Induction of MicroRNA-21 With Exogenous Hydrogen Sulfide Attenuates Myocardial Ischemic and Inflammatory Injury in Mice

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Background—Maintaining physiological levels of hydrogen sulfide during ischemia is necessary to limit injury to the heart. Because of the anti-inflammatory effects of hydrogen sulfide, we proposed that the hydrogen sulfide donor, sodium sulfide (Na2S), would attenuate myocardial injury through upregulation of protective microRNA-21 (miR-21) and suppression of the inflammasome, a macromolecular structure that amplifies inflammation and mediates further injury.

Methods and Results—Na2S-induced miR-21 expression was measured by quantitative polymerase chain reaction in adult primary rat cardiomyocytes and in the mouse heart. We measured inflammasome formation and activity in cardiomyocytes challenged with lipopolysaccharide and ATP or simulated ischemia/reoxygenation and in the heart after regional myocardial ischemia/reperfusion, in the presence or absence of Na2S. To assess the direct anti-inflammatory effects of hydrogen sulfide in vivo, we used a peritonitis model by way of intraperitoneal injection of zymosan A. Na2S attenuated inflammasome formation and activity, measured by counting cytoplasmic aggregates of the scaffold protein apoptosis speck-like protein containing a caspase-recruitment domain (−57%) and caspase-1 activity (−50%) in isolated cardiomyocytes and in the mouse heart (all P<0.05). Na2S also inhibited apoptosis (−38%) and necrosis (−43%) in cardiomyocytes in vitro and reduced myocardial infarct size (−63%) after ischemia/reperfusion injury in vivo (all P<0.05). These protective effects were absent in cells treated with the miR-21 eraser, antagomiR-21, and in miR-21 knockout mice. Na2S also limited the severity of inflammasome-dependent inflammation in the model of peritonitis (P<0.05) in wild-type but not in miR-21 knockout mice.


Key Words: hydrogen sulfide ▪ inflammasomes ▪ microRNAs ▪ myocardial infarction

Ischemic injury to the heart is followed by an intense inflammatory response that begets further injury and loss of viable myocardium.1 Ischemia itself and the debris released during cell death affect infiltrating and resident cells by promoting the formation of the inflammasome, a macromolecular structure formed by apoptosis speck-like protein containing a caspase-recruitment domain (ASC), the Nod like receptor containing a pyrin domain-3 (NLRP3, or cryopyrin), and caspase-1, which is responsible for the amplification of the inflammatory response.2 Caspase-1 is the effector enzyme of the inflammasome which is primarily responsible for the processing and release of interleukin-1β (IL-1β); as well as IL-18 and induction of inflammatory cell death.2 Formation of the inflammasome and increased caspase-1 activity during acute myocardial infarction promote cell death, adverse cardiac remodeling, and heart failure in mice.3-7

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Hydrogen sulfide (H2S) is an endogenous gasotransmitter known to influence a multitude of physiological and pathological processes, including protection against ischemia,8-11 pressure overload,12 doxorubicin toxicity,13 inflammation,14,15 arterial contraction,16 blood vessel relaxation,17,18 and insulin release.19 In addition to the benefits of exogenous administration of H2S by way of donors, endogenous H2S also seems to mediate the cardioprotective effects of cGMP regulating drugs, including phosphodiesterase-5 inhibitors and the NO-independent guanylate cyclase activator, cinaciguat.20,21

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Although the mechanisms of cardioprotection by H₂S remain under investigation, several published studies suggest that opening of K⁺<sub>ATP</sub> channels (sarcolemmal and mitochondrial), activation of protein kinase C, and RAC-alpha serine/threonine-protein kinase (AKT) are considered the potential targets. Nevertheless, the role of microRNA and regulation of inflammasome formation in mediating the cardioprotective effect of H₂S are currently unknown. To this context, we considered the possible role of microRNA-21 (miR-21) in cardioprotection. Prosurvival Akt signaling has long been established in mediating the cardioprotective effects of ischemic preconditioning (IPC) whereby IPC increased Akt phosphorylation in the heart and phosphoinositide 3-kinase (PI3K) inhibitors abolished cardioprotection with IPC. Therefore, we sought to examine whether miR-21 mediates the opening of K⁺<sub>ATP</sub> channels (sarcolemmal and mitochondrial), under investigation, several published studies suggest that interestingly, Akt has been shown to positively regulate miR-21 expression in cardiomyocytes. Moreover, miR-21 expression in the infarcted area was significantly downregulated, whereas IPC inhibited this downregulation. We and others have recently demonstrated the cardioprotective role of miR-21 against ischemia/reperfusion (I/R) injury whereby miR-21 levels were shown to decline in the setting of I/R and that attempts to restore miR-21 in such stresses have proved beneficial in attenuating injury. Interestingly, Akt has been shown to positively regulate miR-21 in the heart. Additionally, H₂S has been shown to have an anti-inflammatory effect in animal models. Based on this compelling rationale, we hypothesized that H₂S may provide a protective effect in the heart during myocardial I/R by inhibiting the formation and activation of the inflammasome in an miR-21-dependent mechanism. We therefore sought to examine whether miR-21 mediates the cardioprotective effect of H₂S against I/R injury.

