Hydrogen sulfide alleviates cardiac contractile dysfunction in an Akt2-knockout murine model of insulin resistance: role of mitochondrial injury and apoptosis

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Hydrogen sulfide alleviates cardiac contractile dysfunction in an Akt2-knockout murine model of insulin resistance: role of mitochondrial injury and apoptosis. Am J Physiol Regul Integr Comp Physiol 306: R761–R771, 2014. First published March 12, 2014; doi:10.1152/ajpregu.00327.2013.—Hydrogen sulfide (H2S) is a toxic gas now being recognized as an endogenous signaling molecule in multiple organ systems, in particular, the cardiovascular system. H2S is known to regulate cardiac function and protect against ischemic injury. However, little information is available regarding the effect of H2S on cardiac function in insulin resistance. This study was designed to examine the impact of H2S supplementation on cardiac function using an Akt2 knockout model of insulin resistance. Wild-type and Akt2 knockout mice were treated with NaHS (50 µM·kg−1·day−1 ip for 10 days) prior to evaluation of echocardiographic, cardiomyocyte contractile, and intracellular Ca2+ properties, apoptosis, and mitochondrial damage. Our results revealed that Akt2 ablation led to overtly enlarged ventricular end-systolic diameter, reduced myocardial and cardiomyocyte contractile function, and disrupted intracellular Ca2+ homeostasis and apoptosis, the effects of which were ameliorated by H2S. Furthermore, Akt2 knockout displayed upregulated apoptotic protein markers (Bax, caspase-3, caspase-9, and caspase-12) and mitochondrial damage (reduced aconitase activity and NAD+, elevated cytochrome-c release from mitochondria) along with reduced phosphorylation of PTEN, Akt, and GSK3β in the absence of changes in pan protein expression, the effects of which were abolished or significantly ameliorated by H2S treatment. In vitro data revealed that H2S-induced beneficial effect against Akt2 ablation was obliterated by mitochondrial uncoupling. Taken together, our findings suggest the H2S may reconcile Akt2 knockout-induced myocardial contractile defect and intracellular Ca2+ mishandling, possibly via attenuation of mitochondrial injury and apoptosis.

hydrogen sulfide; Akt2; insulin resistance; mitochondria; apoptosis; cardiomyocyte

Although produced mainly in gastrointestinal and nervous systems, hydrogen sulfide (H2S), a known toxic gas, plays a crucial role as a signaling molecule (34, 49). Recent studies have established a role for H2S as the third endogenous gaseous transmitter in mammals, besides nitric oxide (NO) and carbon monoxide (CO), in the regulation of biological function in multiple organ systems (8, 45, 53, 61). Accumulating evidence has revealed low circulating levels of H2S in diabetic patients and streptozotocin (STZ)-induced experimental diabetes models (24). Not surprisingly, H2S deficiency has been postulated to contribute to the pathogenesis of endothelial dysfunction, nephropathy, and cardiomyopathy in diabetes (29, 50), implicating the potential benefit for H2S in the management of diabetes and diabetic complications. This is supported by various cardiovascular actions of H2S, including the Akt-dependent proangiogenic property (6, 52), antiapoptosis (57), and inhibition of L-type Ca2+ channels (46) in cardiomyocytes.

Insulin resistance is attributed to the obesity pandemic and drastically increases the prevalence of cardiovascular diseases (4). Although a number of scenarios have been postulated for insulin resistance-induced cardiovascular anomalies, including dyslipidemia, inflammation, endoplasmic reticulum, and oxidative stress (21, 26–28), the precise mechanisms behind the cardiac dysfunction induced by insulin resistance still remain controversial, thus making adequate clinical management somewhat challenging. Insulin signaling plays an essential role in the regulation of myocardial oxidative phosphorylation and myocardial contractile function (38). This is supported by the fact that insulin-receptor knockout dampens the oxidative phosphorylation and exacerbates cardiac dysfunction (1, 42). Although insulin signaling is rather complex, involving a large cascade of signaling molecules, the phosphatidylinositol 3-kinase (PI3K)-Akt cascade is deemed perhaps the main player governing the majority of metabolic properties of insulin (14, 30). Akt is a serine/threonine kinase directly downstream of PI3K to mediate the metabolic actions of insulin (14). Interestingly, impaired insulin-stimulated PI3K/Akt has also been implicated in a number of pathological conditions accompanied by insulin resistance, such as obesity, inflammation, cardiovascular and renal complications of diabetes, as well as cancer (41, 55, 58). As a matter of fact, the onset of insulin resistance and later on diabetes mellitus is often linked to changes in Akt phosphorylation. Akt regulates glucose uptake in muscle and adipocytes through stimulating the translocation of GLUT4 glucose transporter to the plasma membrane (20). The identification of a dominant negative Akt2 mutation (R274H), which leads to severe hyperinsulinemia and diabetes in humans, has consolidated a permissive role for Akt2 in metabolic regulation (18). In particular, Akt2 knockout mice exhibit overt global insulin resistance as manifested by decreased glucose uptake into muscle and adipose cells, despite the normal growth and development (9, 17, 39).

