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Sulfide intoxication induced circulatory failure is mediated by a depression in cardiac contractility

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Abstract

Hydrogen sulfide (H₂S) intoxication produces a rapid cardio-circulatory failure leading to cardiac arrest. In non-lethal forms of sulfide exposure, the presence of a circulatory shock is associated with long-term neurological sequelae. Our aim was to clarify the mechanisms of H₂S-induced circulatory failure.

In anesthetized paralyzed and mechanically ventilated rats, cardiac output, arterial pressure and ventricular pressures were determined while NaHS was infused to increase arterial concentration of soluble H₂S (CgH₂S) from undetectable to levels leading to circulatory failure.

Compared to control/saline infusion, blood pressure started to decrease significantly along with a modest drop in peripheral vascular resistance ($-19 \pm 5\%$, $P < 0.01$), when CgH₂S reached about 1 microM. As CgH₂S exceeded 2-3 microM, parameters of ventricular contractility diminished with no further reduction in peripheral resistance. Whenever H₂S exposure was maintained at a higher level (CgH₂S over 7 microM), a clear inhibition of cardiac contractility was observed, leading to asystole within minutes, but with no evidence of peripheral vasoplegia.

The immediate and long-term neurological effects of specifically counteracting sulfide induced cardiac contractility depression following H₂S exposure remain to be investigated.

Keywords

cardiac toxicity; H₂S; vascular blood flow; vasodilation

Introduction

Hydrogen sulfide (H₂S) intoxication produces a coma associated to a depression in breathing and a profound circulatory failure [1]. This gas remains a potential source of dreadful intoxication in oil and gas industry [2] and has more recently used as a method of suicide [3]. In subjects or animals surviving the initial exposure, various neurological after-effects can occur spanning from minor defects -such as a persistent anosmia- to major cognitive and motor dysfunctions [4]. These neurological lesions appears to be provoked by

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the presence of a circulatory shock induced brain ischemia rather than by the direct exotoxicity of H₂S alone, as shown for instance by Baldelli et al [5].

The mechanisms of the circulatory shock produced during H₂S intoxication remains however poorly understood. Indeed, an increasing number of in-vitro studies are supporting the view that H₂S has vasodilatory properties, akin to the effects of nitric oxide (NO) [6-8]. In most of these studies, soluble H₂S at a concentration of 10 microM or above have been shown to produce a potent vasodilation [6-8]. Since similar blood levels of sulfide are sufficient to produce a terminal apnea in-vivo [9, 10], these observations suggest that exogenous H₂S-induced vasodilation is an effect of sulfide toxicity. On the other hand, H₂S has also been shown to reduce cardiac contractility, at least in-vitro, by acting like a calcium channel blocker [11, 12]. Since H₂S-induced circulatory failure seems to be the major determinant of the neurological outcome of patients surviving H₂S exposure [5], establishing the mechanisms of H₂S-induced circulatory failure, i.e. vasoplegia versus cardiogenic shock, will help us defining the strategy to be used in the symptomatic treatment of sulfide exposure and hopefully limit its potential devastating neurological sequelae of hydrogen sulfide, which appear to be greatly potentiated by the presence of low perfusion of the brain [4]. To clarify this outstanding issue, we determined the hemodynamic changes, i.e. cardiac output, peripheral arterial blood flow, intracardiac and arterial pressures and stroke volume along with the change in peripheral resistances and lactate production as a function of the concentrations of H₂S in the blood during stepwise increase or constant rate of sulfide infusion in anesthetized mechanically ventilated rats.

In this manuscript, the term sulfide or H₂S will be used interchangeably, as dissolved/ soluble H₂S when in solution in the blood or in cells is, at physiological pH, mainly (70-80%) in the form of the anion HS⁻, while the gaseous form of H₂S, which is responsible for H₂S partial pressure and thus its diffusion across membranes, is only 20-30% of the total pool of free sulfide [13, 14]. Of note, is that majority of H₂S produced or infused is combined with proteins and metallo-compounds sulfide (see [9, 10] and [15] for review and references). To describe the toxicology of the members of this “family” of molecules, H₂S is often used for convenience.

Material and Methods

Animal preparation

Adult male Sprague-Dawley rats weighing 490 ± 84 g (Charles River) were studied. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, 8th Edition (National Research Council (US) Institute for Laboratory Animal Research).

The study was approved by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee. All rats were housed in cages on a temperature-controlled room (24 ± 1°C) with 12 hour light/dark cycle, and were provided with standard laboratory diet and water ad libitum.

Anesthesia was induced with inhalation of isoflurane 3.5% in O₂, followed by the urethane (1.2 g/kg, i.p.). Rat were tracheostomized (14G surflo catheter, Terumo, Tokyo, Japan) and mechanically ventilated (frequency of breathing: 80 breaths/min, minute ventilation: ~350 ml/min) to maintain PaO₂ around 80 mmHg. To prevent any spontaneous breathing movement, a muscular relaxant was infused via tail vein (10 mg/kg/h, Rocuronium Bromide, Hospira, Inc. Lake Forest, IL). A catheter (PE-50 tubing) was inserted into the right femoral artery for continuous monitoring of systemic arterial blood pressure. Additional catheters were inserted into the right femoral vein for NaHS infusion and left carotid artery for arterial blood sampling. Adequate ventilation was monitored by periodic arterial blood gas measurements. Arterial blood (0.1 ml) was sampled and the gases partial pressures in O₂ (PaO₂), CO₂ (PaCO₂), and blood lactate concentration were measured using i-STAT1 blood gas analyzer (Abaxis, Union City, CA).

The left femoral artery was isolated and a transonic flow probe (MA1PRB, Transonic Systems Inc. Ithaca, NY) was placed around the artery.

Our initial purpose was to determine the change in cardiac output and peripheral resistance. Therefore, in a first group of rats (see protocol section for further information), a catheter (20G surflo catheter, TERUMO, Tokyo, Japan) was inserted into the right ventricle through the right jugular vein for continuous monitoring of right ventricular pressure. Cardiac output was determined as follows: The 1st and 2nd rib on the left side were partially resected and left upper lobe of the lung was retracted to expose the pulmonary artery trunk. The small incision of the pericardium was performed and the pulmonary artery was isolated from surrounding tissues. A transonic flow probe (MA2PSB) was placed around the isolated pulmonary trunk just above the right ventricle.

To further quantify the effects of sulfide on the left ventricle function, a separate group of rats was equipped with a catheter (PE-50 tubing) inserted into *left* ventricle through the right carotid artery. This allowed the continuous monitoring of left ventricular pressure while measuring cardiac output via the determination of aortic blood. A median sternotomy and a small incision of the pericardium were performed to expose the ascending aorta. A transonic flow probe (MA2PSB) was placed around the ascending aorta just above the coronary arteries. Great care was taken to prevent compression of vessels or obstruction of circulation during the procedure.

At the end of the experiment, rats were euthanized by injection of a lethal dose of barbiturate (200 mg/kg) into the right heart through the jugular catheter, followed by aortic dissection.

Measurements

The arterial and ventricular catheters were connected to pressure transducer (BD DTXPlus Transducer, Becton Dickinson, Franklin Lakes, NJ; amplifier TA-100, CWE, Ardmore, PA) to monitor blood pressure.