Our results show that H₂S reduces myocardial I/R injury as demonstrated by reduction of infarct size and preservation of left ventricular (LV) function. Moreover, H₂S increased miR-21 in the heart and cardiomyocytes and attenuated inflammasome formation and caspase-1 activity through an miR-21–dependent mechanism.

Materials and Methods

Animals

Adult male C57BL mice were supplied by The Jackson Laboratories (Bar Harbor, ME); the mean body weight was 32.4±0.9 g. miR-21 knockout breeding pairs were purchased from Dr Eric Olson at The University of Texas Southwestern Medical Center, Dallas, TX. Adult male Wistar rats (300 g) were purchased from Harlan Sprague-Dawley, Inc (Indianapolis, IN). All animal experiments were conducted under the guidelines on humane use and care of laboratory animals for biomedical research published by the National Institutes of Health (No. 85-23, revised 1996).

Drugs and Chemicals

Sodium sulfide (Na₂S), triphenyltetrazolium chloride, lipopolysaccharides from <i>Escherichia coli</i> 0111:B4 (lipopolysaccharide), ATP, zymosan A from <i>Saccharomycyes cerevisiae</i>, and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO). Phthalal blue dye was purchased from Quantum Ink Company (Louisville, KY). The adenoviral vector expressing the miR-21 eraser, antiagomiR-21, was obtained from Dr Maha Abdellatif at the University of Medicine and Dentistry of New Jersey. Detailed information is included in Data Supplement.

To detect ASC localization, we used the combination of a rabbit anti–mouse-ASC (Sigma-Aldrich) with a donkey anti-rabbit conjugated with an Alexa Fluor 594 (Life Technologies, Grand Island, NY) and a goat anti-mouse-cardiac actin (Santa-Diago) with a donkey anti-goat conjugated with an Alexa Fluor 488 (Life Technologies). For the in vitro primary adult rat cardiomyocyte culture, a rabbit anti-rat-ASC (Santa-Diago) was used in combination with a goat anti-rabbit Alexa Fluor 594.

Adult Primary Cardiomyocyte Preparation

Adult rat ventricular cardiomyocytes were isolated using an enzymatic technique as previously reported. The simulated ischemia/reoxygenation protocol is described in detail in Data Supplement.

Real-Time Polymerase Chain Reaction

Cardiomyocyte and myocardial miR-21 levels were assessed by real-time polymerase chain reaction as described in Data Supplement.

Experimental Groups (In Vitro)

Six groups were used. (1) Control: cardiomyocytes were isolated and subjected to SI/RO; (2) Na₂S: cardiomyocytes were treated with 10 μmol/L Na₂S 1 hour before simulated-ischemia/reoxygenation (SI/RO); (3) AntagomiR-21+Na₂S: cardiomyocytes were infected with antagomiR-21 (1.5×10⁶ plaque forming units [pfu]) for 24 hours before treatment with 10 μmol/L Na₂S followed by SI/RO 1 hour later; (4) AntagomiR-21 control: cardiomyocytes were infected with antagomiR-21 for 24 hours before SI/RO; (5) Empty viral vector+Na₂S: cardiomyocytes were infected with empty vector (1.5×10⁶ pfu) for 24 hours before treatment with 10 μmol/L Na₂S followed by SI/RO 1 hour later; (6) Empty viral vector control: cardiomyocytes were infected with empty vector for 24 hours before SI/RO.

Assessment of Cell Death

Trypan blue exclusion assay was used to assess loss of cell membrane integrity as seen in oncotic cell death (necrosis) or inflammatory cell death (pyroptosis) after 2 hours of RO. Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling assay (TUNEL) was used to detect nuclear DNA fragmentation as seen in apoptosis or pyroptosis after 18 hours of RO.

Study of the Inflammasome in Cardiomyocytes

Cardiomyocytes were challenged with lipopolysaccharide (100 ng/mL, for 2 hours) and ATP (5 mmol/L, for 1 hour) in the absence or presence of Na₂S treatment (10 μmol/L 5 minutes before lipopolysaccharide challenge). Cardiomyocytes were infected with antagomiR-21 (1×10⁷ pfu/cell) or the empty vector 1 hour after isolation. After 24 hours, the cells were incubated with Na₂S (10 μmol/L) for 5 minutes. Subsequently, the cells were challenged with lipopolysaccharide (100 ng/mL, for 2 hours) and ATP (5 mmol/L, for 1 hour) as follows: (1) empty viral vector control; (2) empty viral vector+Na₂S; (3) empty viral vector+lipopolysaccharide+ATP; (4) empty viral vector+Na₂S and lipopolysaccharide+ATP; (5) antagomiR-21 control; (6) antagomiR-21+Na₂S; (7) antagomiR-21+lipopolysaccharide+ATP; (8) antagomiR-21+Na₂S and lipopolysaccharide+ATP. Where applicable, Na₂S was administered 5 minutes before lipopolysaccharide.