Given that cardiac dysfunction is a major complication of insulin resistance where Akt signaling plays a key role in the maintenance of cardiac homeostasis (11, 15, 58), this study
was undertaken to examine the effect of Akt2 knockout on myocardial function, and the impact of H$_2$S supplement on Akt2 knockout-induced myocardial anomalies, if any. In an effort to better elucidate the mechanisms involved in Akt2 knockout and H$_2$S supplement-induced myocardial function, mitochondrial integrity, crucial signaling molecules of insulin signaling, such as Akt, phosphatase and tensin homolog on chromosome 10 (PTEN), glycogen synthase kinase 3β (GSK3β), and protein phosphatase, which usually negatively regulates insulin signaling (44), were examined in hearts from wild-type (WT) and Akt2 knockout (KO) mice.

MATERIALS AND METHODS

Experimental animals and H$_2$S treatment. The animal procedures described in this study were approved by the Institutional Animal Use and Care Committee at the University of Wyoming (Laramie, WY). The Akt2 knockout mice were obtained from Prof. Morris Birnbaum at the University of Pennsylvania (Philadelphia, PA) and were characterized as described previously (9). Wild-type and Akt2KO mice (10 per group) were treated with NaHS (50 μM·kg$^{-1}$·day$^{-1}$ ip for 10 days) according to the dosage and duration for NaHS reported previously (6, 40). NaHS-untreated mice (10 mice per group) received equal volume of PBS as the vehicle control. All mice were maintained at 22°C with a 12:12-h light-dark cycle and received lab chow and water ad libitum.

Intraperitoneal glucose tolerance test. All mice were fasted for 12 h and given an intraperitoneal injection of glucose (2 g/kg body wt ip). Blood samples were drawn from the tail, and glucose levels were determined immediately before glucose challenge, as well as 30, 60, 90, and 120 min thereafter using an Accu-Chek III glucose analyzer (15).

Tissue cystathionine γ-lyase activity. Cardiac cystathionine γ-lyase (CSE) activity was measured according to the Stipanuk method, as described previously (43, 59). Ventricular tissues and plasma were collected at the end of 10-day H$_2$S treatment and were stored at −80°C. Measurement of H$_2$S was performed 1 wk following sample collection. Briefly, frozen tissues (50 mg) were homogenized in 0.5 ml ice-cold 100 mM potassium phosphate buffer (pH 7.4), then centrifuged at 4°C. 10,000 g for 10 min. The clear supernatant was transferred to an Eppendorf tube before being mixed with cystathionine (2 mM) and pyridoxal-5'-phosphate (0.25 mM) in 100 mM Tris-HCl buffer (pH 8.3) for 60 min at 37°C. Trichloroacetic acid (TCA; 10% vol/vol) was added into the reaction mixture to terminate the reaction. Following centrifugation, supernatants were mixed with 1% ninhydrin reagent and were incubated for 5 min in a boiling-water bath. After heating, the solution was cooled on ice for 2 min, and the color reaction development were assayed for 20 min at 455 nm with a spectrophotometer. CSE activity was assessed by cystathionine consumption, and enzyme activity was expressed as nanomoles of

Table 1. General characteristics of WT and Akt2KO mice treated with or without NaHS

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>WT H$_2$S</th>
<th>Akt2KO</th>
<th>Akt2KO H$_2$S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>24.8 ± 0.7</td>
<td>24.9 ± 0.4</td>
<td>24.6 ± 0.6</td>
<td>25.0 ± 0.7</td>
</tr>
<tr>
<td>Heart weight, mg</td>
<td>134 ± 5</td>
<td>136 ± 6</td>
<td>137 ± 6</td>
<td>136 ± 6</td>
</tr>
<tr>
<td>Liver weight, g</td>
<td>1.34 ± 0.06</td>
<td>1.30 ± 0.03</td>
<td>1.36 ± 0.07</td>
<td>1.35 ± 0.05</td>
</tr>
<tr>
<td>Kidney weight, mg</td>
<td>330 ± 15</td>
<td>324 ± 13</td>
<td>328 ± 16</td>
<td>328 ± 14</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>497 ± 27</td>
<td>485 ± 35</td>
<td>489 ± 37</td>
<td>476 ± 25</td>
</tr>
<tr>
<td>Wall thickness, mm</td>
<td>0.89 ± 0.02</td>
<td>0.95 ± 0.03</td>
<td>0.87 ± 0.03</td>
<td>0.93 ± 0.02</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>2.46 ± 0.13</td>
<td>2.43 ± 0.16</td>
<td>2.62 ± 0.17</td>
<td>2.34 ± 0.11</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>1.11 ± 0.10</td>
<td>1.10 ± 0.08</td>
<td>1.63 ± 0.14*</td>
<td>1.02 ± 0.07#</td>
</tr>
<tr>
<td>Fractional Shortening, %</td>
<td>53.7 ± 1.8</td>
<td>55.1 ± 2.2</td>
<td>38.0 ± 1.9*</td>
<td>56.2 ± 2.4#</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE; n = 7 or 8 mice per group. Dosage for NaHS treatment was 50 μM·kg$^{-1}$·day$^{-1}$ ip for 10 days. LV, left ventricular; EDD, end-diastolic diameter; ESD, end-systolic diameter. *P < 0.05 vs. WT group. #P < 0.05 vs. Akt2KO group.
cystathionine consumed per milligram of total protein per hour of incubation.

