Is exogenous hydrogen sulfide a relevant tool to address physiological questions on hydrogen sulfide?

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Summary

This review challenges the use of solutions of dissolved exogenous H_2S in the literature as a tool to determine the potential physiological functions of endogenous H_2S as well as its putative therapeutic applications.

Our major point of contention is that solutions of dissolved H_2S are used in vitro at concentrations, within the high microM range, which are above the concentrations of dissolved H_2S found in blood and tissues during H_2S lethal exposure in vivo. In addition, since the levels of toxicity are extremely variable among cell types, a property that is seldom acknowledged, the physiological relevance of data obtained after local or in-vitro administrations of H_2S at concentrations of few microM is far from certain. Conversely, the rate of disappearance of the dissolved pool of H_2S in the body (being trapped or oxidized), which we found to be at least of several micromoles/kg/min, is so rapid in vivo that if relatively low *quantities* of H_2S, i.e. few micromoles for instance, are administered, no change in H_2S concentrations in the body is to be expected, unless toxic levels are used. Protocols looking at the effects of compounds slowly releasing H_2S must also resolve a similar conundrum, as their effects must be reconciled with the unique ability of the blood and tissues to get rid of H_2S and the steepness of the dose-toxic effects relationship.

Only by developing a comprehensive framework in which H_2S metabolism and toxicity will be used as a rationale to develop and justify any experimental approach will we be able to bring definitive evidence supporting a protective role for exogenous H_2S, if any, and its putative function as an endogenous mediator.

Keywords

Hydrogen sulfide; gaseous transmitter; Toxicology

What effects can we expect to be produced by a 5 microliter solution containing dissolved hydrogen sulfide (H_2S/HS^-) at the concentration of 2.5 *milliM* directly administered into the

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cerebro-ventricles of a rat? It is, in substance, one of the fundamental questions raised by Li et al. (Li et al., 2016) in their recent study published in this journal.

The approach consisting in injecting high microM or even milliM solutions of dissolved H_2S directly into an organ or in the milieu bathing cells in culture has been repeatedly used in the literature (see for list of references (Nicholson and Calvert, 2010; Szabo, 2007; Szabo et al., 2011) with 2 main objectives: (1) determine whether exogenous H_2S possess therapeutic properties, such as for instance limiting the consequences of an hypoxic/anoxic insult or (2) speculate about the possible role of "endogenous" H_2S or its products of oxidation.

For all intents and purposes, the argument presented in this viewpoint is that solutions of exogenous H_2S above few microM, when in direct contact with cells or tissues produce effects that are toxic or even lethal in vivo (cardiac arrest, coma with neuronal necrosis). In contrast, cells or tissues containing large amount of metallo-compounds or expressing high level of sulfide quinone reductase activity, e.g. colonocytes or heptacoytes, are able to trap or oxidize very large amount of H_2S. These specific cells are immune to both the toxicity as well as "physiological" effects produced by sulfide in other cells or tissues. Conversely, low doses injected in vivo are probably not able to increase H_2S in a significant manner, since the blood (hemoglobin and proteins) can prevent any free sulfide to diffuse in a measurable manner into the tissues, unless toxic levels are used. Perhaps more importantly, due to the multifarious effects of H_2S, it is, for now, very difficult to define a specific function for H_2S in vivo based on any given defined H_2S-target interaction described from in vitro experiments.

1- What concentration of H_2S is toxic?

Solutions containing H_2S, prepared from NaSH or Na_2S, have been used as a source of sulfide to test the effects of hydrogen sulfide in various in vitro or in vivo studies [see for general review and discussion (Deleon et al., 2011; Furne et al., 2008; Levitt et al., 2010; Olson, 2011a, b). The sulfides dissolved in these solutions are, at a physiological pH of about 7, composed in large part of HS^- in equilibrium with a smaller portion of dissolved/ free gaseous H_2S (Almgren et al., 1976.; Carroll and Mather, 1989; Douabul and Riley, 1979). When used in cell cultures or isolated tissues, as well as when directly injected into an organ, the amount of H_2S able to diffuse into cells is therefore proportional to the partial pressure of the gaseous form of H_2S and thus the concentration of total free/dissolved H_2S/HS^-. Impurities and products of sulfide oxidation may be present in solution, their roles remain to be clarified (Nagy, 2015). Of note, when prepared without agitation and immediately before the experiment using a sealed container, concentration of gaseous H_2S remains relatively stable over time (at least for one hour) (Van de Louw and Haouzi, 2013). As soon as the solution of H_2S is added into a dish, evaporation of gaseous H_2S takes place and the concentration of soluble H_2S/HS^- exposed to the cells or tissue decreases in the milieu depending on many factors including the temperature, the pH, the surface of exchange and the level of agitation. In most experimental conditions, if the solution is neither agitated nor ventilated, a concentration in high microM or milliM range is going to remain present within the same order of magnitude for many minutes, a time long enough to

produce a lethal effect (Judenherc-Haouzi et al., 2016). Many of these points have been discussed in details in recent reviews (Olson, 2011a; Olson et al., 2014).

