scale bars: 5mM

(C) Ejection fraction (EF, %) and LV fractional shortening (FS, %) (n =5-7) analyzed by M-mode images of the LV from PBS and arsenite group 1 day after myocardial I/R. The data were analyzed using t-test, ** $P< 0.01$. As, Sodium Arsenite.

(D) and (E) The heart rates, systolic pressure (SP) and diastolic pressure (DP) were measured by placing a catheter into the femoral artery of rats at 24 hour after myocardial I/R. The pulse pressure was calculated by SP and DP. There is no significant statistic difference of these parameters in each group. As, Sodium Arsenite.

Figure 4. Arsenite-mediated IF2α phosphorylation and HO-1 induction in myocardial tissue

(A) Western blot analysis of total protein extracted from heart tissue of mice injected with 4.5mg /kg sodium arsenite for the indicated time. (B) Western blot analysis of total protein extracted from HeLa cells treated with 4.5μg /ml $NaAsO_2$ for the indicated time. (C) RT-PCR analysis of indicated genes in SMMC-7721cells treated with $NaAsO_2$ for the indicated times. (D) SD rats were intraperitoneally injected with 4.5mg /kg $NaAsO_2$ for 7 hours. After that total RNA was isolated from heart , liver and brain tissues and stress related genes were analyzed by RT-PCR.

Figures/Tables

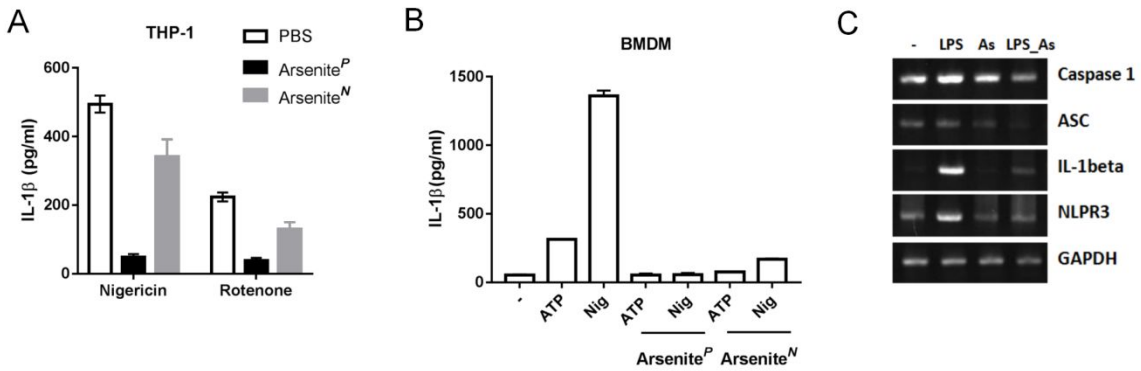
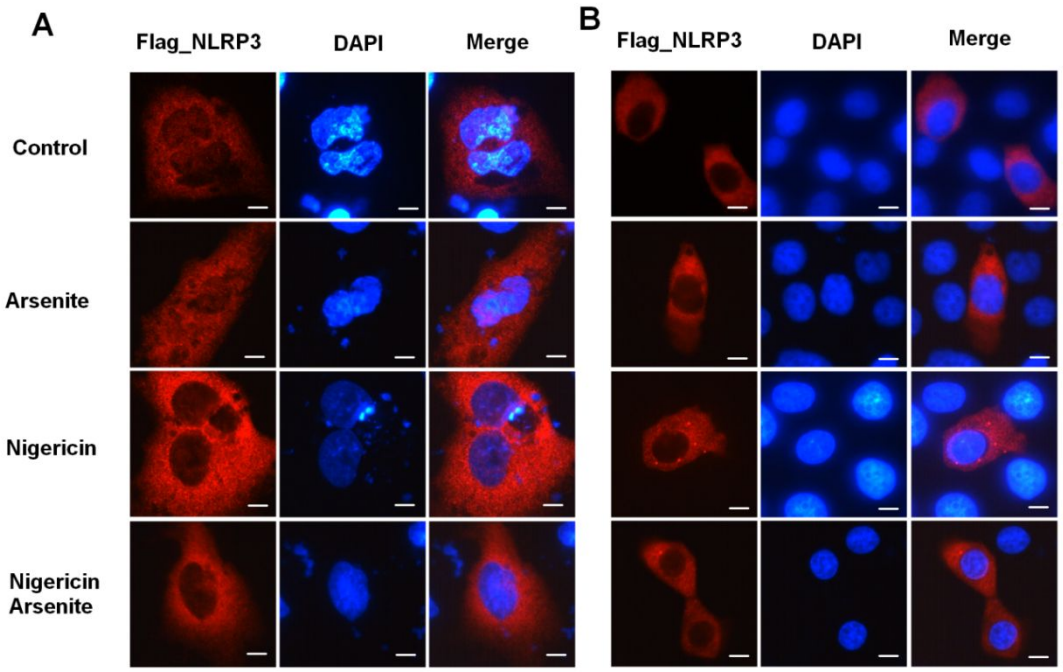