The blood flow probes were connected to the transit-time flowmeter (TS420, Transonic Systems Inc., Ithaca, NY).

Expiratory flow was measured using a pneumotachograph (1100 Series, Hans Rudolph, Inc. Shawnee, KS), as previously described [9]. Mixed-diluted expired O_2 ($F_{ch}O_2$), CO_2 ($F_{ch}CO_2$) and H_2S fractions ($F_{ch}H_2S$) were measured continuously using O_2 (Oxystar-100, CWE Inc. Ardmore, PA), CO_2 (model 17630, VacuMed, Ventura, CA) and H_2S (InterScan RM series; range: 0-200 ppm, InterScan Corporation, Simi Valley, CA) analyzers.

All signals were digitized at 400 Hz using an analog-to-digital data acquisition system (Power Lab 16/35, AD Instruments, Inc. Colorado Springs, CO) and were visualized on-line. All data were stored for further analysis by LabChart7 (AD Instruments, Inc. Colorado Springs, CO).

Data Analysis

Blood flow was determined by positive integration of the flow signals every 3 seconds then expressed in ml/min. Total peripheral resistance and hindlimb peripheral resistance were computed by dividing mean arterial pressure by mean cardiac output or mean femoral artery blood flow respectively. For the evaluation of left ventricular function, left ventricular systolic pressure (LVSP) and heart rate (HR) were determined by peak detection, and dP/dt max was analyzed by derivative of the left ventricular pressure signal.

Breathing frequency (f) and tidal volume (VT) was determined using peak detection and integration of the expiratory flow signal, respectively, and minute ventilation (\dot{V}_E) was computed as $f \times VT$ in body temperature and pressure, saturated (BTPS) conditions.

Mixed expired fraction of H_2S (FE_{H_2S}) were computed as previously described [9]. The partial pressure of expired H_2S (PE_{H_2S}) was then calculated as $FE_{H_2S} \times$ barometric pressure (PB, 760 mmHg). Then alveolar partial pressure of H_2S (PA_{H_2S}) assimilated to the arterial partial pressure of H_2S (Pa_{H_2S}) was computed [9]. Finally the concentration of gaseous H_2S in the blood (Cg_{H_2S}) was then calculated as: $Cg_{H_2S} = 0.00012 \times Pa_{H_2S}$, with 0.00012 being the coefficient of solubility of H_2S (0.09 mole/l/760 mmHg at 37°C in saline). Assuming that H_2S is under the form of H_2S gas and its sulfhydryl anion HS^- at a ratio of 1/3 and 2/3 in the arterial blood [16], the concentration of dissolved H_2S could be estimated as three times Cg_{H_2S} .

Experimental protocols: intravenous NaHS infusion

H_2S solution was prepared using sodium hydrosulfide hydrate (NaHS, Sigma, St. Louis, MO). The molecular weight of the “hydrated” form of NaHS was 74.08 g/mol as the crystals used for preparing the solution contained 75% NaHS - 25% H_2O . NaHS was diluted in saline at a concentration of 0.8 mg/ml (10.8 mM), and prepared immediately prior each experiment and kept in airtight syringes. The level of exposure to H_2S was determined based on the relationship we have previously established between the blood concentrations, rate of exogenously administered H_2S , and the corresponding clinical symptoms, i.e., H_2S -induced apnea using continuous infusion of NaHS solution [9, 10].

First, a group of 14 rats was divided into 2 sets, randomly selected, one received NaHS infusion ($n = 7$) while the other received saline ($n = 7$). After 10 minute of stable recording, intravenous NaHS infusion was started at the rate of 0.05 ml/min (0.5 micromoles/min)

using a syringe pump (Fusion 100, Chemyx Inc., Stafford, TX). Infusion rate was increased by 0.05 ml/min every 2 minutes until 0.6 ml/min (6.5 micromoles/min). Based on our previous experiments, the rate of 0.6 ml/min will increase CgH_2S by more than 2 microM and produce a breathing stimulation [9, 10]. To limit the number of animals that were sacrificed and after having observed that the cardiovascular effects were extremely short lasting (few minutes at the best after the cessation of NaHS infusion all circulatory parameters returned to normal), two of seven rats in both group received a second series of saline or NaHS infusion. Animals were then given a recovery period long enough so that blood pressure and flow returned to normal before repeating the same infusion. All parameters were monitored continuously and averaged over the last 30 seconds of each step of infusion. Arterial blood was sampled before and after the periods of NaHS infusion. Blood gas analysis was performed immediately after sampling. Corresponding volume of saline was infused at the same rate of NaHS infusion in the control group.

In an additional group of 6 rats, left ventricular pressure and aortic flow were determined while NaHS was infused at a constant rate just above the level found to decrease cardiac output (0.7 ml/min see result section), in keeping with the data obtained in the protocol described above.

Finally, at the end of the experiment, NaHS was infused at a higher rate of 2.0 ml/min in 5 rats while the changes in cardiac function versus peripheral resistance were determined.

Statistical Analysis

All results are presented as mean \pm SD. All variables of interest were compared between the vehicle control group and the group receiving NaHS at the corresponding rate of infusion using two-way ANOVA for repeated measurements. Post-hoc comparisons were performed using a Bonferroni correction. $P < 0.05$ was regarded as significant for all these comparisons. Data were compared before and during each rate of infusion using ANOVA for repeated measurements. For left ventricular function determined during constant infusion, the values were compared between baseline and 2 minutes into NaHS infusion, using paired t test. All statistical analyses were conducted using GraphPad Prism 6 (Graphpad Software, La Jolla, CA).

Results

1- Effect of incremental infusion of saline vs NaHS (from 0 to 6 micromoles/min) on circulation

Figure 1 shows an example of the effects of saline versus NaHS infusion on mean arterial blood pressure (Mean ABP), mean right ventricular pressure (Mean RV pressure), mean cardiac output, total peripheral vascular resistance, and estimated CgH_2S (displayed in linear and log scale) in 2 different rats. Averaged data are shown on figures 2-4. At the end of the protocol, the total volume of fluid infused to each animal of both group was the same (7.8 ml, ~ 15 ml/kg).

1-a) Effects of saline infusion (control)

Hemodynamic changes: As illustrated on figure 1-3, mean ABP, mean RV pressure, and cardiac output increased progressively during vehicle saline infusion in keeping to the rate of sulfide infusion. Mean ABP, which baseline levels averaged 79 ± 12 mmHg, rose throughout the period of infusion becoming significantly higher than the pre-infusion values at 0.40 ml/min ($P < 0.01$). Mean ABP increased by 9 ± 10 mmHg at 0.6 ml/min of infusion ($P < 0.01$) as shown on figure 2. This increase in mean ABP was clearly related to a rise in cardiac output, which (baseline 55 ± 14 ml/min) rose by $23 \pm 18\%$ at 0.6 ml/min of infusion ($P < 0.01$). Baseline heart rate was 402 ± 125 bpm and did not change significantly throughout the period of infusion (fig. 3), as a result, the rise in cardiac output was exclusively related an increase in stroke volume (fig. 3): stroke volume averaged 0.147 ± 0.047 ml at baseline, and increased significantly at 0.50 ml/min infusion ($P < 0.01$) to reached $+22 \pm 21\%$ at 0.6 ml/min infusion ($P < 0.01$). This increase in cardiac output related to volume that was infused was associated to a modest reduction in peripheral resistance; total peripheral vascular resistance (fig. 2) decreased by $-7 \pm 12\%$ at 0.6 ml/min (not significant). Femoral flow did not change significantly throughout the infusion period (fig. 2).