It should be kept in mind that H_2S is one of the most toxic mitochondrial poisons, even more toxic than cyanide on a mole-to-mole basis. *In vitro*, the activity of the mitochondrial cytochrome *c* oxidase is abolished by a solution of H_2S at concentrations of H_2S/HS^- ranging from *10 to 30 microM* (Cooper and Brown, 2008; Leschelle et al., 2005; Yong and Searcy, 2001). This effect appears however to develop at much lower concentrations in certain tissues (neurons), while cells like the colonocytes can survive exposure to milliM concentrations of free H_2S for long period of time. *In vivo*, a depression in respiratory medullary neurons (leading to a fatal apnea within minutes) and a severe depression in cardiac contractility (leading to a terminal asystole within seconds) can be produced in rodents and in large mammals (figure 1) by infusing or inhaling H_2S at levels yielding blood concentrations of gaseous H_2S between *2–5 microM* (Haouzi et al., 2014; Klingerman et al., 2013; Sonobe and Haouzi, 2016), corresponding to level of total dissolved/free sulfide of less than 20 microM (Almgren et al., 1976 ; Carroll and Mather, 1989; Douabul and Riley, 1979). For instance, whenever solutions of H_2S at concentrations above 50 microM are used on an isolated heart or isolated cardiac cells, the effect is always a depression in cardiac contractility (Geng et al., 2004; Sun et al., 2008; Yong et al., 2008; Zhang et al., 2012). This depression is an obvious pathological and toxic change in the function at both the level of individual cardiac cells and the whole heart (Judenherc-Haouzi et al., 2016), akin to the effects observed in vivo during life threatening H_2S exposure (figure 2). In a recent study, we found that the ejection fraction of the left ventricle started to decrease for an average level of gaseous H_2S of 7.09 ± 3.53 microM, while blood pressure is already decreased around 5 microM. This corresponds to a total level dissolved H_2S/HS^- of about 15–20 microM (assuming that 2/3 of total dissolved sulfide present is in the form of HS^- at physiological pH). Finally, Extensive necrosis of the cortical and subcortical neurons is observed in about 30% of animals surviving H_2S induced coma (Sonobe et al., 2015), which can develop after exposure to concentrations of free H_2S in the blood in the *microM* range for few minutes only. Interpretation of the effects of exogenous H2S must consider the fact that effects of H_2S on, for instance, neurons, cardiomyocytes, hepatocytes or cancer cells - the latter relying on a glycolytic activity for ATP production- are not interchangeable. In addition, the level of metabolism of cells in culture and in vivo can be very different. A model allowing us to translate the effects of H_2S in a dish to those in vivo is therefore cruelly missing, but few microM concentrations of dissolved H_2S does produce toxic effects in most tissues in vivo, affecting vital respiratory and cardio-vascular functions.

2- Why are toxic levels of H_2S used in physiological studies?

The rational for using high levels, i.e. microM or even mM, of H_2S to study and predict physiological effects relies on initial reports, wherein H_2S concentrations were found to be in the high micromolar range in the blood or in tissues under conditions assumed to be endogenous H_2S production only [see for discussion (Furne et al., 2008)]. Furne and Levitt (Furne et al., 2008; Levitt et al., 2010) followed by works from Olson's group (Whitfield et al., 2008) have shown that these figures, i.e. high microM concentrations of free/soluble H_2S, are unrealistically high by several orders of magnitude. Two main reasons have been

