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Autore/i: Vellecco V, Mancini A, Ianaro A, Calderone V, Attanasio C, Cantalupo A, Andria B, Savoia G, Panza E, Di Martino

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Original Paper

Cystathionine β-synthase-derived hydrogen sulfide is involved in human malignant hyperthermia

Valentina Vellecco*[1], Antonio Mancini†[1], Angela Ianaro*, Vincenzo Calderone‡, Chiara Attanasio§, Anna Cantalupo*¶, Barbara Andria†, Gennaro Savoia‖, Elisabetta Panza*, Antonietta Di Martino†, Giuseppe Cirino* and Mariarosaria Bucci*

*Department of Pharmacy, University of Naples 'Federico II', Naples, Italy
†Centre of Biotechnology, Cardarelli Hospital, Naples, Italy
‡Department of Pharmacy, University of Pisa, Pisa, Italy
§Centre for Advanced Biomaterials for Health Care (CRIB), Italian Institute of Technology, Naples, Italy
¶Weill Cornell University Medical College, New York City, NY 10065, U.S.A.
‖Unit of Anaesthesiology and Resuscitation IV, Cardarelli Hospital, Naples, Italy

Abstract

Hydrogen sulfide is an endogenous gasotransmitter and its mechanism of action involves activation of ATP-sensitive K^+ channels and phosphodiesterase inhibition. As both mechanisms are potentially involved in malignant hyperthermia (MH), in the present study we addressed the involvement of the L-cysteine/hydrogen sulfide pathway in MH. Skeletal muscle biopsies obtained from 25 MH-susceptible (MHS) and 56 MH-negative (MHN) individuals have been used to perform the *in vitro* contracture test (IVCT). Quantitative real-time PCR (qPCR) and Western blotting studies have also been performed. Hydrogen sulfide levels are measured in both tissue samples and plasma. In MHS biopsies an increase in cystathionine β-synthase (CBS) occurs, as both mRNA and protein expression compared with MHN biopsies. Hydrogen sulfide biosynthesis is increased in MHS biopsies (0.128 ± 0.12 compared with 0.943 ± 0.13 nmol/mg of protein per min for MHN and MHS biopsies, respectively; $P < 0.01$). Addition of sodium hydrosulfide (NaHS) to MHS samples evokes a response similar, in the IVCT, to that elicited by either caffeine or halothane. Incubation of MHN biopsies with NaHS, before caffeine or halothane challenge, switches an MHN to an MHS response. In conclusion we demonstrate the involvement of the L-cysteine/hydrogen sulfide pathway in MH, giving new insight into MH molecular mechanisms. This finding has potential implications for clinical care and could help to define less invasive diagnostic procedures.

Key words: cystathionine β-synthase, L-cysteine, gasotransmitter, hydrogen sulfide, malignant hyperthermia, skeletal muscle.

INTRODUCTION

Malignant hyperthermia (MH) is a rare pharmacogenetic disorder of skeletal muscle triggered by volatile anaesthetics and depolarizing muscle relaxants. It is characterized by hypermetabolism, hypercapnia, tachycardia, hypoxia, muscle rigidity, rhabdomyolysis and rapid increase in body temperature, which can lead to death unless immediately reversed [1–4]. The reported incidence of MH ranges from 1:5000 to 1:50 000 anaesthetic episodes, although these values may underestimate the true prevalence [5,6]. Although the mortality rate has settled at around 10%, the morbidity rate is higher (35%), with several complications that range from cardiac and/or renal dysfunctions to disseminated intravascular coagulation [7]. Molecular genetic studies have established the ryanodine receptor 1 (*RYR1*) gene, on chromosome 19q13.1, as the primary locus for MH susceptibility. To date, more than 300 *RYR1* mutations have been associated with MH, but a causative role has been demonstrated for only 30 [3,8–10]. However, less than 50% of MH-susceptible (MHS) individuals have mutations in the *RYR1* gene [11–13]. In addition, mutations in the *CACNA1S* gene on chromosome 1q32 have been linked to MH [14,15]. In spite of these advances in MH molecular genetics,