Figure 1. Arsenite blocks NLRP3 inflammasome activation



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357 **Figure 2. NLRP3 aggregation is compromised by arsenite**

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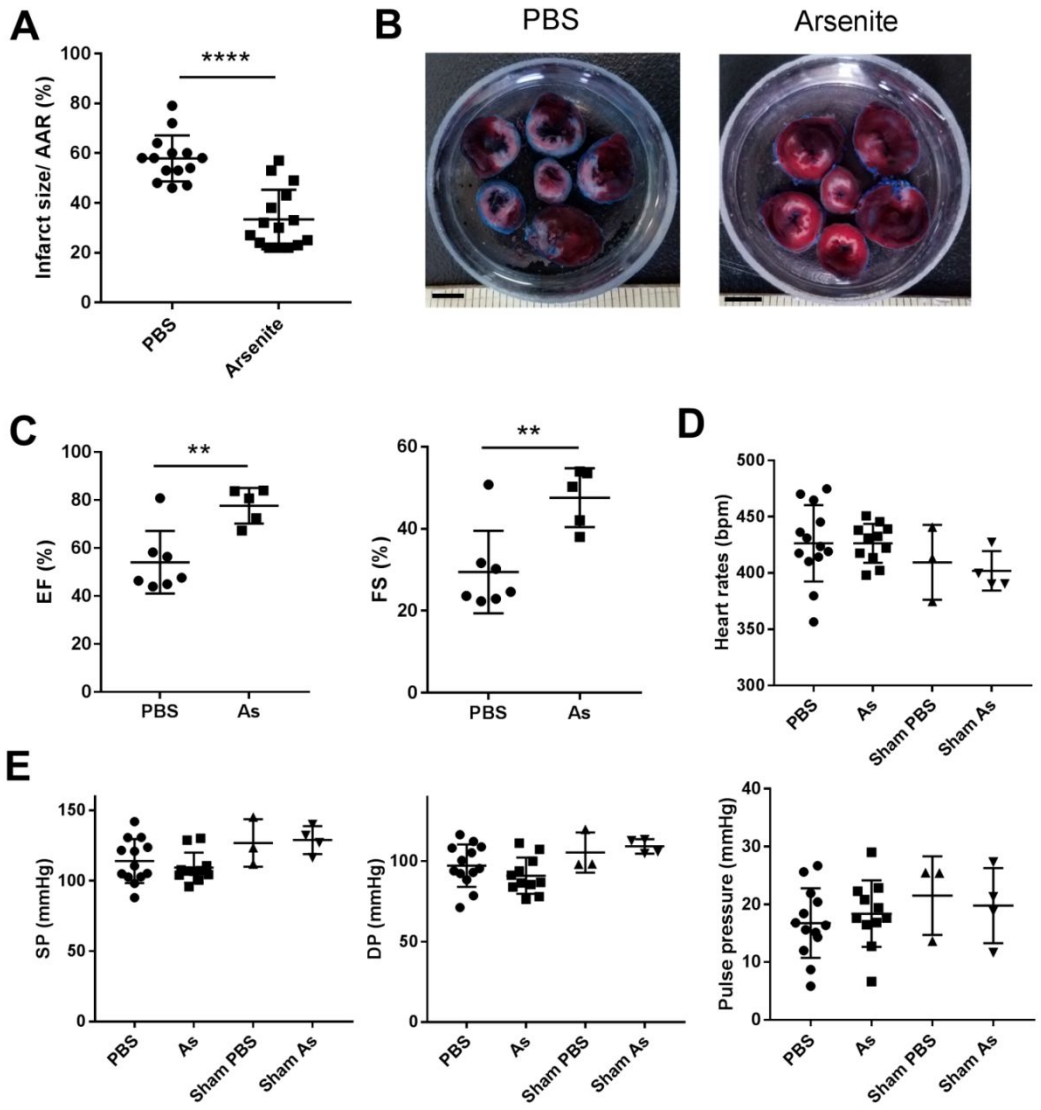
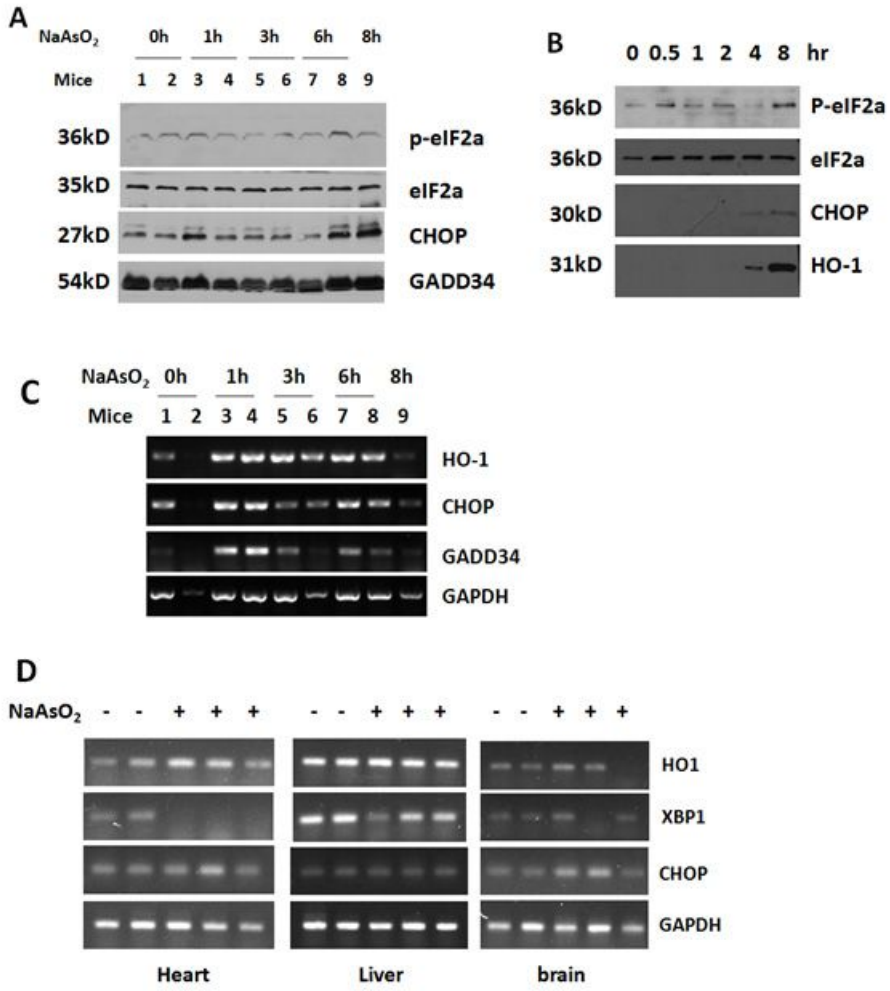


Figure 3. Arsenite therapy in myocardial IR



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