Blood gas and lactate: Baseline PaO_2 and PaCO_2 were 79 ± 16 mmHg and 34 ± 6 mmHg, respectively, and were not affected by the saline infusion. At the end of period of infusion PaO_2 and PaCO_2 averaged 75 ± 11 mmHg and 36 ± 7 mmHg, respectively. Blood lactate at the end of saline infusion was 1.69 ± 0.29 mM.

1-b) Effects of NaHS infusion

CgH₂S during NaHS infusion: As illustrated in figure 1 and figure 4, CgH₂S increased in keeping with the rate of NaHS infusion following an exponential pattern.

Hemodynamic changes: Baseline levels of mean ABP (83 ± 18 mmHg), stroke volume (0.124 ± 0.034 ml), cardiac output (53 ± 12 ml/min), and femoral flow (3.5 ± 1.7 ml/min) were not different from the saline group.

When compared to saline infusion, the effects of H₂S on circulation can be described in keeping with the rate of infusion as follows:

- a. Up to 0.35 ml/min (~ 3.8 micromoles/min)

The changes in mean ABP, blood flow, pressure in the heart and peripheral resistance were indistinguishable from the effects of saline infusion. Mean ABP increased by 5 ± 7 mmHg from baseline i.e. within the same range as saline infusion (fig. 2 and 3). CgH₂S reached a value, which averaged 0.31 microM.

- b. From 0.35 – 0.55 ml/min (3.8-5.9 micromoles/min)

Mean ABP during NaHS infusion diverged from saline infusion and decreased significantly along with a reduction in peripheral resistance. This change in mean ABP was significantly different from the saline group above 0.45 ml/min infusion (4.9 micromoles/min, $P < 0.05$). Cardiac output continuously increased by $20 \pm 10\%$ of the baseline value at 0.55 ml/min (5.9 micromoles/min, $P < 0.05$ from baseline).

Femoral flow also increased by $14 \pm 25\%$ from baseline at 0.55 ml/min (although this difference was not significant). Total vascular resistance showed significant decrease from baseline at 0.45 ml/min ($P < 0.05$), and continued to decrease reaching $-19 \pm 5\%$ at 0.55 ml/min ($P < 0.01$). All the changes in vascular resistance were significantly different from saline group between 0.45-0.55 ml/min of NaHS infusion ($P < 0.01$). This progressive reduction in mean ABP and peripheral resistance was associated with an increase in stroke volume ($19 \pm 16\%$ at 0.55 ml/min, $P < 0.05$). These effects were observed at CgH_2S between 0.31-1.36 microM.

c. From 0.55-0.6 ml/min (5.9- 6.4micromoles/min)

Figure 5 displays an example of the effects of infusion during the transition from 0.55 to 0.6 ml/min on arterial pressure, cardiac output, femoral flow, and CgH_2S in one rat. On average, blood pressure continued to decrease (fig. 2), but this time along with a decrease in systolic RV pressure, stroke volume (fig. 3) and cardiac output as soon as the rate of infusion was above 0.55 ml/min. These effects were observed for CgH_2S , which averaged 1.36 microM. The most striking change was an increase in diastolic right ventricular pressure and drop in systolic RV pressure (fig. 1 and 3), which was significant at 0.6 ml/min, reflecting a reduction in cardiac performance. Infusion was stopped at this rate and both blood pressure and cardiac output were able to recover very rapidly i.e. as soon as the infusion was interrupted.

Blood gas and lactate: Baseline PaO_2 and $PaCO_2$ were 87 ± 11 mmHg and 35 ± 6 mmHg respectively. After the period of NaHS infusion, PaO_2 and $PaCO_2$ were 82 ± 8 mmHg and 36 ± 8 mmHg, respectively, and therefore were not affected by NaHS infusion, while blood lactate averaged 2.59 ± 0.69 mM ($P < 0.01$, vs saline).

2- Effect of H_2S infusion on left ventricular function a rate of 0.7 ml/min (7.5 micromoles/min)

We found that left ventricular and blood pressure dropped along with cardiac output at a rate of 0.7 ml/min (fig. 6 and 7). Cardiac output dropped by half (152 ± 47 to 75 ± 33 ml/min/kg) 88-141 seconds (median 108 seconds) after the onset of sulfide infusion. At the same time, left ventricular systolic pressure (LVSP) decreased from 94 ± 19 to 62 ± 11 mmHg ($P < 0.01$), and heart rate decreased from 433 ± 78 bpm to 311 ± 100 bpm ($P < 0.01$); dP/dt max also dropped by about half from 3260 ± 946 to 1626 ± 432 mmHg/s ($P < 0.01$). This severe reduction in cardiac contractility occurred when H_2S concentration in the arterial blood reached 7.6 ± 0.6 microM.

CgH_2S and circulatory effects of H_2S —Figure 8 displays all individual data obtained from all NaHS infusion tests, showing the change in cardiac output and blood pressure as a function of gaseous H_2S concentration from infusions ranging from 0 to 0.7 ml/min. Clearly, the decrease in blood pressure was associated with a drop in cardiac output as soon as CgH_2S increased above 2-3 microM.

Finally, In 5 rats, the infusion of NaHS was resumed at the end of the experiment and maintained at about twice the levels required to affect cardiac function. Both arterial

pressure and cardiac output dropped dramatically (fig. 9A). In all cases, effective cardiac contractions stopped being discernible on the arterial pressure or cardiac output signals after 3 minutes, without any reduction in peripheral vascular resistance (fig. 9B). In other words, at the time required for cardiac output to drop by half when a rate of sulfide infusion of 7-8 micromoles/min was used, 15-16 micromoles/min led to a complete cessation of cardiac contractions.

Discussion

Intravenous infusion of NaHS produces a small (less than 20%), but significant, reduction in peripheral vascular resistance in anesthetized ventilated rats at concentrations of gaseous H₂S ranging between 0.5-1.0 microM. Below these levels, no circulatory changes were observed, whereas, as soon as CgH₂S increased above 2-3 microM, which corresponds to levels not even associated with symptoms of toxicity [9, 10], cardiac contractility decreased very rapidly, leading to a dramatic reduction in cardiac output but with no additional reduction in peripheral vascular resistance.