Two additional subsets of cells were subjected to SI/RO to study the inflammasome in this model: (1) control: cardiomyocytes were isolated and subjected to SI/RO; (2) Na₂S: cardiomyocytes were treated with 10 μmol/L Na₂S 1 hour before SI/RO.

All experiments were repeated 3 times, and each single group was run in triplicate. Immunofluorescence was used to detect the presence of ASC aggregates, reflecting formation of the inflammasome, with
a rabbit anti-rat-ASC antibody (1:200, overnight) used in combination with a goat anti-rabbit Alexa Fluor 594. In a similar experiment, caspase-1 activity, the effector enzyme in the active inflamma-
some, was measured using a fluorescent labeled inhibitor of caspase (FLICA)-based assay (Axxora LLC, Farmingdale, NY) following the supplier’s instructions. In a parallel experiment, we used trypan blue staining to measure cell death as previously described.3

Myocardial Infarction Protocol and Infarct Size Measurement
The methodology of myocardial infarction was described previously.20 A brief summary of myocardial infarction and infarct size measurement is in Data Supplement.

Experimental Groups (In Vivo)
Five groups were used. (1) Saline (control): Each C57BL wild-type mouse received 0.2 mL (IP) normal saline 1 hour before I/R; (2) Na2S: C57BL mice received 100 µg/kg Na2S (IP) 1 hour before I/R; (3) miR-21 knockout control: miR-21 knockout mice received 100 µg/kg Na2S (IP) 1 hour before I/R; (4) Na2S+miR-21 knockout: miR-21 knockout mice received 100 µg/kg Na2S (IP) 1 hour before I/R. In all groups, infarct size was measured 24 hours after I/R. Before euthanization, LV function was analyzed using echocardiography. Six mice in each group were used for infarct size assessment and for functional analysis using echocardiography. The detailed experimental protocol is shown in Figure 1.

Survival
Survival rate was determined based on the animals that survived the experimental protocol starting at recovery after surgery until 24 hours after infarction.

Echocardiography
Echocardiography was performed using the Vevo770 imaging system (VisualSonics Inc, Toronto, Ontario, Canada) before surgery (baseline) and 24 hours after surgery before euthanizing the animal. Pentobarbital (30 mg/kg IP) was used for anesthesia and the procedure was performed as previously described17 to measure LV end-diastolic diameter and end-systolic diameter. LV fractional shortening was calculated as (LV end-diastolic diameter−LV end-systolic diameter/LV end-diastolic diameter×100).

Western Blotting
Expression of phospho- and total Akt was assessed using Western blot analysis as described in Data Supplement.

Measurement of the Inflammasome in the Heart
After I/R protocol, tissue slides (3-µm thick slices) were prepared from formalin-fixed and paraffin-embedded hearts. The sections were deparaffinized and rehydrated using sequential washes in xylene and decreasing concentrations of ethanol. After antigen retrieval with 0.01 mol/L citrate buffer (pH 6.0) for 20 minutes, the slides were blocked with 1% normal swine serum in tris buffered saline (TBS) for 15 minutes. For characterization of cardiomyocyte-specific expression of the aggregates of the scaffold protein ASC, which is indicative of inflammasome formation, the double immunofluorescence technique was used. After blocking, the slides were incubated with primary antibody for ASC (1:50, Sigma-Aldrich) overnight at 4°C. Anti-rabbit Alexa Fluor 594–conjugated secondary antibody (1:100) was applied for 4 hours at room temperature, then slides were incubated with a primary antibody for cardiac actin (1:200, Sigma-Aldrich) overnight at 4°C, and Alexa Fluor 488–conjugated secondary antibody (1:100, Invitrogen) was applied for 4 hours at room temperature.2 The cell counterstaining was performed with 4′,6-diamidino-2-phenylindole (DAPI) 1:1000 for 5 minutes and the slides were coverslipped with SlowFade Antifade (both Invitrogen). Negative controls with nonspecific IgG were run in parallel. Images were acquired with an IX70 microscope and the MagnaFire 1.1 software (both Olympus) using a ×40 objective (×400 magnification). Color composite images were generated with ImageJ software.

Measurements of ASC in the infarct area were performed by 2 investigators who were blinded to treatment allocation and the expression was quantified using a semiquantitative scale ranging from 0 (no expression) to 1+ (minimal expression meaning either few aggregates [<1 per high power field] or mild diffuse stain without aggregates), 2+ (moderate expression meaning either 1–5 aggregates per high power field or diffuse stain with few aggregates), 3+ (diffuse intense staining with many cytoplasmic aggregates [5 per high power field]).