put forward to explain why high microM levels of "endogenous" H_2S were initially (and are still) reported in the blood and tissues. The first reason relates to the nature of the pools of sulfide present in the blood and in tissues found in post mortem conditions. No relevant (or trivial) levels of free/dissolved H_2S ([Furne et al., 2008; Levitt et al., 2010]), which should smell like rotten eggs, can be found in the blood, the brain ([Furne et al., 2008; Ishigami et al., 2009; Levitt et al., 2011]) or heart ([Levitt et al., 2011; Ubuka et al., 2001]) homogenates. However, significant amount of H_2S can be mobilized after exposing these tissues to a strong acid (bringing down pH of the tissues below 2) and can be measured after evaporating from the brain ([Goodwin et al., 1989; Ishigami et al., 2009; Levitt et al., 2011; Ogasawara et al., 1994]) or the heart ([Levitt et al., 2011 Ogasawara, 1994 #1383; Ubuka et al., 2001]). This pool of H_2S represents several micromoles of sulfide per kg of tissue ([Goodwin et al., 1989; Ishigami et al., 2009; Levitt et al., 2011; Ogasawara et al., 1994; Ubuka et al., 2001]), but is "trapped", literally fossilized, in the form for instance of metalo-sulfide (including Fe, Zn, Co etc..). Similarly, strong reducing agents can release H_2S from a solution of proteins or from various tissues, where H_2S is present under the form protein-bound thiols ([Ogasawara et al., 1994; Warenycia et al., 1990]). This effect can be produced by using large concentrations of DTT and TCEP (although DTT sometimes used as a reducing agent can per se also release H_2S ([Togawa et al., 1992])). The presence of H_2S evaporating after such reactions should not be confused with the normal presence of dissolved H_2S, able to freely diffuse and to potentially act as a gaseous-transmitter. The conditions leading to the release of free H_2S by a powerful reducing agent or a strong acid are utterly artificial and can obviously never be spontaneously reproduced in vivo. The source of these "hidden" pools of H_2S is far from being understood and could originate from numerous extra and intracellular thiol compounds and cysteine residues of almost all proteins (when exposed to a reducing agent) or sulfide combined with metallo-proteins (when exposed to a strong acid). The second reason accounting for these artificially high levels of free H_2S reported in the blood and tissues relate to errors produced by the methodology of determination of the pool of diffusible/dissolved H_2S in a biological milieu, either directly or after trapping it with Zn, using for instance techniques relying on the production of methylene blue based spectrophotometry method, which level of detection is relatively low and is affected by the turbidity of the milieu ([Van de Louw and Haouzi, 2012]).

3- Low levels of H_2S administered in vivo are unlikely to increase concentrations of H_2S in tissues

In contrast to the fate of a solution of H_2S in vitro, the majority of H_2S present in the blood during an intravenous (IV), or following an intra-peritoneal (IP), injections in vivo is immediately oxidized or combined with metallo-proteins (hemoglobin), dropping the level of free H_2S to almost zero, unless lethal levels are used ([Haouzi and Klingerman, 2013; Insko et al., 2009; Klingerman et al., 2013; Wintner et al., 2010]). There is a very fine margin between the concentrations producing no effect and concentrations capable to kill. More specifically until the level of IV infusion reaches 3–5 micromol/kg/min in rat ([Haouzi and Klingerman, 2013; Insko et al., 2009; Klingerman et al., 2013; Wintner et al., 2010]) or large mammals ([Haouzi et al., 2014]), soluble H_2S concentration barely increases in the blood remaining in the nanoM range. Whenever the amount of H_2S administered IV at a rate that

would be less than 5 micromole/kg/min, trivial diffusion of H_2S into the tissues is therefore to be expected. More importantly, as all free H_2S disappears from the body within one minute after an infusion of exogenous H_2S is stopped (Haouzi and Klingerman, 2013; Insko et al., 2009; Klingerman et al., 2013; Wintner et al., 2010) due to the high rate of H_2S oxidation in the mitochondria, it is unlikely that any IV injection of few micromoles/kg of H_2S could significantly affect the levels of free H_2S in the tissues in any given organ. Conversely, since the relationship dose effect is extremely steep for H2S, studies using a rate of H_2S higher than 20 micromole/kg/min should be regarded as extremely toxic producing the dreadful effects on most physiological functions, this level of toxicity can be produced by an IP injection of 300 micromol/kg (Sonobe et al., 2015; Sonobe and Haouzi, 2015). As displayed in the lists of studies reviewed for instance by Szabo et al (Szabo, 2007; Szabo et al., 2011), the concentrations of H_2S used among experiments are so variable that their results in toxic levels or levels of H_2S unable to change H_2S concentration in tissues. This issue is extremely relevant to the use of compounds which are slowly releasing H2S, for which no change in H_2S in the blood and tissue might be expected if the rate of H_2S "release" is too slow, while if higher rates of release are present, toxic levels can be reached in certain tissues like neurons for instance, with almost no transition from innocuous concentrations. The very short life (less than one minute) of free sulfide in the blood and the tissues {Haouzi, 2014 #1370} makes this argument even more compelling. This is not to say that no "therapeutic" window can not be found, but with respect to H_2S, this window is expected to be extremely narrow varying form tissue to tissue in keeping with multiple factors and interactions much too complex to be inventoried yet (from content in metallo-proteins to sulfide quinone reductase activity)

4- Can we separate wheat from chaff?