H₂S and peripheral vascular resistance

We found that H₂S decreased the peripheral vascular resistance by an average of 19%, leading to a relative reduction in ABP (in keeping to the effect of saline infusion) only in a narrow and low ranges of H₂S infusion and concentrations (fig. 1-3). Cardiac output increases along with the rate of infusion in both saline and NaHS infusion. These results are qualitatively consistent with earlier findings showing that H₂S can produce a vascular relaxation in various in-vitro vascular preparations [8]. As discussed in the following paragraphs, it should be pointed out that the concentrations required to produce such an effect, in-vitro, were one to two orders of magnitude higher than the concentrations found to be effective in our in-vivo experiments. Previous data obtained in small animals using H₂S intravenous injection have resulted in a drop of blood pressure [6, 7]. The mechanism, which has been put forward to account for the effects of H₂S, relate to the activation of K-ATP channels in smooth muscle [17]. In addition, H₂S induced vaso-relaxation, although not directly mediated by cyclic guanosine 5'-monophosphate (cGMP), appears to be potentiated by the NO pathway [18, 19]. Our present findings depart from most in-vitro studies, in the sense that the effects we observed remain very modest and are only produced in a very small range of diffusible H₂S concentrations in contact with the vessels. Certainly, various factors could have blunted or limited the vascular of H₂S in-vivo, one of them being the stimulation of the chemoreceptors, which occurs in the same ranges of CgH₂S as those producing a reduction in peripheral resistance [9]. Such stimulation could have activated the sympathetic system triggering in turn a classical vasoconstriction, which can oppose any direct vascular relaxation effects (chemoreceptor induced sympathetic stimulation [20, 21]). Intravenous bolus injection of an exogenous H₂S solution has long been shown to increase breathing by stimulation of carotid body chemoreceptor [22, 23]. In our study, the rats were mechanically ventilated and were given a muscular relaxant to prevent the confounding effects of spontaneous breathing on circulation. As a consequence, the determination of expired H₂S and blood flow were not influenced by the changes in breathing pattern. Still H₂S-induced direct stimulation of chemoreceptors, cardiac receptors, or even lung receptors might have

affected peripheral blood flow regulation [21, 24]. Incidentally, the ratio between systolic RV pressure and pulmonary blood flow, a marker of right ventricular afterload, was not different between the two groups. This is consistent with the study using human pulmonary artery which could only be dilated with a concentration 200 times higher than the concentrations we used in-vivo (500 microM of H₂S [25]). Sun et al. [26] also found differences in the response to NaHS between rat aortic and pulmonary arterial rings, the latter being less responsive to NaHS than aorta. They also found that pulmonary artery rings could produce a vasoconstriction after 2 minutes of administration of very large concentrations of soluble NaHS (50-200 microM).

Toxicity of H₂S for the heart, depression of cardiac contractility

H₂S at levels, lower than those we previously found to be able to depress breathing [9], decreases cardiac contractility (see also fig. 8). As soon as level of soluble H₂S in the blood increases to higher concentrations and reaches about 7 microM, cardiac output was severely depressed leading to asystole within a few minutes.

H₂S can affect cardiac contractility via a direct inhibition of ATP production [27] or an inhibition of L-type calcium channels [11, 12], producing in turn a negative inotropic effect. In addition, H₂S appears to potentiate the effect of NO [18, 28, 29]. NO is thought to be one of the main mechanisms of cardiac depression in various conditions wherein a cardiogenic shock can develop, e.g. septic shock [30, 31]. The “blocking” effect of H₂S on calcium channels appears to be the major factor leading to the reduction in the contractility of the cardiomyocytes [11, 12]. At least 2 different mechanisms could be put forward to account for this effect. The first one relates the sulfhydration of cysteine residues on calcium channels [11]. This mechanism has important potential implications since sulfhydration could be antagonized through the creation of a reducing environment of the heart to “release” H₂S. Second, calcium channel activity seems to be able to be modulated by the red-ox environment of the cells [32], the latter could be greatly affected by the presence of H₂S. Of note, is that all the circulatory parameters returned to their baseline values very rapidly following the cessation of H₂S exposure, suggesting that the mechanisms of depression in cardiac contractility is rapidly reversible, and thus compatible with the very rapid oxidation of H₂S [10, 33] as well as the trapping of free H₂S by the metallo-compounds [34] present in the heart (myoglobin). Maintaining H₂S exposure or leading to levels of more than 5-10 microM, will lead to major cardiac depression.

Circulatory effects as a function of H₂S levels

In our previous studies [9], we found that breathing was stimulated in sedated rats by H₂S at CgH₂S of about 1 microM, while a terminal apnea occurred around 8 microM, a level that would be also associated with a severe reduction in cardiac contractility. In in-vitro experiments, H₂S is usually diluted in saline, or phosphate buffered solution, and is therefore only present in soluble forms (H₂S and HS⁻). In contrast, in the blood, a large part of H₂S that can be measured for instance using monobromobimane technique [9, 35], is combined with plasma proteins or hemoglobin (and thus is not diffusible). We used the level of alveolar H₂S to estimate the gaseous and thus soluble forms of H₂S interacting with the heart or the vascular structure in-vivo. Assuming that H₂S is under the form of H₂S gas and

HS⁻ at a ratio of 1/3 in the arterial blood [13, 14], the total free concentration of H₂S and HS⁻ was therefore approximately 4 microM, when the cardiac function was affected. This effect seems therefore to develop very “early” during H₂S intoxication. These data are consistent with results obtained in the perfused rat heart, wherein a reduction in cardiac function can be observed starting at only 1 microM of NaHS [36]. Incidentally, such low microM levels of H₂S have also been shown to be able to already affect the cytochrome c oxidase activity [27, 37].

Model limitations

The modality of sulfide exposure used in this study, i.e. intravenous infusion of sulfide, is not faithful to a clinical scenario of H₂S intoxication, wherein H₂S is typically inhaled. When in solution of NaHS, sulfides are actually present under the form of H₂S/HS⁻, i.e. in gaseous form (20-30%) and under the form of HS⁻ (70-80%) [13, 14, 16]. These 2 forms also constitute the pool of soluble sulfide (and in the same proportion) in the blood and in the cells. Indeed, as mentioned in the introduction, whether “H₂S” is produced endogenously, inhaled, infused or formed from a slow releasing agent, it is never present in its gaseous form only: as soon as H₂S reaches the blood or increases in the tissues, the majority of soluble H₂S is under the form of HS⁻, while an even larger pool combined with proteins. All these pools are always in equilibrium in keeping with the level of H₂S partial pressure [9]. The general term sulfide should always be preferable to the use of H₂S, as H₂S in its gaseous form is never present alone in a biological milieu (it would be present under a pure gaseous form in a very acidic solution).

Certainly the toxicity of sulfide towards the lungs and the upper-airways [38, 39] is expected to be blunted, at any given level of sulfide in the blood, if sulfides are infused rather than inhaled [40]. However, as soon as H₂S reaches the blood, the original site of diffusion, whether the alveolar regions to or a direct venous infusion, is not going to affect the distribution of the various pools of sulfide in the blood and the concentration of H₂S in the tissues (see above paragraph and ref for discussion [9, 10, 35]). It is however likely that when infused into the venous blood, concentrations of free H₂S reaching the right heart would be higher than those entering the left heart (exposed to a lower concentration after a passage through the lungs), while the opposite is expected to occur during H₂S inhalation, i.e. when sulfide reached directly the left heart from the pulmonary circulation. Finally, recent studies have been using promising slow H₂S releasing agent such as GYY4137 [41] to study the physiological effects of sulfide on vessels. The interest of using these compounds in investigating sulfide toxicity is yet to be established. Indeed, a slow H₂S releasing compound [41] is not faithful to a real life scenario created by an exposure to toxic levels of H₂S, in keeping with the very fast kinetics of H₂S toxicity (coma, respiratory arrest or circulatory failure), which can be produced within seconds during inhalation of H₂S or infusion of solution of sulfide obtained from NaHS [40, 42]. Perhaps more importantly, whenever GYY4137 “releases” H₂S, the latter will, for most of it, be immediately transformed into HS⁻ along with the creation of a pool of sulfides combined to proteins in various ways, including in sulfhydration form [15, 43], just like any sources of exogenous or endogenous H₂S. For all intends and purposes, the reduction in left cardiac contractility during H₂S infusion that we are reporting in the present study appears to be an early sign of

sulfide toxicity and thus represents a relevant mechanism responsible of the circulatory failure that occur very early during sulfide inhalation. The limits of using an sedated animal has been discussed in details in one of our previous paper [44].