Caspase-1 Activity in the Heart
An additional subset of mice were euthanized 24 hours after surgery (n=4–6 per treatment group). The heart was removed, rinsed in PBS, and snap-frozen in liquid nitrogen. Caspase-1 activity was measured in the tissue using a fluorogenic substrate Ac-Tyr-Val-Ala-Asp-7-amino-4-methyl coumarin (Ac-YVAD-AMC) specific for caspase-1 (Enzo Life Sciences, Farmingdale, NY).18 After homogenization using radio-immuno precipitation assay (RIPA) buffer containing a cocktail of protease inhibitors and centrifugation at 16000 rpm for 20 minutes, 50 µg of protein from each sample was used for the assay as previously described.1 Fluorescence was measured after 60 minutes and was expressed as arbitrary fluorescence units produced by 1 µg of sample per minute (fluorescence/µg per minute) and calculated as fold change compared with the caspase-1 activity in homogenates of the hearts of sham-operated mice, whereby oral in-
tubation and a left thoracotomy were performed and a 7-0 silk suture was placed around the left coronary artery but not tightened.

Inflammasome-Dependent Peritonitis Model
To determine the effects of H2S on the inflammasome in vivo independently of its effects on ischemia or infarction, we used a model of peritonitis induced by the intraperitoneal injection of zymosan A (1 mg/mouse), which induces the activation of the cryopyrin inflamma-
some.19 After 6 hours, peritoneal lavage was performed with sterile NaCl 0.9% (7 mL), and the fluid was assessed for leukocyte content using the Thoma chamber. The total amount of leukocytes was determined in the presence or absence of Na2S (100 µg/kg). Glyburide (500 mg/kg), a specific cryopyrin inhibitor,29 or IL-1 receptor antagonist (20 mg/kg), which inhibits the IL-1–dependent peritoneal migration of leukocytes, was used as a control agent of inflamma-
some-related activity. An equal volume of normal saline was used in the control group.
Statistics
All measurements were expressed as group means±SE. The data were analyzed by a nonparametric Kruskal–Wallis rank-sum test, which is a generalization of the Wilcoxon rank-sum test for ≥2 groups. We used the Kruskal–Wallis rank-sum test in cases of ≥2 groups to remain consistent across all the experiments. All the data analyses were performed through the statistical software R version 3.0.3 using functions in the base package.

The authors had full access to the data and take responsibility for its integrity. All the authors have read and agreed to the article as written.

Results
H₂S Donor, Na₂S, Induces miR-21 in Primary Cardiomyocytes and the Heart
To test the effect of H₂S on miR-21, the cardiomyocytes were incubated with Na₂S (10 μmol/L) or control medium for 1 hour before measuring miR-21 expression. Figure 2A shows a significant increase in miR-21 in Na₂S-treated cardiomyocytes compared with controls. Similarly, miR-21 increased in the heart after treatment with Na₂S (100 μg/kg IP) as shown in Figure 2B. This increase in miR-21 at 1 hour was not paralleled by an increase in Akt phosphorylation (Figure 2C), ruling out its potential role in the acute induction of miR-21.

Na₂S Attenuates Cardiomyocyte Injury After Ischemia/Reoxygenation
Primary rat cardiomyocytes treated with Na₂S or control medium were exposed to 90 minutes of simulated ischemia followed by 2 (for necrosis) or 18 (for apoptosis) hours of reoxygenation. Trypan blue permeability and TUNEL positivity were measured as markers of necrotic cell death and DNA fragmentation as reported in Figure 3A and 3B. The treatment with Na₂S significantly reduced cell death and DNA fragmentation, indicating its protective role against SI/RO injury. Interestingly, antagomiR-21 blunted the decrease in necrosis and apoptosis observed with Na₂S treatment.

Na₂S Improves Survival After I/R Injury
A total of 134 mice were used in this study. Fifteen of 18 C57BL mice survived with Na₂S (83%) compared with 14 of 25 with saline (56%). The survival rate was 100% in sham-operated mice. Fourteen of 29 miR-21 knockout mice survived in the Na₂S group (48%), and 14 of 28 survived in the saline group (50%). This suggests that the survival benefits observed with Na₂S are dependent on miR-21.

Na₂S Protects Against I/R Injury Through miR-21
To demonstrate the cardioprotective role of H₂S in vivo and to determine the role of miR-21 in protection, wild-type and miR-21 knockout mice were treated with Na₂S or saline before I/R injury (Figure 4A and 4B). Na₂S significantly reduced infarct size to 16.3±1.5% compared with 44.4±1.6% in wild-type mice, confirming the protective action of Na₂S observed in cardiomyocytes. Interestingly, Na₂S treatment in miR-21 knockout mice failed to reduce infarct size, suggesting a central role of miR-21 in mediating cardioprotection with H₂S.

The beneficial effect of H₂S treatment was reflected also at functional level, as demonstrated by preservation of LV fractional shortening in wild-type mice (Figure 4C). The absence of miR-21 per se did not alter baseline fractional shortening. However, Na₂S-treated miR-21 knockout mice did not show improvement in ventricular function after I/R injury, confirming an indispensable role of miR-21 in mediating cardioprotection with H₂S.

Interestingly, Na₂S-treated mice exhibited a significant increase in myocardial Akt phosphorylation 24 hours after I/R injury compared with saline-treated mice (Figure 4D).