Abbreviations: 3-MST, 3-mercaptopyruvate sulfurtransferase; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; DPD, *N*,*N*-dimethyl-*p*-phenylendiamine sulfate; IVCT, *in vitro* contracture test; K_{ATP} channel, ATP-sensitive K^+ channel; MH, malignant hyperthermia; MHN, malignant hyperthermia-negative; MHS, malignant hyperthermia-susceptible; PDE, phosphodiesterase; PLP, pyridoxal phosphate; qPCR, quantitative real-time PCR; RYR1, ryanodine receptor 1; TCA, trichloroacetic acid.

[1] These authors contributed equally to this work.

Correspondence: Professor Giuseppe Cirino (email cirino@unina.it).

the diagnostic gold standard for detecting MH susceptibility still relies on a bioassay, namely the *in vitro* contracture test (IVCT) [16–19]. IVCT is an invasive diagnostic procedure that requires a biopsy from the quadriceps muscle to assess skeletal muscle contractility in response to halothane and caffeine, *in vitro*.

Hydrogen sulfide (H_2S) is endogenously produced in mammalian cells from L-cysteine through the action of cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), both pyridoxal phosphate (PLP)-dependent enzymes. There is also a PLP-independent third pathway involving 3-mercaptopyruvate sulfurtransferase (3-MST). These enzymes are widely distributed in the body [20–22] and the L-cysteine/H_2S pathway has been involved in several physiopathological processes [23]. As described above, IVCT requires exposure to halothane, the mechanism of action for which involves the activation of several channels, including ATP-sensitive K^+ (K_{ATP}) channels [24–28], and to caffeine, which is known to be a non-specific phosphodiesterase (PDE) inhibitor [29]. As the H_2S mechanism(s) of action involves either (i) activation of K_{ATP} channels [30,31], or (ii) inhibition of PDE activity [32], we have studied the possible involvement of the L-cysteine/H_2S pathway in MH susceptibility.

MATERIALS AND METHODS

The Cardarelli Hospital Centre for the Study of Malignant Hyperthermia has operated since December 1989. In 23 years (1989–2012), 1686 patients have been identified, 1465 of whom have been outpatients for clinic assessment. An accurate anamnesis, both personal and familial, has been carried out to identify potential muscular disorders, problems that occurred during previous general anaesthetic exposures and sudden neonatal death. Contextually, a clinical assessment revealing musculoskeletal alterations, such as kyphoscoliosis, club foot or flying scapulae, was performed together with a clinical evaluation of squint, cryptorchidism, palpebral ptosis, and inguinal and/or disc hernia. As eligible participants, 695 underwent a muscle biopsy for the IVCT. The average results were: 60% MH-negative (MHN) individuals diagnosed (417 participants) and 40% MHS individuals diagnosed (278 participants). Following approval by the Cardarelli Ethics Committee, written informed consent for the different investigations was obtained from the patients or their parents, as appropriate.

IVCT

The IVCT procedure was performed using either caffeine or halothane, according to the European group protocol for investigation of malignant hyperthermia susceptibility (see the Supplementary Online Data for details and for 'malignant hyperthermia sensitivity diagnosis').

In vitro study on MHN samples

A functional analysis has been carried out on muscle bundles not required for diagnostic purposes, i.e. after diagnosis has been made. To evaluate the possible interference of sodium hydrosulfide (NaHS) with muscle functionality, strips obtained from MHN patients were exposed to NaHS at different concentrations. The resting tension trend of the bundles was thoroughly observed for 20 min.