Conclusion

Continuous intravenous infusion of a sulfide solution prepared from NaHS produces a modest reduction in peripheral vascular resistance in rats, which occurs when CgH_2S reaches 0.5 to 1 microM (2-4 microM of total soluble sulfide, i.e. comprising H_2S and HS^-) in the arterial blood, corresponding to levels of sulfide we previously found capable of stimulating breathing. However, the range of exogenous H_2S able to produce this effect is relatively narrow and as soon as gaseous H_2S concentration reaches 2-3 microM, i.e. still below levels of sulfide producing an apnea, a significant depression in cardiac contractility occurs leading to a fall in cardiac output and blood pressure as sulfide concentrations increases. This finding supports the view that H_2S -induced circulatory failure is predominantly related to a cardiogenic shock, with little involvement of the peripheral vascular network.

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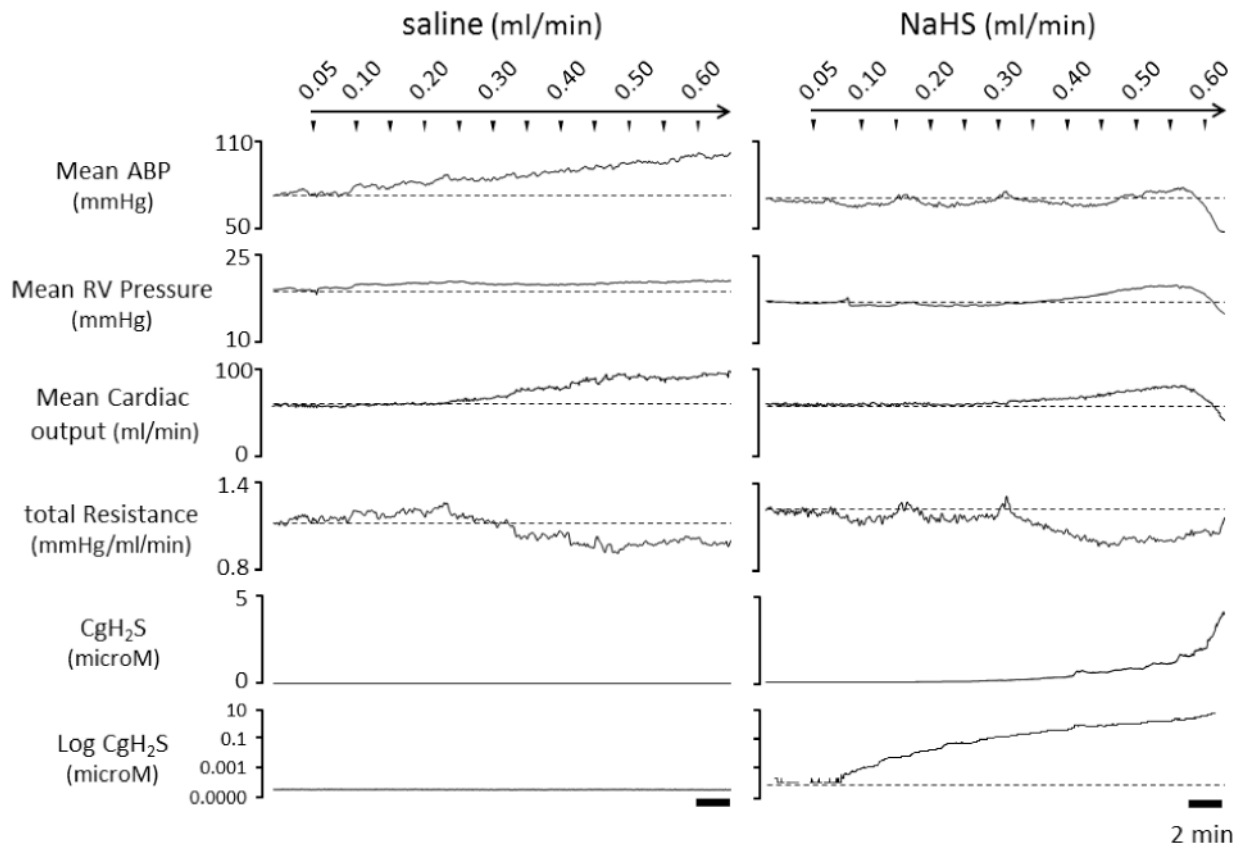


Figure 1.

Example of the effects of saline (left panel) and sulfide infusion (right panel) on mean arterial blood pressure (Mean ABP), mean right ventricular pressure (Mean RV pressure), mean cardiac output, total peripheral vascular resistance (total Resistance), and estimated CgH_2S (displayed in normal and log scale) in 2 different rats for rate up to 0.6 ml/min. Mean ABP and cardiac output displayed a progressive and continuous rise during saline infusion, with a modest reduction in total resistance. During sulfide infusion (10.8 micromoles/ml), the normal increase in mean ABP was virtually abolished which was associated with the reduction of total resistance, which was clearly visible above 0.4 ml/min. A very rapid drop in mean ABP, mean RV pressure, and mean cardiac output was observed after 0.55 ml/min (5.9 micromoles/min) of NaHS infusion. CgH_2S increased exponentially during NaHS infusion.

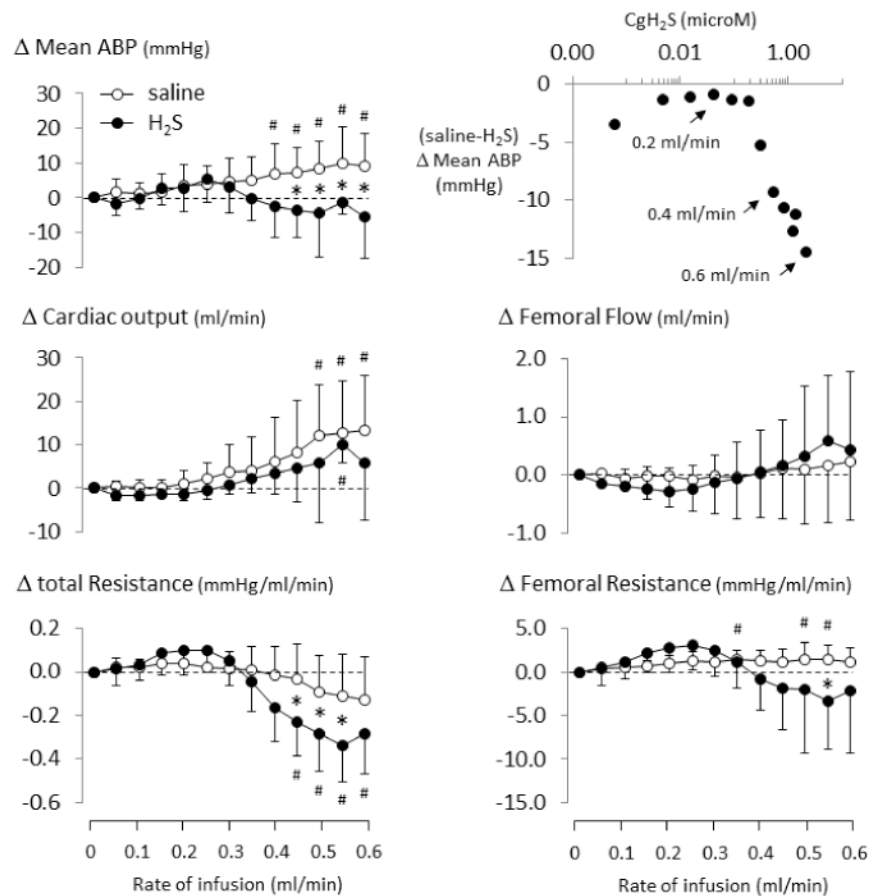


Figure 2.