Investigating the role of a given molecule as a neuro-mediator or a gaseous-transmitter, relies on two prerequisites: 1- the effect of this molecule is only dictated by the limited numbers of accessible receptors or targets and the affinity for these targets; 2- the molecule of interest has no relevant effects outside its interaction with its receptors or its targets, at least within the range of concentrations of interest. As a fundamental corollary, the responses observed in vivo must be predicted or anticipated from those produced in vitro, and vice versa. For instance, applying dopamine or morphine directly into a tissue or in cell cultures will produce effects that can be understood in light of the interactions between dopamine and dopaminergic and adrenergic receptors or between morphine and opioid receptors, in a dose-dependent manner. These responses can be translated in vivo into a series of physiological effects and functions. However, H_2S does not fit into this paradigm. Indeed at any given concentration, exogenously applied H_2S is going to interact with a large number of receptors and metabolic pathways comprising interactions with cysteine residues of proteins and a pool of countless metallo-proteins; ATP regeneration will be impeded (even at very low levels) while producing free radicals and affecting the activity of many ion channels, with an alarming overlap between concentrations producing toxic or "non-toxic" effects. This makes extremely difficult, even in reduced preparations, to establish a causal link between a perturbation and a H_2S-target interaction, unless all these interactions are understood and could be dissociated into elementary responses, which is, for now, totally

unrealistic. This notion remains valid whether the described mode of action is a receptor-molecule interaction or a cascade of signaling pathways initiated or modulated by soluble H_2S or any products of sulfide oxidation. Another level of confusion has aroused from the fact that the frame of reference used to rationalize experimental findings about hydrogen sulfide has been constantly changing (Haouzi et al., 2011). For instance, H_2S has been claimed to be a gaseous-transmitter in large part based on the premise that free H_2S is released from tissues and can be measured in conditions susceptible to increase its concentration in tissues (see (Olson, 2008) for discussion on the effects of hypoxia). If true, it should be possible to decrease H_2S concentrations in cells by increasing the ability of H_2S to diffuse more rapidly through for instance the trapping of H_2S as soon as it diffuses in the blood or even directly into tissues (Haouzi, 2011). However the above approach becomes obsolete, if data supporting the diffusion of free H_2S in and outside cells or tissues result from an artifact (Olson, 2011a), if H2S would be trapped as soon as it diffuses in the blood in baseline conditions. The implications of such a debate are rarely acknowledged but are quite simple, rejecting data obtained from the measurement of H_2S diffusing outside various tissues should make us refute the original hypothesis, which was the rational for measuring H_2S in the first place, that is H_2S is a gaseous transmitter. The confusion here does not result from a difference in opinion but from a constantly evolving framework to understand what level of H_2S is required to produce a claimed effect. This process, which should be regarded as a self-correcting process in any scientific endeavor, has resulted in the creation of an untestable hypothesis. The analogy made between the presence and role of H_2S and the search for "an invisible, incorporeal, floating dragon who spits heatless fire hired in Sagan's garage (Sagan, 1997)" still holds (Haouzi, 2011). A "dragon" with such characteristics makes no difference with "no dragon at all". Until each experiment using exogenous H_2S can define what concentrations of H_2S are really present in the tissues and what effects are expected to be produced in keeping with sulfide toxicity and its rapid disappearance from the body or in vitro solution, no proper case can be made to support the role of H_2S as a neuro-mediator.

5 - Final words

Finding a compound/molecule, which possesses genuine protective properties against the effects of anoxia, is certainly the Holy Grail of critical care research. However, if one wants to offer a mitochondrial poison like H_2S as a possible treatment of the dreadful consequences of hypoxia-induced neuronal injury for instance, not only should the "limitations" of such an idea be presented in a comprehensive way, but undisputable evidence supporting this disturbing claim should be provided, unless by protection we imply that repetitive anoxic insults produced by H_2S toxicity may, through some form of preconditioning, limit the effects of future hypoxia or ischemia.