Na₂S Inhibits Formation of the Inflammasome in Cardiomyocytes In Vitro
Na₂S attenuated the formation of the inflammasome (measured as ASC aggregates by 57%; *P<0.05; Figure 5A and 5B) in cardiomyocytes challenged with lipopolysaccharide+ATP compared with control cells and also in cardiomyocytes subjected to SI/RO (55% reduction; *P<0.05; Figure 5C). The

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Real-time polymerase chain reaction analysis showing significant increase in microRNA-21 (miR-21) expression in adult primary rat cardiomyocytes (A) and in the mouse heart (B) 1 hour after treatment with sodium sulfide (Na₂S) compared with control groups. C, Western blots and densitometry showing no change in phospho-Akt (p-Akt) to total Akt ratio at 1 hour after Na₂S treatment compared with saline control. Horizontal lines represent means±SE.
Na₂S inhibits inflammasome formation in the heart after I/R injury. Treatment with Na₂S blunted inflammasome formation in the heart (ASC aggregates in the zone bordering the infarct [by 37%; $P<0.05$; Figure 7A and 7B]) and caspase-1 activity (Figure 8A) after myocardial I/R injury. In contrast, miR-21 knockout mice exhibited a trend toward elevated basal caspase-1 activity, which increased with I/R injury and was not attenuated with Na₂S treatment.

Na₂S inhibits inflammasome-mediated peritonitis in the mouse. To further demonstrate the cause and effect relationship of miR-21 in H₂S-induced attenuation of inflammasome function in vivo, we used the zymosan A–induced peritonitis model, an inflammatory model in which leukocyte migration into the peritoneal cavity is mediated by the inflammasome. Pretreatment with Na₂S significantly reduced the leukocyte infiltrate in the peritoneal lavage after challenge with zymosan A (45% reduction; Figure 8B). This effect was similarly reproduced by the inflammasome inhibitor glyburide and by the IL-1 blocker IL-1 receptor antagonist (anakinra, not shown). However, Na₂S treatment did not prevent the leukocyte increase observed with zymosan A in miR-21 knockout mice, supporting a pivotal role of miR-21 in mediating the anti-inflammatory effects of H₂S. Interestingly, glyburide and anakinra were still capable of reducing leukocyte infiltration after zymosan challenge in the miR-21 knockout mouse (not shown).

Discussion

H₂S is produced endogenously and maintained at physiological concentrations in mammalian systems. The concentration of H₂S has been shown to influence a wide range of physiological processes, and exogenous administration of H₂S by way of donors (ie, Na₂S) has shown to modulate the course of several acute and chronic illnesses. In the current study, we show that administration of an H₂S donor during I/R in mice prevents the formation and the activation of the cryopyrin inflammasome, a macromolecular complex responsible for sensing tissue injury or danger, amplifying the inflammatory response, and inducing cell death. Na₂S prevented the formation of the inflammasome aggregates in cardiomyocytes in vitro and in the heart in vivo after both ischemic and nonischemic injuries (ie, lipopolysaccharide/ATP in vitro, peritonitis in vivo), thus demonstrating a specific anti-inflammasome effect of Na₂S that cannot be solely attributed to the reduction in ischemic injury.
The effects of Na$_2$S on inflammation have been widely reported. In a recent study, H$_2$S was used to treat arthritis in an inflammasome-mediated mouse model. However, the mechanisms by which H$_2$S exerts its anti-inflammatory effects remain poorly understood. Because of its lipophilic structure as a gasotransmitter, H$_2$S is likely to have many and variable targets, because it lacks a true receptor. Prior studies have shown that H$_2$S activates prosurvival Akt, which is a key regulator of miR-21 expression. It is noteworthy that miR-21 has been implicated in the endogenous mechanisms of cardioprotection after ischemic or pharmacological preconditioning as well as in anti-inflammatory pathways. For this reason, we determined whether H$_2$S also positively regulates miR-21. Our results show that Na$_2$S significantly induced miR-21 expression in primary adult rat cardiomyocytes (2.3-fold increase) and in the intact mouse heart (2.7-fold increase). The same in vitro and in vivo doses of Na$_2$S caused a significant cytoprotective effect after ischemia. However, contrary to our initial hypothesis, Na$_2$S treatment did not alter the phosphorylation status of Akt at 1 hour after treatment, which rules out its role in acute miR-21 induction. Nonetheless, we did detect an increase in Akt phosphorylation at 24 hours after I/R in the Na$_2$S-treated group versus control. Other studies have reported that miR-21 itself can also increase Akt phosphorylation by inhibiting phosphatase and tensin homolog deleted on chromosome 10, one of the known targets of miR-21. Although it remains unclear how H$_2$S increases miR-21 expression acutely, we think that increased Akt phosphorylation with Na$_2$S at 24 hours after I/R may be responsible for maintaining miR-21 levels in the H$_2$S group, which may initiate a self-propagating cycle of Akt activation and miR-21 induction. More in-depth studies involving multiple time points during and after ischemia are warranted to further examine the mechanism of miR-21 induction with Na$_2$S. miR-21 has several known targets that support its protective role against ischemia, including phosphatase and tensin homolog deleted on chromosome 10, programmed cell death 4, Toll-interleukin receptor, transforming growth factor-β receptor II, and myeloid differentiation factor 88. However, investigations into the direct or indirect target(s) of miR-21 that are responsible for the infarct-sparing effect of Na$_2$S (which occurs within a few hours of MI) are also needed.