Averaged blood pressure, blood flow, and vascular resistance in the groups of rats receiving saline (open circle) vs NaHS (closed circle) infusion as a function of rate of infusion (for rate up to 0.6 ml/min). Mean arterial blood pressure (ABP) and cardiac output are increasing throughout the period of saline infusion, without major changes in femoral blood flow. This resulted in a modest reduction of total peripheral resistance. NaHS infusion induced similar changes in all parameters between 0-0.35 ml/min (3.8 micromoles/min), above these levels, mean ABP, femoral resistance, and total resistance departed from saline infusion showing a significantly lower blood pressure associate to a decrease in peripheral resistance. At 0.6 ml/min (6.5 micromoles/min) there was a trend for a decrease in cardiac output in the NaHS group. The iso-rate (iso-volume) difference in the variation in mean ABP between saline and H₂S group is illustrated as a function of CgH₂S (top right). Compared to saline infusion, NaHS infusion clearly reduced blood pressure when CgH₂S increased. Values are shown in mean \pm SD. *significantly different from saline/control at $P < 0.05$. #significantly different from baseline at $P < 0.05$.

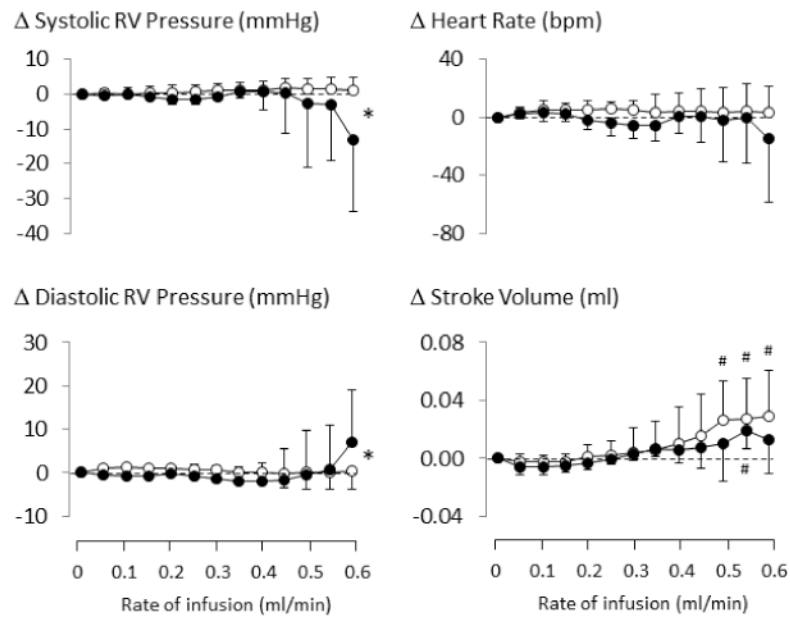


Figure 3.

Averaged cardiac parameters (stroke volume, heart rate, and right ventricular (RV) pressure) in the groups of rats receiving saline (open circle) vs NaHS (closed circle) infusion as a function of rate of infusion, for rate up to 0.6 ml/min. During saline infusion, stroke volume increased according to infusion rate, but systolic RV pressure, diastolic RV pressure, and heart rate did not change. The only significant changes produced by NaHS infusion were observed at 0.6 ml/min (6.5 micromoles/min) wherein systolic RV pressure decreased and diastolic RV pressure significantly increased, associated to a non-significant decrease in stroke volume. Values are shown in mean \pm SD. *significantly different from saline/control at $P < 0.05$. #significantly different from baseline at $P < 0.05$.

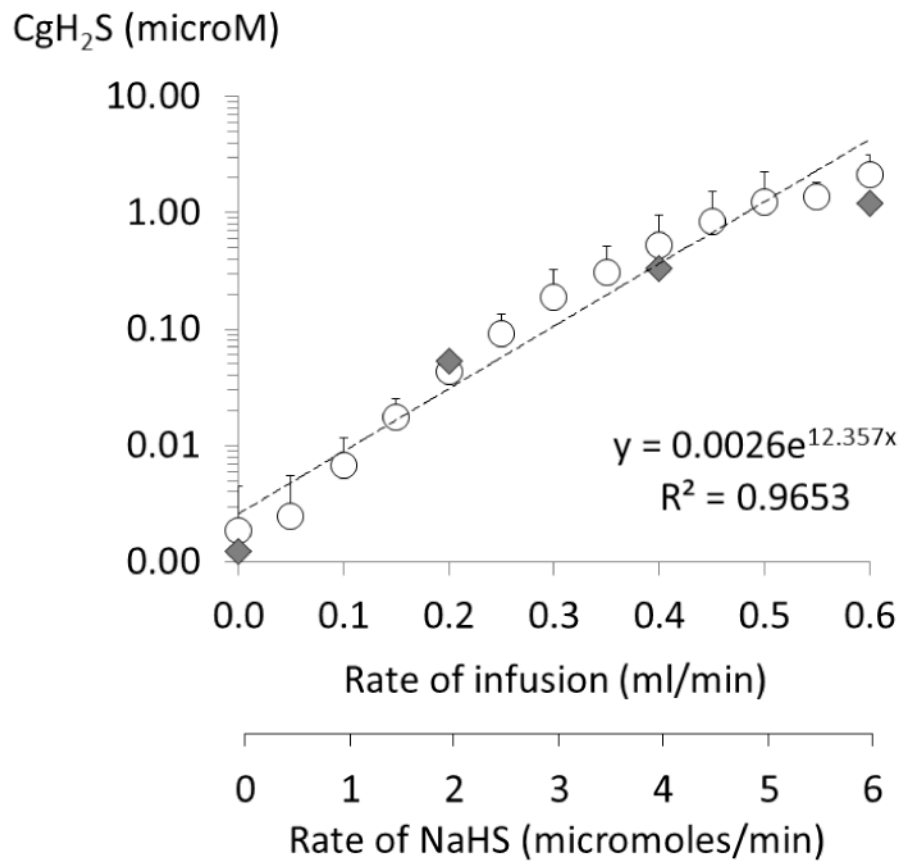


Figure 4. CgH₂S as a function of the rate of NaHS infusion, for rate up to 0.6 ml/min). CgH₂S showed exponential-like increase during NaHS infusion in keeping with the rate of infusion. The data from the previous study (Klingerman et al. 2013, gray diamond) fits with the relationship in this study. Values are shown as mean \pm SD.