In another set of experiments, we evaluated the possible interference of NaHS with either caffeine or halothane by using muscle strips obtained from MHN patients. Tissues were incubated for 5 min with NaHS at different concentrations (0.5, 1 and 3 mM) or NaCl at equimolar doses, then caffeine (2 mM) or 2% halothane (0.4 mM) was added, and the bundles' resting tension trend was observed thoroughly for 10 min.

In vitro study on MHS samples

The functional analysis was also carried out on muscle bundles not required for diagnostic purposes, i.e. after diagnosis has been made. Muscle strips obtained from MHS patients were exposed to NaHS (3 mM), then the bundles' resting tension trend was observed thoroughly for 20 min. In another set of experiments, MHS muscle bundles were incubated with glibenclamide, a selective K_{ATP} channel blocker (10 mM). After 15 min of exposure, 2% halothane was added and the bundles' resting tension trend was observed thoroughly for 10 min.

H_2S quantification assays

Methylene Blue assay

H_2S determination was performed according to the following protocol [33–35]. Briefly, muscle samples were homogenized in a lysis buffer (100 mM potassium phosphate buffer, pH 7.4, 10 mM sodium orthovanadate and a cocktail of protease inhibitors, 1% v/v) and the protein concentration determined using the Bradford assay (Bio-Rad Laboratories). The lysates were added to a reaction mixture (total volume 500 μl) containing pyridoxal 5′-phosphate (2 mM, 20 μl), L-cysteine (10 mM, 20 μl) and saline (30 μl). The reaction was performed in Parafilm-sealed Eppendorf tubes and initiated by transfer of tubes from ice to a 37 °C water bath. After a 30-min incubation, zinc acetate (1%, 250 μl) was added to trap evolved H_2S, followed by trichloroacetic acid (TCA; 10%, 250 μl). Subsequently, *N*,*N*-dimethyl-*p*-phenylendiamine sulfate (DPD; 20 μM, 133 μl) in 7.2 M HCl and $FeCl_3$ (30 μM, 133 μl) in 1.2 M HCl were added. After 20 min, the absorbance values were measured at a wavelength of 650 nm. All samples were assayed in duplicate, and the H_2S concentration was calculated against a calibration curve of NaHS (3.12–250 μM). The results were expressed as nmol/mg of protein per min.

H_2S determination in plasma samples was performed as follows: samples (200 μl) were added to Eppendorf tubes containing TCA (10%, 300 μl), to allow protein precipitation. Supernatant was collected after centrifugation and zinc acetate (1% ,150 μl) then added. Subsequently, DPD (20 mM, 100 μl) in 7.2 M HCl and $FeCl_3$ (30 mM, 133 μl) in 1.2 M HCl were added to the reaction mixture, and the absorbance was measured after 20 min at a wavelength of 650 nm. All samples were assayed in duplicate, and the H_2S concentration was calculated against a calibration curve of NaHS (3.12–250 μM).

Amperometric assay

The amperomentric assay was performed by an Apollo-4000 Free Radical Analyzer (WPI) detector and 2-mm H_2S-selective minielectrodes [35,36]. The H_2S-selective minielectrode was

equilibrated in a 20-ml organ bath filled with Kreb's solution oxygenated with carbogen (95% oxygen and 5% carbon dioxide mixture) at 37 °C. Once a stable baseline had been achieved, an aqueous solution of NaHS (20 mM) was added. The H_2S generation was monitored for 10 min.