Figure 4. A, Representative heart sections stained with phthalo blue to demarcate the nonrisk area and triphenyltetrazolium chloride to identify viable tissue. B, Myocardial infarct size (% of risk area [RA]) measured 24 hours post–myocardial infarction (MI) in the various groups. Note that sodium sulfide (Na$_2$S) treatment exhibited a smaller infarct size after ischemia/reperfusion (I/R) compared with saline controls. The infarct-sparing effect of Na$_2$S was abolished in microRNA-21 (miR-21) knockout (KO) mice. The area at risk, expressed as percent of left ventricle (LV), was similar in all groups. C, Cardiac function measured as LV fractional shortening was preserved after I/R injury with Na$_2$S compared with saline controls. This benefit was lost in miR-21 KO mice. D, Western blots and densitometry showing a significant increase in myocardial phospho-Akt (p-Akt) to total Akt ratio at 24 hours after I/R in Na$_2$S–treated mice compared with saline-treated controls. Horizontal lines represent mean±SE.
The protective effects of Na₂S were not observed in miR-21 knockout mice. A recent study reported that miR-21 can suppress cystathionine beta-synthase (CSE) expression in dedifferentiated human aorta smooth muscle cells and injured mouse carotid arteries. Although this may seem in apparent contrast with our signaling cascade, our study focuses on primary adult cardiomyocytes and the heart as a whole. However, even if these observations were to hold true in our model, we are not relying on endogenous production of H₂S but on H₂S donor therapy. In fact, this observation may represent

Figure 5. A, Representative fields showing apoptosis speck-like protein containing a caspase-recruitment domain (ASC) aggregates (red) in adult primary cardiomyocytes. Adenoviral antagomiR-21, a miR-21 eraser used to inhibit miR-21, was used to test the role of microRNA-21 (miR-21) in mediating the anti-inflammasome effects of sodium sulfide (Na₂S), whereas an empty virus was used as control (CTRL). B, Lipopolysaccharide (LPS)+ATP caused a significant increase in ASC aggregates which was attenuated by Na₂S. This anti-inflammatory effect of Na₂S was abolished by antagomiR-21. C, Subjecting adult primary cardiomyocytes to simulated-ischemia/reoxygenation (SI/RO) also caused a significant increase in ASC aggregates, which was attenuated by Na₂S treatment. Horizontal lines represent mean±SE. DAPI indicates 4',6-diamidino-2-phenylindole. A.U. indicates arbitrary units.

Figure 6. A, Representative fields illustrating caspase-1 activity (red) in adult primary cardiomyocytes. B, Lipopolysaccharide (LPS)+ATP significantly increased caspase-1 activity which was attenuated by sodium sulfide (Na₂S). C, Na₂S also significantly attenuated necrotic cell death caused by LPS+ATP. Horizontal lines represent mean±SE. CTRL indicates control. TB indicates Trypan Blue staining.
knockout mice treated with Na₂S. The results of the current study confirm the essential role of miR-21 not only in mediating the infarct-sparing effects of H₂S, but also in attenuating myocardial inflammasome formation and its central role in postinfarction adverse remodeling, which might explain the chronic benefits of Na₂S shown to suppress toll-like receptor signaling and lung inflammation in mice and in human peripheral blood mononuclear cells. Interestingly, a recent study demonstrated that hepatitis C virus–induced miR-21 contributes to evasion of host immune system by targeting myeloid differentiation factor 406.

The exact mechanisms by which H₂S inhibits the formation of the inflammasome and subsequent caspase-1 activation in the heart during acute myocardial infarction are unknown. Our results show that both formation of the inflammasome and activation of caspase-1 are impaired by H₂S, suggesting that it may be acting upstream of the aggregation of the multimers. This could be at the level of priming of the inflammasome (by inhibiting expression of key components) or at the level of triggering (by inducing structural changes in the sensor of the inflammasome). Of utmost importance is the fact that the inflammasome-inhibiting effects of H₂S were completely abolished with deletion of miR-21 by using 2 different and alternative approaches (genetically engineered mice and antagomiR treatment). This suggests that miR-21 is essential for the protective effects of H₂S. MicroRNAs are tight controllers of gene expression and may well explain the many and variable effects seen with H₂S. In particular, miR-21 has been shown to suppress toll-like receptor signaling and lung inflammation in mice and in human peripheral blood mononuclear cells. Interestingly, a recent study demonstrated that hepatitis C virus–induced miR-21 contributes to evasion of host immune system by targeting myeloid differentiation factor.
88 and IL-1 receptor–associated kinase 1 in human hepatoma and embryonic kidney cells.35 These reports are in agreement with our findings as they support the anti-inflammatory role of miR-21, especially by targeting upstream pathways that lead to inflammasome formation.