**Plasma H$_2$S levels.** Plasma H$_2$S levels were determined using previously described methods (2, 63). Plasma samples (120 μl) were mixed with 100 μl water and 120 μl TCA (10% vol/vol), reacted for 10 min at room temperature, and then centrifuged at 4°C, 14,000 g for 10 min. The clear supernatant was transferred to an Eppendorf tube containing zinc acetate (1% 60 μl). Subsequently, N, N-dimethylphenylendiamine sulfate 40 μl (20 mM in 7.2 M HCl) and FeCl$_3$ 40 μl (30 mM in 1.2 M HCl) were added to the reaction mixture for 20 min at room temperature. The absorbance was measured at a wavelength of 670 nm with a spectrophotometer. The plasma H$_2$S concentration was calculated against the calibration curve of standard H$_2$S solutions, and all samples were assayed in duplicate.

**Echocardiographic assessment.** Cardiac geometry and function were evaluated in anesthetized (ketamine 80 mg/kg and xylazine 12 mg/kg ip) mice using the two-dimensional guided M-mode echocardiography (Phillips Sonos 5500) equipped with a 15–16-MHz linear transducer (Phillips Medical Systems, Andover, MD). Left ventricular anterior and posterior wall dimensions during diastole and systole were recorded from three consecutive cycles in M mode using methods adopted by the American Society of Echocardiography. Fractional shortening was calculated from LV end-diastolic (EDD) and end-systolic (ESD) diameters using the equation (EDD − ESD)/EDD. Heart rate was averaged over 10 cardiac cycles (23).

**Isolation of cardiomyocytes.** Murine cardiomyocytes were isolated as described by Ceylan-Isik et al. (7). After ketamine/xylazine sedation, hearts were removed and perfused with Ca$^{2+}$-free Tyrode’s solution containing (in mM): 135 NaCl, 4.0 KCl, 1.0 MgCl$_2$, 10 HEPES, 0.33 NaH$_2$PO$_4$, 10 glucose, 10 butanedione monoxime, and the solution was gassed with 5% CO$_2$–95% O$_2$. Hearts were digested with Liberase Blendzyme 4 (Hoffmann-La Roche, Indianapolis, IN) for 20 min. Extracellular Ca$^{2+}$ was added incrementally back to 1.20 mM over a period of 30 min. Isolated myocytes were used within 8 h of isolation. Normally, a yield of 50–60% viable rod-shaped cardiomyocytes with clear sarcomere striations was achieved. Only rod-shaped myocytes with clear edges were selected for mechanical study. To discern the role of mitochondria in H$_2$S supplementation’s protective effect against Akt2 ablation-induced cardiomyocyte contractile dysfunction, freshly isolated murine cardiomyocytes from wild-type and Akt2KO mice were pretreated with the mitochondrial uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP; 1 μM) (54) for 1 h prior to the exposure to NaHS (50 μM) (6) for an additional 2 h.

**Cell shortening/relengthening.** Mechanical properties of cardiomyocytes were assessed using a SoftEdge MyoCam system (IonOptix, Milton, MA). In brief, cardiomyocytes were placed in a Warner chamber mounted onto the stage of an inverted microscope (Olympus IX-70, Olympus, Tokyo, Japan) and superfused (1 ml/min at 25°C) with a buffer containing (in mM) 131 NaCl, 4 KCl, 1 CaCl$_2$, 1 MgCl$_2$, 10 glucose, and 10 HEPES at pH 7.4. The cells were field-stimulated with suprathreshold voltage at a frequency of 0.5 Hz using a pair of platinum wires placed on opposite sides of the chamber connected to a Pulsar 6bp bipolar stimulator (FHC, Brunswick, NJ). The myocyte being studied was displayed on the computer monitor using an IonOptix MyoCam camera. An IonOptix SoftEdge software was used to capture changes in cell length during shortening and relengthening. Cell shortening and relengthening were assessed using the following

![Graph](https://via.placeholder.com/150)

**Fig. 2.** Effect of NaHS (50 μM·kg$^{-1}$·day$^{-1}$ ip for 10 days) on cardiomyocyte contractile properties in WT and Akt2KO mice. **A:** resting cell length. **B:** peak shortening (% of resting cell length). **C:** maximal velocity of shortening (+dL/dt); **D:** maximal velocity of relengthening (−dL/dt); **E:** time-to-peak shortening (TPS). **F:** time-to-90% relengthening (TR$_{90}$). Values are expressed as means ± SE; n = 90–100 cells from three mice per group, *P < 0.05 vs. WT group.
indices: resting cell length, peak shortening (PS), time-to-PS (TPS),
time-to-90% relengthening (TR90), and maximal velocity of shorten-
ing/relengthening (± dL/dt) (22).