Western blotting

Muscle samples were homogenized in modified RIPA buffer (50 mM Tris/HCl, pH 7.4, 1% Triton X-100, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 10 mM sodium orthovanadate and a cocktail of protease inhibitors, 1%, v/v) using a micropestle. After centrifugation of homogenates at 8050 *g* for 15 min, the protein concentration was determined using the Bradford assay with BSA as a standard (Bio-Rad Laboratories). The denatured proteins (40 μg) were separated on SDS/10% or 8% PAGE and transferred on to a PVDF membrane. Membranes were blocked in PBS/Tween 20 (0.1%, v/v) containing 3% non-fat dry milk for 1 h at room temperature, and then incubated with anti-CSE (1:500 dilution), anti-CBS (1:1000 dilution) or anti-3-MST (1:500 dilution) overnight at 4 °C. The filters were washed with PBS/Tween 20 (0.1%, v/v) extensively for 30 min, before incubation, for 2 h at 4 °C, with the secondary antibody (1:5000 dilution) conjugated with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG. The membranes were then washed and the immunoreactive bands visualized using Enhanced Chemiluminescence Substrate (ECL; GE Healthcare) with ImageQuant 400 equipped with Software ImageQuant Capture (GE Healthcare) and analysed using Quantity One Software version 4.6.3.

RNA purification and qPCR

Total RNA is isolated from tissues using the TRI Reagent (Sigma–Aldrich), according to the manufacturer's instructions, followed by spectrophotometric quantification. Final preparation of RNA is considered to be DNA- and protein-free if the ratio between readings at 260 and 280 nm is $\geqslant$1.7. Isolated mRNA was reverse-transcribed using iScript Reverse Transcription Supermix for quantitative real-time PCR (qPCR; Bio-Rad Laboratories). The qPCR was carried out in an CFX384 real-time PCR detection system (Bio-Rad Laboratories) with specific primers through the use of the SYBR Green Master Mix kit (Bio-Rad Laboratories). Samples were amplified simultaneously in triplicate in a one-assay run, with a non-template control blank for each primer pair to control for contamination or primer–dimer formation, and the CT (cycle threshold) value for each experimental group was determined. The housekeeping gene (ribosomal protein S16) was used as an internal control to normalize the CT values, using the $2^{-\Delta C T}$ formula.

Reagents

Caffeine, halothane, DMSO, DPD, pyridoxal 5′-phosphate hydrate, $FeCl_3$, zinc acetate, NaHS, TCA, glibenclamide and NaCl were purchased from Sigma Chemical Co. DPD was dissolved in 7.2 M HCl, $FeCl_3$ was dissolved in 1.2 M HCl and all other drugs were dissolved in distilled water. The anti-CSE (M02) antibody was purchased from Abnova, the anti-CBS (H-300) antibody was from Santa Cruz Biotechnology, the anti-3-MST antibody was from Abcam and the anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody from Sigma Chemical Co.

Statistical analysis

Data are expressed as means $\pm$ S.D. and analysed for statistical significance using Student's t test or one-way ANOVA, followed by Dunnett's post-hoc test via use of GraphPad Prism Software. $P < 0.05$ was considered significant.

RESULTS

The study has been performed on skeletal muscle biopsies obtained from 56 MHN- and 25 MHS-diagnosed participants.

CBS expression is enhanced in MHS muscle biopsies

As shown in Figure 1(A), qPCR reveals an enhanced mRNA expression of CBS in MHS samples – the opposite of MHN. CBS Western blot analysis demonstrates that the enhanced mRNA is consistent with a significant protein increase (Figure 1B, $P < 0.001$). CSE and 3-MST mRNA levels did not differ between MHN and MHS tissues. Conversely, protein expression of CSE (Figure 1C) and 3-MST (Figure 1D) was reduced in MHS compared with MHN participants.

Hydrogen sulfide content is enhanced in MHS muscle biopsies

As shown in Figure 2(A), in MHN samples there is a small but detectable basal (without substrate) production of H_2S. Incubation of MHN tissue samples with the substrate L-cysteine caused a significant increase in H_2S generation. In MHS samples, basal and L-cysteine-stimulated H_2S biosynthesis were both significantly higher when compared with MHN tissue samples (Figure 2A). Notably, MHS samples showed an approximately 10-fold increase in basal content of H_2S compared with MHN samples. This effect is congruent with the increase in mRNA (Figure 1A) and protein expression observed (Figure 1B). Conversely, plasma levels of H_2S were not significantly different between MHN and MHS participants (Figure 2B).