The use of adenoviral antagomiR-21 and genetically engineered miR-21 knockout mice are, however, also fraught with limitations. For instance, genetically engineered animals may have other alterations or compensatory mechanisms that are phenotypically masked and the use of antagonist may trigger off-target effects. In fact, Thum et al51 demonstrated that miR-21 mediates cardiac fibrosis in a pressure-overload mouse model by augmenting extracellular-regulated kinase–mitogen-activated protein kinase activity through inhibition of sprouty homolog 1 in cardiac fibroblasts. Moreover, in vivo systemic delivery of antagomiR-21 reduced extracellular-regulated kinase–mitogen-activated protein kinase activity, inhibited interstitial fibrosis, and attenuated cardiac dysfunction.52 However, a more recent study in miR-21 knockout mice revealed that stress-dependent adverse cardiac remodeling develops in the absence of miR-21, suggesting the lack of its role in pathological cardiac remodeling.53 Although these findings are in apparent contrast, the question of antagonist specificity and compensatory modifications after genetic deletion remains unanswered. Moreover, the pressure-overload model with a focus on cardiac fibroblasts is different from our model of myocardial I/R. Nevertheless, the use of complementary approaches of knockout mice and antagonist provide conclusive evidence for the role of miR-21 in contributing to the anti-inflammatory effect of H2S during ischemia. Moreover, other studies have illustrated that overexpression of miR-21 could also reduce fibrosis during ischemic heart disease or failure by decreasing myocyte death and, thus, inflammatory cell infiltration and fibroblast proliferation.30

In conclusion, H2S exerts its cardioprotective effects and inhibits the formation and activation of the inflammasome through signaling pathways requiring miR-21. H2S therapy or modulation of endogenous production of H2S may be an attractive approach in reducing injury and inflammasome assembly/activation in the heart and other organs. Moreover, the important role that miR-21 plays in mediating cardioprotection with H2S will enable us to design therapies using small molecules that would likely exhibit a more linear pharmacokinetic profile, as it has been shown for other microRNA targets.53

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Disclosures

None.

References

Disruption of endogenous hydrogen sulfide (H$_2$S) levels has been implicated in a wide variety of ailments ranging from cardiovascular disease and heart failure to nervous system disorders and inflammatory syndromes. Exogenous supplementation of H$_2$S by way of donors in experimental models has been shown to attenuate injury secondary to ischemia in the heart and other organs; however, the long-term benefits and mechanisms remain unknown. Here, we present a novel mechanism through which microRNA-21 (miR-21) mediates the cardioprotective effects of H$_2$S in reducing infarct size, preserving left ventricular function, and attenuating infammasome formation in the postischemic heart. Continuous infammasome activation in the heart leads to worsening of cardiac function and heart failure; however, no known inhibitors of the infammasome exist. Our study suggests that H$_2$S inhibits the formation and activation of the infammasome in the presence or absence of myocardial infarction, which may explain its benefits in models of inflammatory disease. A key discovery is the important role that miR-21 plays in mediating the infarct-sparing and anti-inflammasome effects of H$_2$S in our study. We took advantage of adenoviral vectors encoding miR-21 antisense (antagomir) as well as miR-21 knockout mice to confirm our findings. These results highlight the need to develop and test safe and effective H$_2$S donors for use in patients and further endorse H$_2$S therapy for cardiovascular and inflammatory diseases. Moreover, the cardioprotective role of miR-21 may enable us to design therapies to treat cardiovascular disease using a targeted approach with small molecules.
Induction of MicroRNA-21 With Exogenous Hydrogen Sulfide Attenuates Myocardial Ischemic and Inflammatory Injury in Mice

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SUPPLEMENTAL MATERIAL

AntagomiR-21 Viral Vector

A 320-bp sequence encompassing the stem-loop of miR-21 was amplified from mouse genomic DNA by PCR using the following primers: 5′-CCTGCCTGAGCACCTCGTGC-3′ and 5′-GACTGTGACGACTACCCCAA-3′ and cloned into recombinant adenovirus downstream of a cytomegalovirus (CMV) promoter. The miR-21 eraser was synthesized in the form of a tandem repeat antisense sequence of mature miR-21 terminating in (T)6. This construct was cloned into recombinant adenovirus downstream of a U6 promoter. The cells were infected with ad.antagomiR-21 immediately after plating for 24 hours before SI/RO or treatment with LPS+ATP.