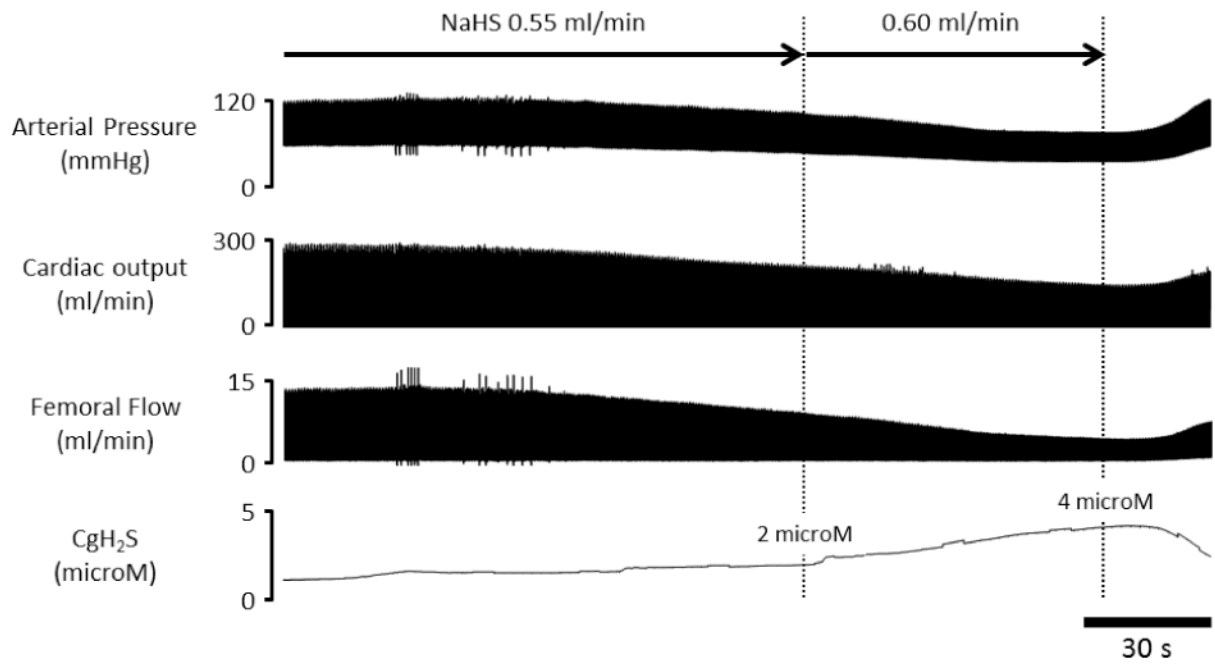


Figure 5.

Example of the effects of NaHS infusion at 0.55-0.60 ml/min (5.9-6.5 micromoles/min) on arterial pressure, cardiac output, femoral flow, and CgH₂S in one rat. Both blood pressure and blood flow started to drop when CgH₂S increased above 2 microM.

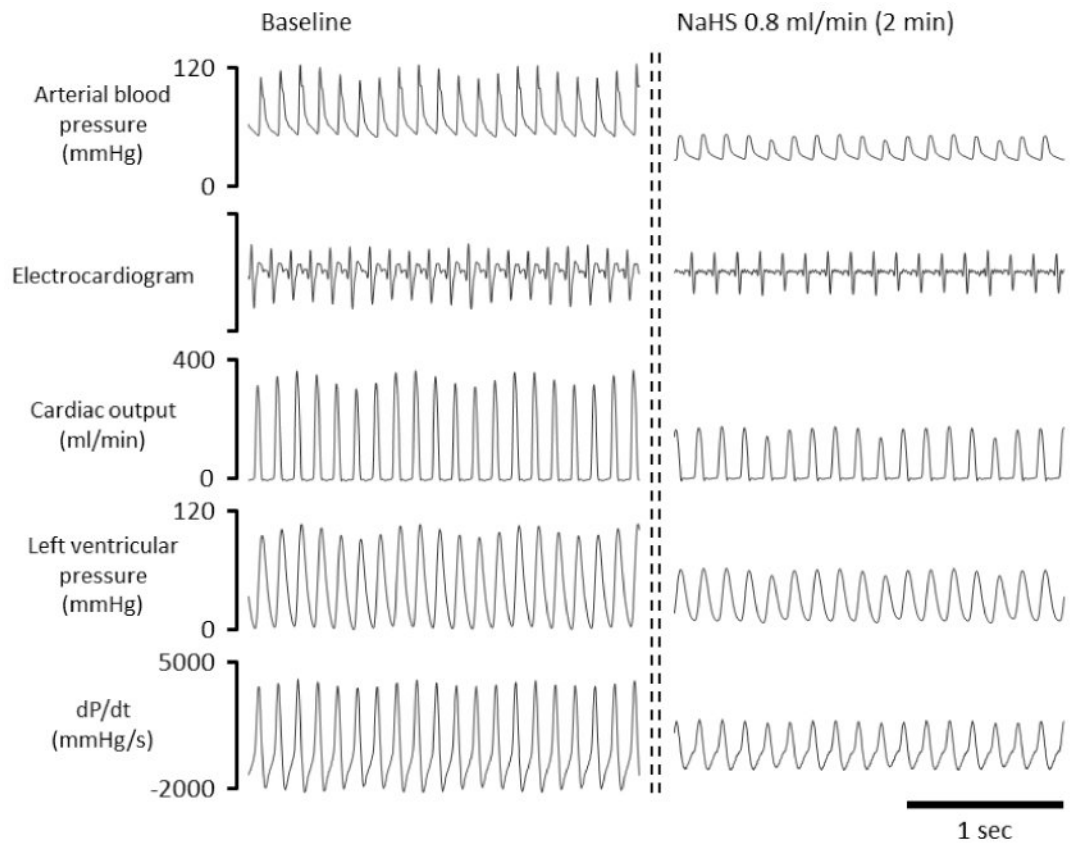


Figure 6. Example of effects of NaHS infusion at a rate of 0.8 ml/min (8.6 micromoles/min) on the arterial blood pressure, electrocardiogram, cardiac output, and left ventricular pressure in one rat. Two to three minutes into NaHS infusion, arterial blood pressure, cardiac output, and left ventricular pressure largely decreased compared to baseline.