Intracellular Ca^{2+} transients. A cohort of myocytes was loaded
with Fura-2 AM (0.5 μM) for 10 min, and fluorescence intensity was
recorded with a dual-excitation fluorescence photomultiplier system
(IonOptix). Myocytes were placed onto an Olympus IX-70 inverted
microscope and imaged through a Fluor 40 oil objective. Cells were
exposed to light emitted by a 75-W lamp and passed through either a
360- or a 380-nm filter, while being stimulated to contract at 0.5 Hz.
Fluorescence emissions were detected between 480 and 520 nm, and
qualitative change in Fura-2 AM fluorescence intensity (FFI) was
inferred from the FFI ratio at the two wavelengths (360/380). Flu-
orescence decay time was measured as an indication of the intracellular
Ca^{2+} clearing rate. Single exponential curve fit was used to calculate
the intracellular Ca^{2+} decay constant (22).

Caspase-3 assay. Caspase-3 is an enzyme activated during induc-
ton of apoptosis. In brief, 1 ml of PBS was added to flasks containing
mouse cardiomyocytes, and the monolayer was scraped and collected
in a microfuge tube. The cells were centrifuged at 10,000 g
for 10 min, and cell pellets were lysed in 100 μl of ice-cold cell lysis
buffer (50 mM HEPES, 0.1% CHAPS, 1 mM dithiothreitol, 0.1 mM
EDTA, 0.1% NP40). After cells were lysed, 70 μl of reaction buffer
was added to cell lystate (30 μl), followed by an additional 20 μl of
caspase-3 colorimetric substrate (Ac-DEVD-pNA) and incubated at
37°C for 1 h, during which time the caspase in the sample was allowed
to cleave the chromophore pNA from the substrate molecule.
The samples were then read with a microplate reader at 405 nm. Caspase-3
activity was expressed as picomoles of pNA released per micromgram
of protein per minute (60).

Determination of NAD^{+}. NAD^{+} was extracted from frozen ven-
tricular tissues using perchloric acid (10, 16). For these determi-
inations, 30 mg of fresh frozen tissue was powdered in a mortar under
liquid nitrogen and thoroughly mixed with 150 μl of 6 M hydroxyl aci-
cid. The mixture was then homogenized, neutralized with 150
potassium hydroxide, and centrifuged. NAD^{+} concentrations were
determined fluorometrically in dilutions of the supernatant sample
using alcohol dehydrogenase (Sigma-Aldrich, St. Louis, MO). Excita-
tion was at 339 nm, and emission wavelength was at 460 nm in a
spectrofluorimeter (Spectra Max Gemini XS, Sunnyvale, CA) (62).

Aconitase activity. Mitochondrial aconitase, an iron-sulfur enzyme
occurring during the citric acid cycle, is readily damaged by oxidative
stress via removal of an iron from [4Fe-4S] cluster. Mitochondrial
fractions prepared from whole heart homogenate were resuspended
in 0.2 mM sodium citrate. Aconitase activity assay (Aconitase activity
assay kit, Aconitase-340 assay, OxisResearch, Portland, OR) was
performed according to manufacturer instructions with minor modi-
fications. Briefly, mitochondrial sample (50 μl) was mixed in a
96-well plate with 50 μl trisodium citrate (substrate) in Tris-HCl pH
7.4, 50 μl isocitrate dehydrogenase (enzyme) in Tris-HCl, and 50 μl
NADP + reagent in Tris-HCl. After incubating for 15 min at 37°C, the
absorbance was dynamically recorded at 434 nm every min for 5 min
with a spectrophotometer. During the assay, citrate is isomerized by
aconitase into isocitrate and eventually α-ketoglutarate. The Acon-
itase-340 assay measures NADPH formation, a product of the oxida-
tion of isocitrate to α-ketoglutarate. Tris-HCl buffer (pH 7.4) was
served as blank (37).