A large body of evidence supporting the "physiological" and protective roles of H_2S has been obtained using solution of free H_2S in the high microM range - levels only found during a lethal intoxication in vivo. Despite the fact that such concentrations of free H_2S, as physiological levels, have been repeatedly shown to be unrealistically high due to possible errors on the nature of the sulfide compounds that could be released from the blood or

tissues, high microM or milliM levels of free sulfide are still used to try to elucidate the properties of endogenous H_2S.

As an answer to the question raised in the introduction, we could offer the following proposition, which, one should admit, remains a pure speculation: injecting 5 μl of a solution with a concentration of 2.5 mM of H_2S/HS^- in the cerebro-ventricles - assuming a *total* CSF volume in rat brain of approximately 90 μl- could seriously intoxicate all the neurons surrounding the site of injection within seconds. In addition, since the cerebrospinal fluid, unlike the blood, contains little metallo-proteins to rapidly "trap" free sulfide, the effect of such a high concentration of H_2S/HS^- on more distant neuronal populations is quite worrisome and must be understood.

Finally, the effect of H_2S cannot be reduced to well-defined physiological compound-target interactions. The reactivity of H_2S with a multitude of critical biological processes is so pervasive at the dose typically used to produce a so-called physiological effect that the data obtained from these studies should in many instances belong to the field of toxicology.

Only the accumulation of experimental evidence based on well validated methodologies and a proper framework allowing us to give meaning to all the effects produced in vivo and in vitro by exogenous H_2S will eventually bring a final answer to the question of the protective role, if any, of H_2S and its potential functions as a endogenous mediator.