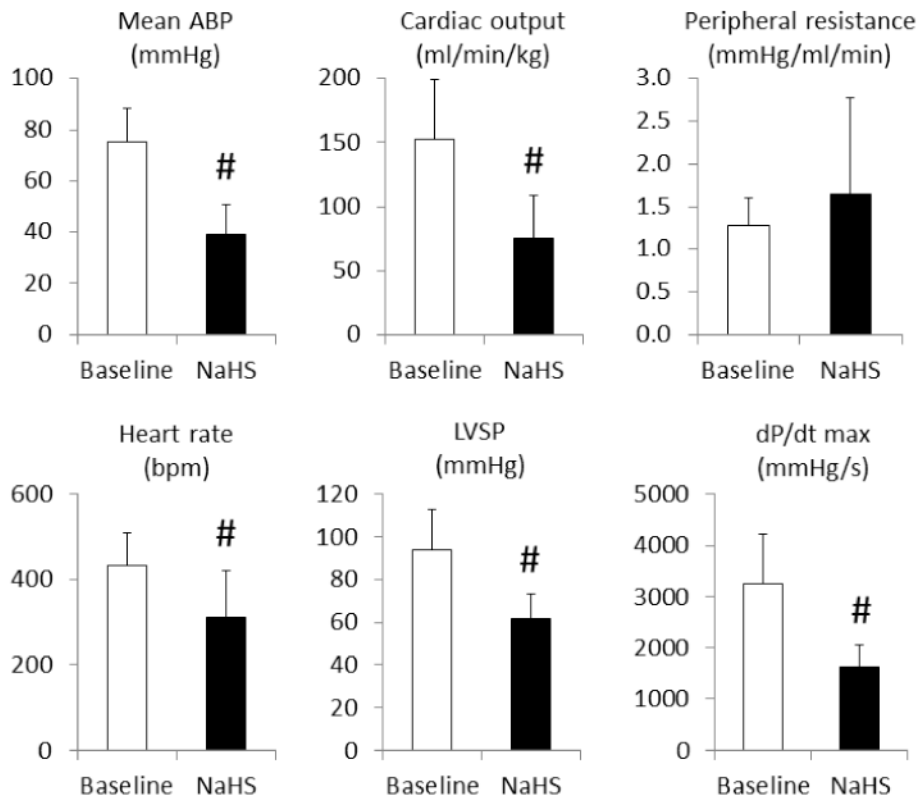


Figure 7.

Mean \pm SD values of left ventricular functions: mean arterial blood pressure (ABP), cardiac output, peripheral resistance, left ventricular systolic pressure (LVSP), dP/dt max, and heart rate at baseline (before infusion) and at 2 minutes into an infusion of 0.6-0.8 ml/min NaHS in 6 rats. All the left ventricular-related parameters significantly dropped during NaHS infusion, without any reduction in peripheral resistance. #significantly different from baseline, $P < 0.01$.

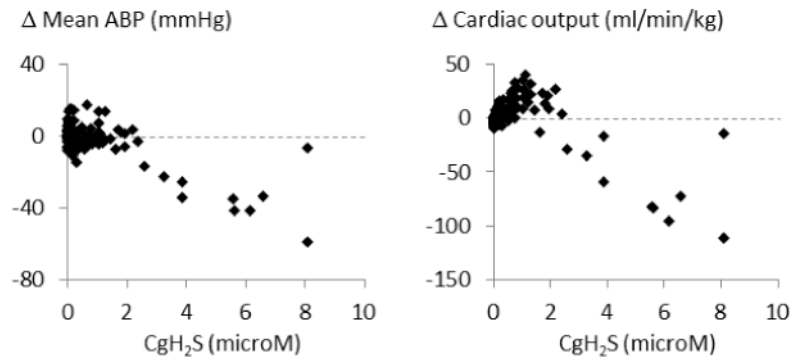


Figure 8.

Individual data points obtained from all NaHS infusion tests. Note that when CgH₂S reached concentrations about 2-3 microM, a progressive in drop blood pressure associated to a reduction in cardiac output was observed leading to sever reduction in cardiac output a soon as CgH₂S was above 3 microM (see discussion section for more details).

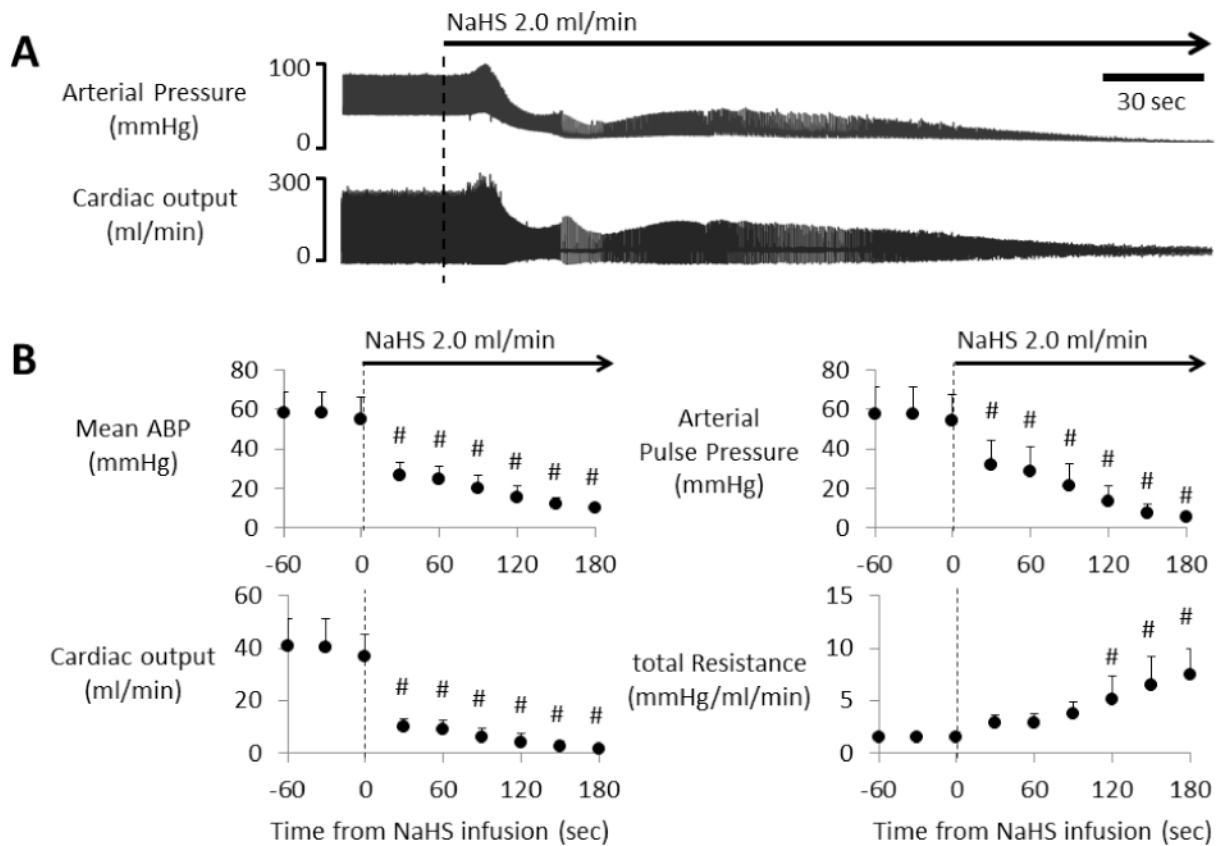


Figure 9.

A) Example of the effects of NaHS infusion at 2.0 ml/min (21.6 micromoles/min) on arterial pressure and cardiac output in one rat. Both blood pressure and cardiac output rapidly dropped during continuous infusion of high-dose of NaHS. Pulse pressure was undetectable within 3 minutes. B) Averaged arterial blood pressure (ABP), arterial pulse pressure, cardiac output, and total peripheral resistance in 5 rats receiving NaHS infusion at 2.0 ml/min. During NaHS infusion, arterial pressure, pulse pressure, and cardiac output significantly dropped within the first minute of infusion, while estimated total peripheral resistance increased until cardiac asystole occurred. Values are shown in mean \pm SD. #significantly different from the value at -60 seconds, $P < 0.05$.