Separation of mitochondrial and cytosolic fractions. Ventricles
were minced and homogenized by Polytron in the ice-cold MES
buffer [220 mM mannitol, 70 mM sucrose, 2 mM EGTA, 5 mM
3-(4-morpholino) propane sulfonic acid (MOPS), at pH 7.4, 0.2% 
BSA] and a protease inhibitor cocktail containing 4-(2-aminoethyl)
benzenesulfonyl fluoride, E-64, bestatin, leupeptin, aprotinin, and
EDTA obtained from Sigma Chemicals. The homogenates were
centrifuged for 10 min at 600 g to remove unbroken tissue and nuclei,
and the supernatants were centrifuged for 10 min at 3,000 g to obtain
cytosolic fraction. The mitochondrial pellet was dissolved in the protein
lysis buffer and centrifuged at 10,000 g for 30 min at 4°C to make a soluble protein. Fifty micrograms of the

Fig. 3. Effect of NaHs (50 μM·kg⁻¹·day⁻¹ ip for 10 days) on intracellular Ca^{2+} transients mea-
sured using Fura-2 AM in cardiomyocytes from WT and Akt2KO mice. A: baseline Fura-2 fluo-
rescence intensity (FFI), B: change in FFI (ΔFFI) in response to electrical stimuli. C: single exponen-
tial intracellular Ca^{2+} decay rate. D: biexponential intracellular Ca^{2+} decay rate. Values are
expressed as means ± SE; n = 75–80 cells from three mice per group, *P < 0.05 vs. WT group,
#P < 0.05 vs. Akt2KO group.
mitochondrial or cytosolic protein was separated by 15% SDS-PAGE for Western blot analysis of cytochrome c (13).

Western blot analysis. Protein samples were prepared as previously described (7). Samples containing an equal amount of proteins were separated on 10% SDS-polyacrylamide gels in a minigel apparatus (Mini-PROTEAN II, Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes. The membranes were blocked with 5% milk in TBS-Tween, and were incubated overnight at 4°C with anti-Akt2, anti-Bax, anti-Bcl-2, anti-cleaved caspase-3, anti-cleaved caspase-9, anti-cleaved caspase-12, anti-PTEN, anti-phospho-PTEN, anti-Akt, anti-phospho-Akt, anti-GSK3β, anti-phospho-GSK3β, and anti-cytochrome c antibodies. After washing blots to remove excessive primary antibody binding, blots were incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (1: 5,000). Antibody binding was detected using enhanced chemiluminescence (Amersham Pharmacia, Piscataway, NJ), film was scanned, and the intensity of immunoblot bands was detected with a Bio-Rad Calibrated Densitometer (model no. GS-800). All tissue samples were run in duplicate. GAPDH was used as the loading control.

Statistical analysis. Data are expressed as means ± SE. Statistical significance (P < 0.05) was estimated by a two-way ANOVA followed by a Bonferroni multicomparison analysis when necessary.

RESULTS

General and echocardiographic properties of WT and Akt2KO mice following H2S supplement. Neither Akt2 knockout nor H2S treatment, or both, significantly affected body, heart, liver, and kidney weights. Echocardiographic assessment revealed comparable heart rate, left ventricular (LV) wall thickness, and LV end-diastolic diameter (LVEDD) among all four mouse groups. However, Akt2 knockout significantly increased LV end-systolic diameter (LVEDD) and lessened fractional shortening, the effect of which was abrogated by H2S supplement. H2S itself did not affect any of the echocardiographic indices tested (Table 1).

Effect of hydrogen sulfide supplement on Akt2 knockout-induced insulin resistance. Intraperitoneal glucose tolerance was performed at the end of the 10-day H2S supplement. Following the intraperitoneal glucose challenge (2 g/kg body wt), serum glucose levels started to decline after peaking at 30 min and returned to near baseline levels at 120 min. To the contrary, Akt2 knockout displayed severe glucose intolerance, as evidenced by a much greater area under the curve for IPGTT, although basal glucose levels were comparable to those of WT mice. H2S supplementation failed to affect basal blood glucose levels or glucose disposal rate following glucose challenge in either WT or Akt2 knockout mice. Plasma H2S levels were significantly lower in Akt2KO mice compared with WT mice, the effect of which was abolished by H2S treatment for 10 days. Likewise, cardiac H2S synthase CSE activity, an indicator for tissue H2S synthesis rate, was significantly lower in Akt2KO mice compared with WT mice, the effect of which...

![Fig. 4. Effect of NaHS (50 μM·kg⁻¹·day⁻¹ ip for 10 days) on caspase-3 activity, cytochrome c distribution, aconitase activity, and NAD⁺ level in WT and Akt2KO mice. Myocardial tissues were separated using differential density centrifugation to yield cytosolic and mitochondrial fractions prior to gel electrophoresis. A: caspase-3 activity. B: representative gel blots depicting level of cytochrome c in cytosol and the mitochondria using respective specific antibodies. GAPDH was used as loading control. C: cytochrome c levels in the cytosol. D: cytochrome c levels in mitochondria. E: aconitase activity. F: NAD⁺ level depicting mitochondrial permeation pore opening. Values are expressed as means ± SE; n = 4 or 5 mice per group. *P < 0.05 vs. WT group, #P < 0.05 vs. Akt2KO group.](http://ajpregu.physiology.org/doi/10.1152/ajpregu.00327.2013)
was removed by the short-term H$_2$S treatment. H$_2$S treatment itself did not overtly affect tissue or plasma H$_2$S levels (Fig. 1).