Acknowledgments

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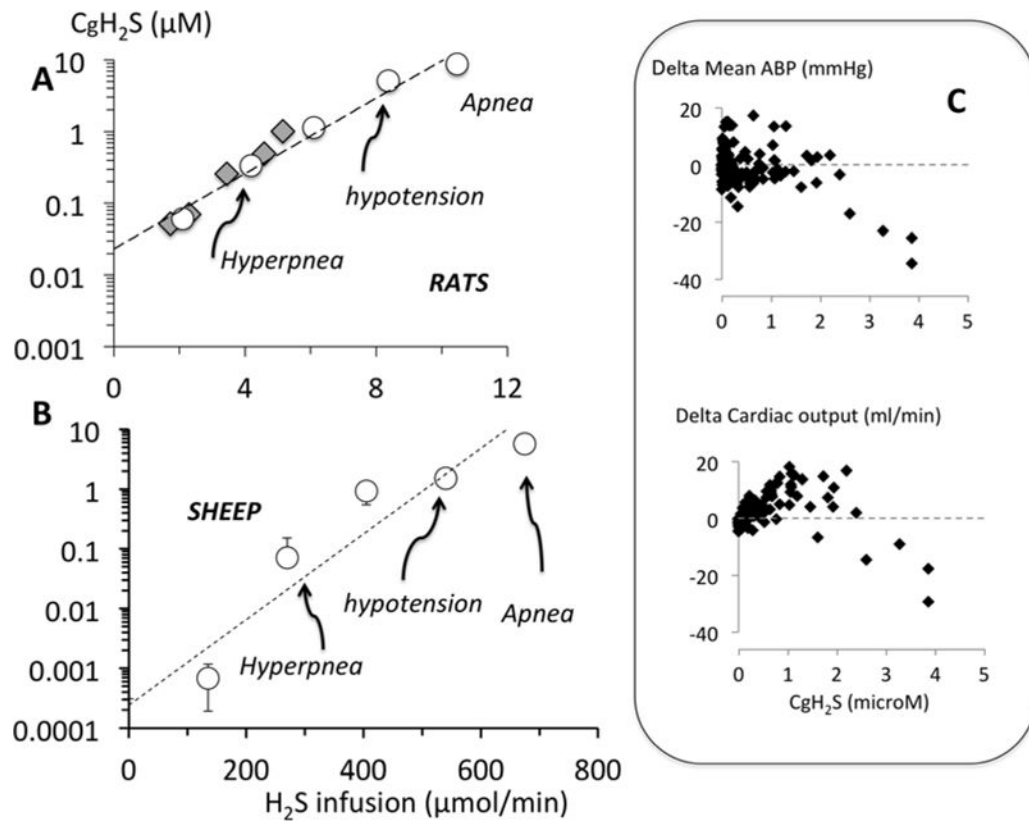
FIGURE 1.
A: Concentration of gaseous H2s in the arterial blood (CgH$_2$S) estimated from alveolar H$_2$S as a function of the rate of H$_2$S infused (only mean values are shown) in a group of rats. Breathing was stimulated at concentrations of gaseous H$_2$S ranging between 0.34 and 1.14 μM while the highest values corresponding to the lethal level ranged from 5.09 to 8.80 μM. The data recomputed form the study of Insko et al ([Insko et al., 2009]) (diamonds) fits with the relationship established in the present study. Modified from ([Klingerman et al., 2013]). Of note these concretion represents 1/3 of total Free H2S, i.e. H2S/HS⁻, present in the blood. **B:** Concentration of gaseous H$_2$S in the arterial blood (CgH$_2$S) estimated from alveolar H2S as a function of the rate of H$_2$S infused in a group of sheep. Breathing stimulation and apnea were produced at levels similar to those found in the rats. Modified from ([Haouzi et al., 2014]) **C**: Individual data points obtained in a group of rats during infusion of NaHS. Note that when CgH$_2$S reached concentrations of about 2–3 microM (less than 10 microM total free sulfide), a progressive drop in blood pressure associated with a reduction in cardiac output was observed. Note the very narrow range of H$_2$S between trivial and life-threatening effects. Modified from ([Sonobe and Haouzi, 2016]).
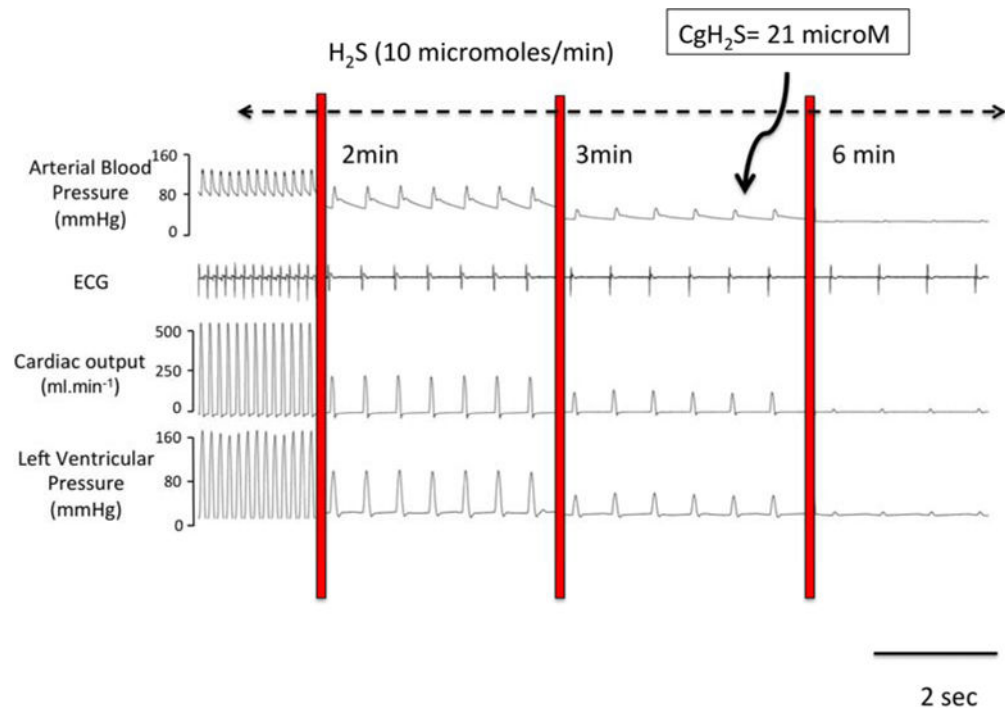
FIGURE 2.
Example of a recording obtained in an anesthetized and mechanically ventilated rat, showing the effects of a continuous infusion of a solution of dissolved H_2S at 10 μmol/min on hemodynamics and cardiac function. Blood pressure, EKG signal, cardiac output and left ventricular pressure are displayed. Within few minutes only, H_2S led to a complete pulseless electrical activity or electromechanical dissociation, as the heart becomes incapable of contracting despite a persistent electrical activity visible of the EKG signal, while CgH2S in the blood reached about 20 microM (60 microM total free H_2S). Modified from (Sonobe and Haouzi, 2015).