Simulated Ischemia/Reoxygenation Protocol

One hour after plating, the cells were subjected to simulated ischemia (SI) for 90 minutes by replacing the cell medium with an “ischemia buffer” that contained 118 mm NaCl, 24 mm NaHCO₃, 1.0 mm NaH₂PO₄, 2.5 mm CaCl₂-2H₂O, 1.2 mm MgCl₂, 20 mm sodium lactate, 16 mm KCl, 10 mm 2-deoxyglucose (pH adjusted to 6.2). The cells were incubated at 37°C in tri-gas incubator adjusting 1-2% O₂ and 5% CO₂ during the entire simulated ischemia (SI) period. Reoxygenation (RO) was accomplished by replacing the ischemic buffer with normal cell medium under normoxic conditions. Cell necrosis and apoptosis were assessed after 2 or 18 hours of reoxygenation, respectively. In this study, we chose adult rat primary cardiomyocytes because the stability of these cells is superior to primary mouse cardiomyocytes especially with the use of adenoviral vectors to modulate miR-21.
Real-Time PCR

Total RNA including small RNA was isolated from frozen control and Na₂S-treated primary rat cardiomyocytes or whole mouse heart tissue using miRNeasy mini kit according to manufacturer’s protocol (QIAGEN Sciences, MD, USA). Concentration and purity of the isolated RNA was checked using Nanodrop ND-1000 spectrophotometer (Agilent technologies, CA, USA). Briefly 10 ng of total RNA were subjected for reverse transcription reaction with miRNA specific RT primer using microRNA reverse transcription kit (Applied Biosystems, CA, USA). Real time PCR was performed using Roche Light cycler 480 II (Roche Applied Science, IN, USA) Taqman miRNA assay probe (Applied Biosystems, CA, USA) to determine the expression level of mir-21-UAGCUUAUCAGACUGAUGUUGA and were normalized using endogenous U6 small RNA. Reverse transcription was performed using stem loop specific microRT primer under following condition : 16°C for 30 minutes ; 42°C for 30 minutes and 85°C for 5 minutes. The obtained cDNA was diluted in 1:3 ratio and subjected to real-time PCR using Taqman amplicon specific assay probe under the following PCR cycle condition: 95°C for 10 minutes; 95°C for 15 seconds and 60°C for 60 seconds.

Myocardial Infarction Protocol

In brief, mice were orotracheally intubated under anesthesia (pentobarbital 50 - 70 mg/kg), placed in the right lateral decubitus position, then subjected to left thoracotomy, pericardiectomy, and ligation of the proximal left coronary artery for 30 minutes by a 7.0 silk ligature that was placed around it and a small piece of polyethylene tubing (PE10) that was positioned on top of it. Reperfusion was established by removing the PE10 tube that was
compressing the coronary artery before closure of the thorax. The animals were extubated and then received analgesia (buprenex; 0.1 mg/kg; sc) and antibiotic (Gentamicin; 0.7 mg/kg; IM).

**Infarct Size Assessment**

After 24 h of reperfusion, the heart was quickly removed and mounted on a Langendorff apparatus. The coronary arteries were perfused with 37°C Krebs-Henseleit buffer. After the blood was washed out, 3 ml of 10% TTC in isotonic phosphate buffer (pH 7.4) at 37°C were infused over several minutes before the ligature was retightened and ~1 ml of 5% Phthalo blue dye was injected as a bolus into the aorta until most of the heart turned blue. The heart was perfused with saline to wash out the excess Phthalo blue. Finally, the heart was removed, frozen, and cut into 8–10 transverse slices from apex to base of equal thickness (~1 mm). The slices were then fixed in 10% neutral buffered formaldehyde for 4 to 24 h with a weight on top to keep the heart slices flat for the initial 30 min. The areas of infarcted tissue, the risk zone, and the whole left ventricle were determined by computer morphometry using ImageJ imaging software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2011).

**Western Blotting**

Isolated LV samples (n = 3/group) were homogenized and centrifuged at 12,000 g for 10 min at 4°C. Total proteins (75 μg) from each sample were separated by SDS-PAGE on 4-20% gradient acrylamide gels, transferred onto a nitrocellulose membrane, and blocked with 5% nonfat dry milk in Tris-buffered saline. Membranes were incubated overnight with mouse monoclonal antibody (dilution 1:1000, Cat# 4691 or 4060; Cell Signaling, MA) specific for total Akt or phospho-Akt, respectively, and goat polyclonal antibody (dilution 1:1,000, Cat # Sc-1616,
Santa cruz , CA) specific for Actin. The blot was then incubated for 1 hour with the corresponding secondary horseradish peroxidase-conjugated antibody and developed using Western Lightning Plus–Ecl substrate (PerkinElmer, MA, USA). The densitometric analysis for the corresponding phospho- and total Akt and beta actin band was done using ImageJ software.

**Figure Legend:**

**Supplemental Figure 1:** Proposed scheme outlining the pathway by which Na$_2$S leads to miR-21 induction and protection against cardiomyocyte cell death, inflammasome formation/activation and LV dysfunction following ischemia/reperfusion injury.
Ischemia-Reperfusion Injury → miR-21

- LV Dysfunction
- Cardiomyocyte Death
- Inflammasome (Cryopyrin, ASC, Caspase-1)

Myocardial Protection