**Effect of hydrogen sulfide on cardiomyocyte contractile and intracellular Ca$^{2+}$ properties.** Neither short-term H$_2$S treatment nor Akt2 knockout overtly affected resting cell length in cardiomyocytes. Cardiomyocytes from Akt2 knockout mice displayed significantly depressed PS and $\pm$dL/dt, as well as prolonged TR$_{90}$ without affecting TPS. Although H$_2$S itself did not affect these mechanical parameters tested, it significantly attenuated Akt2KO-induced mechanical anomalies (Fig. 2). To further understand the possible mechanism of action behind Akt2KO and H$_2$S supplementation-induced cardiac responses, intracellular Ca$^{2+}$ homeostasis was evaluated in cardiomyocytes using the intracellular Ca$^{2+}$ fluorescence dye Fura-2 AM. Data presented in Fig. 3 show that Akt2KO significantly elevated the baseline FFI and suppressed electrically stimulated rise in Fura-2 AM fluorescence intensity ($\Delta$FFI), as well as slowed down intracellular Ca$^{2+}$ decay rate. Although H$_2$S supplementation itself did not affect these intracellular Ca$^{2+}$ parameters, it abolished or significantly attenuated Akt2KO-induced changes in intracellular Ca$^{2+}$ handling.

**Effect of hydrogen sulfide on caspase-3 activity, cytochrome c release, aconitase activity, and mitochondrial permeation pore opening in Akt2KO mice.** Data shown in Fig. 4 depict that Akt2KO significantly triggers apoptosis (as manifested by elevated caspase-3 activity) and promotes mitochondrial cytochrome c release into cytosol (decreasing mitochondrial content and increasing cytosolic cytochrome c content). Although H$_2$S supplementation did not affect apoptosis and mitochondrial cytochrome c release, it significantly attenuated Akt2KO-induced apoptosis and cytochrome c release. Given that aconitase, an iron sulfur enzyme located in citric acid cycle, is closely associated with oxidative stress and mitochondrial function (37), we further evaluated aconitase activity and NAD$^+$ levels, a marker for mitochondrial permeation pore opening. Our data further revealed significantly decreased aconitase activity and NAD$^+$ levels in Akt2KO mouse hearts, indicating mitochondrial injury. While H$_2$S supplementation itself did not affect aconitase activity and NAD$^+$ levels, it effectively rectified Akt2KO-induced decrease in aconitase activity and mitochondrial permeation pore opening (evidenced by reduced NAD$^+$ levels).

**Effect of hydrogen sulfide treatment on pan and phosphorylated Akt, PTEN, and GSK3β in WT and Akt2KO mice.** Our results indicate that although Akt2KO mice had significantly reduced Akt expression, pan protein expression of PTEN and GSK3β was not affected. Moreover, Akt2KO significantly decreased phosphorylation of PTEN, Akt, and GSK3β (absolute or normalized value). Although H$_2$S supplementation failed to alter expression of pan and phosphorylated Akt and GSK3β, it significantly attenuated or ablated Akt2KO-elicited loss in the phosphorylation of Akt, PTEN, and GSK3β (Fig. 5). 

**Effect of hydrogen sulfide treatment on apoptotic protein makers in WT and Akt2KO mice.** Our results shown in Fig. 6 indicate that Akt2 knockout led to significantly upregulated expression of the proapoptotic proteins Bax, Caspase-3, Caspase-9, and the ER-specific caspase-12, as well as downregulated expression of the antiapoptotic protein Bcl-2. Although H$_2$S itself failed to affect the expression of these apoptotic proteins, it significantly attenuated or mitigated

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**Fig. 5.** Effect of NaHS (50 μM·kg$^{-1}$·day$^{-1}$ ip for 10 days) on basal and phosphorylated levels of PTEN, Akt, and GSK3β in myocardium from WT and Akt2KO mice. A: representative gel blots depicting expression of pPTEN, PTEN, pAkt, Akt, pGSK3β, and GSK3β using respective specific antibodies. GAPDH was used as loading control. B: pPTEN-to-PTEN ratio. C: pAkt-to-Akt ratio. D: pGSK3β-to-GSK3β ratio. Values are expressed as mean ± SE; n = 4 or 5 mice per group. *P < 0.05 vs. WT group, #P < 0.05 vs. Akt2KO group.
Akt2KO-elicited responses in Bax, Bcl-2, caspase-3, caspase-9, and caspase-12.

Influence of mitochondrial uncoupling on H2S-induced cardiomyocyte contractile responses. To further discern the role of mitochondria in H2S supplementation’s protective effect against Akt2KO cardiomyocyte anomalies, freshly isolated murine cardiomyocytes from WT and Akt2KO mice were pretreated with the mitochondrial uncoupler FCCP (1 μM) (54) for 1 h prior to the exposure to NaHS (50 μM) (6) for an additional 2 h. Similar to the in vivo observation, short-term coincubation of H2S obliterated or significantly dampened Akt2KO-induced cardiomyocyte contractile function (shown as reduced PS, dL/dt and prolonged TR90) without eliciting any effect itself. Interestingly, the beneficial mechanical effects of H2S were nullified by the mitochondrial uncoupler FCCP. Moreover, FCCP did not produce any further effect on Akt2 ablation-induced cardiomyocyte contractile dysfunction (Fig. 7).

DISCUSSION

The salient findings from our present study revealed that Akt2KO exerts a drop in circulating and cardiac tissue H2S levels, cardiac contractile and intracellular Ca2+ derangement (as evidenced by enlarged LVESD and reduced fraction shortening, depressed peak shortening, maximal velocity of shortening/relengthening, prolonged duration of relengthening, increased basal intracellular Ca2+ levels, and reduced intracellular Ca2+ release in response to electrical stimuli). The compromised cardiac function and intracellular Ca2+ handling in Akt2KO mice were accompanied with upregulated proapoptotic protein markers (Bax, caspase-3, caspase-9, and caspase-12), downregulated anti-apoptotic protein marker Bcl-2, elevated caspase-3 activity, and overt mitochondrial injury (reduced aconitase activity and NAD+ and elevated cytochrome c release from mitochondria). Evaluation of cell signaling mechanism revealed that Akt2KO-induced insulin resistance suppressed phosphorylation of PTEN, Akt, and GSK-3β in the myocardium, the effect of which was attenuated or reversed by H2S supplementation. These data implicate a possible role of Akt-GSK3β signaling in H2S-offered cardioprotection. These findings have indicated a favorable effect of H2S supplementation against myocardial anomalies in Akt2KO-induced insulin resistance.

Our findings suggest that knockout of Akt2 elicits overt glucose intolerance, as manifested by increased area under the curve for IPGTT, which is associated with unchanged baseline blood glucose levels (except for the presence of outright diabetes), validating the murine model of insulin resistance (9). Our data further revealed that knockout of Akt2 promoted myocardial contractile anomalies, including enlarged LVESD; reduced fractional shortening, peak shortening, and maximal velocity of shortening/relengthening (dL/dt), and prolonged duration of lengthening (TR90), which is associated with the unchanged LV wall thickness, LVEDD, cardiomyocyte cell length, and duration of shortening (TPS). Our data revealed impaired intracellular Ca2+ handling, which was manifested as elevated baseline intracellular Ca2+ levels, reduced intracellular Ca2+ rise (∆FFI), and prolonged intracel-

Fig. 6. Effect of NaHS (50 μM·kg−1·day−1 ip for 10 days) on apoptotic proteins Bax, Bcl-2, caspase-9, caspase-12, and caspase-3 in WT and Akt2KO mice. A: representative gel blots depicting expression of Bax, Bcl-2, cleaved caspase-9, caspase-12, and caspase-3 using respective specific antibodies. GAPDH was used as loading control. B: Bax expression. C: Bcl-2 expression. D: cleaved caspase-9 expression. E: cleaved caspase-12 expression. F: cleaved caspase-3 expression. Values are expressed as means ± SE; n = 4 or 5 per group. *P < 0.05 vs. WT group, #P < 0.05 vs. Akt2KO group.
lular Ca\(^{2+}\) clearance in cardiomyocytes from Akt2KO mice, indicating an essential role of intracellular Ca\(^{2+}\) mishandling in Akt2KO-induced myocardial mechanical defects. These findings are somewhat consistent with our earlier findings using a high-sucrose or high-fat diet-induced insulin resistance model (11, 12, 15, 31) and confirm a pivotal role of Akt in the regulation of glucose metabolism and myocardial function (25, 41, 58).

In our hands, Akt2KO-induced insulin resistance is associated with a significant reduction in both plasma and tissue H\(_2\)S levels, consistent with the findings from both genetically predisposed (nonobese diabetic) (5) and chemically induced (STZ) (24) experimental diabetes models. After the 10-day NaHS treatment, Akt2KO-induced decrease in plasma and tissue H\(_2\)S levels (or synthesis rate) was significantly attenuated or ablated, in a manner somewhat consistent with the finding reported in experimental diabetes (32).

Perhaps the most intriguing finding from our study is that H\(_2\)S supplementation effectively ameliorated Akt2KO-induced glucose intolerance and myocardial anomalies. These results are in line with the earlier report that H\(_2\)S may facilitate glucose uptake, insulin receptor sensitivity, and phosphorylation of PI3K/Akt in muscles (56). Beneficial properties of chronic H\(_2\)S supplementation are also present in vasculatures, including promoted migration and tube formation in vascular endothelial cells (6). Given the multiple protective roles for H\(_2\)S in the cardiovascular system (8, 45, 53, 61), it is plausible to speculate that reduced circulating and cardiac tissue H\(_2\)S levels may contribute to the development of insulin resistance-induced myocardial anomalies. Nonetheless, further study is warranted to elucidate the mechanism of action behind H\(_2\)S deficiency-induced cardiac pathology.

Although the precise mechanism(s) of action behind H\(_2\)S supplement ion’s protective action remains somewhat elusive, several possible scenarios may be considered. Our findings exhibited that H\(_2\)S is capable of alleviating Akt2KO-induced mitochondrial injury. Emerging evidence has indicated a unique role for mitochondrial integrity in the pathogenesis and management of cardiac dysfunction in insulin resistance (38). Mitochondria exerts a key role in the control of energy metabolism, cell survival, and myocardial function (38). Our observation of reduced aconitase levels and elevated release of cytochrome c, an essential component of the electron transport...
chain in mitochondria (51), in hearts from Akt2KO mice supports a role of mitochondrial injury in insulin resistance-triggered cardiac anomalies. Adequate Akt signaling is indispensable in the regulation of mitochondrial function in the heart. The Akt downstream signaling molecules hexokinase and Pim-1 kinase are known to preserve mitochondrial function (47). Moreover, Akt suppresses mitochondrial permeation pore (mPTP) opening, thus protecting mitochondrial integrity via phosphorylation of GSK-3β (60). In our study, NAD+ was measured to indirectly assess mPTP opening. Di Lisa et al. (10) reported that cyclosporin A, a potent mPTP inhibitor, prevents NAD+ depletion, considering that NAD+ loss can be directly attributed to mPTP opening. In our hands, knockout of Akt2 lowered phosphorylation of PTEN, which would lead to a higher kinase activity of PTEN, a negative regulator of Akt. This effect, in conjunction with dampened Akt phosphorylation, contributes to the Akt2KO-induced GSK-3β dephosphorylation, en route to mitochondrial injury. H2S supplementation reversed or partially restored Akt2KO-induced loss of phosphorylation in PTEN, Akt, and GSK-3β to exert its beneficial effect on preservation of mitochondrial integrity in the heart.

Our in vitro finding that mitochondrial uncoupler FCCP mitigated H2S-induced beneficial effects further strengthened a cause-effect relationship of mitochondrial integrity in H2S-offered protection.

Our results also demonstrated that H2S exerts protective effects against insulin resistance-induced apoptosis of cardiomyocytes, in a manner similar to mechanical and intracellular Ca2+ responses, favoring a role of lessened apoptosis in H2S-offered beneficial effect in Akt2KO mice. In addition, data from cytochrome c release further supported H2S-induced antiapoptotic responses. As an intermediate in apoptosis and a controlled form of cell death in the process of development or in response to infection or DNA damage (33), cytochrome c is released from the mitochondria in response to proapoptotic stimuli (3). This release of cytochrome c, in turn, activates caspase-9, and subsequently caspase-3, leading to ultimate cell death (19). Our finding of elevated levels of caspase-3, caspase-9, and caspase-12 along with caspase-3 activity supports a likely role for apoptosis in Akt2KO-induced cardiac mechanical anomalies. Along the same line, H2S was found to protect against myocardial injury in diabetes through alleviating apoptosis and oxidative stress (36, 48). Finally, H2S is considered a classic cytochrome c oxidase inhibitor and an in vitro oxidase substrate (35). Similar to other low-molecular-weight messengers and oxidase inhibitors, including NO and CO, H2S may bind to oxidase sites prior to oxidation to generate persulfide species. Thus, sulfide may rapidly inhibit mitochondrial cytochrome c oxidase (35). It is possible that persulfidation of phosphatases and/or inhibition of mitochondrial cytochrome c oxidase may contribute to H2S-induced beneficial effects, although this is beyond the scope of this study.

**Perspectives and Significance**

Insulin resistance is a major independent risk factor for diabetes and cardiovascular diseases. Our article reveals that H2S may provide rescue from glucose intolerance and cardiac contractile and intracellular Ca2+ anomalies in Akt2KO-induced insulin resistance, depicting a favorable role for H2S in insulin resistance and associated cardiac complications. Ample data have revealed low levels of H2S in insulin resistance and diabetes, which may contribute to cardiac complications in these comorbidities (36). Given the impaired PI3K/Akt signaling in insulin resistance and diabetes mellitus, the jury is still out as to whether decreased H2S levels are directly responsible for compromised insulin sensitivity and ultimately insulin resistance. This study, using Akt2KO model, provides the first evidence that H2S supplementation is sufficient to reverse insulin resistance-induced cardiomyopathy. Our results support the notion that H2S benefits cardiac function in insulin resistance through alleviation of PTEN-Akt-GSK3β-mediated mitochondrial injury and apoptosis. Since evidence from human subjects is still lacking with regard to the interplay between Akt2 gene polymorphism and cardiac function, caution must be taken when extrapolating the H2S findings obtained using the Akt2 ablation murine model to the more broadly defined “insulin resistance” model.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

Author contributions: N.H. conception and design of research; N.H. and M.D. performed experiments; N.H. and J.R. analyzed data; N.H. and M.D. prepared figures; N.H. and J.R. analyzed data; N.H. and J.R. edited and revised manuscript; N.H. and J.R. approved final version of the manuscript.

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