H_2S triggers a contractile response in MHS but not in MHN samples

Through use of the IVCT, we tested whether the concentration of H_2S used could influence the contractility of skeletal muscle biopsies. As the isolated muscle bundle technique requires a robust bubbling of Kreb's buffer with carbogen (95% oxygen and 5% carbon dioxide mixture), we assessed the real concentration of H_2S developed in the organ bath after NaHS administration. To do so, we used an amperometric assay that could detect H_2S. Under our experimental conditions, addition of NaHS provided a release of H_2S in the organ bath that was almost 10-fold less. Therefore, the real concentrations of H_2S released by NaHS in the organ bath were 0.5, 1 and 3 mM. NaHS up to a concentration of 3 mM neither affected tissue viability nor changed baseline tension within the assay timeframe, i.e. 20 min (Figure 3A). Conversely, exposure of MHS biopsies to NaHS caused an increase

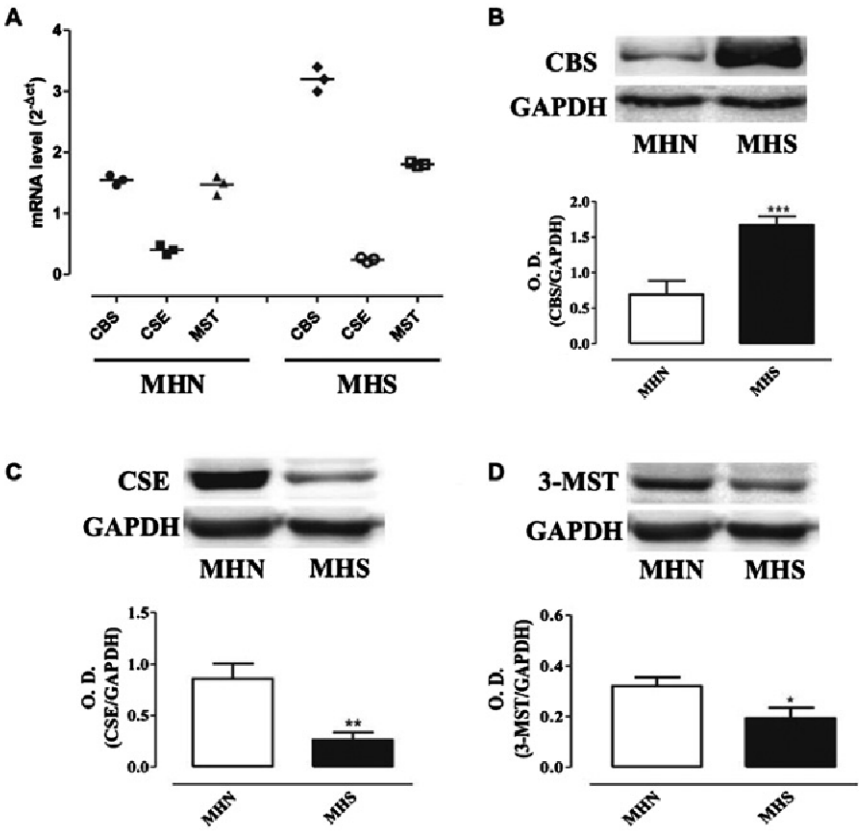

Figure 1 **CBS expression is enhanced in MHS muscle biopsies**

(**A**) qPCR reveals a significantly increased CBS expression in MHS samples compared with MHN. The mRNA levels of both CSE and 3-MST were not significantly different between MHN and MHS groups. (**B**) Western blotting reveals significantly increased CBS expression in MHS samples compared with MHN samples. $^{***}P < 0.001$, $n = 6$ for each group. The bands represent three different experiments. (**C**) Western blotting reveals a significant reduced CSE expression in MHS samples compared with MHN samples. $^{**}P < 0.01$, $n = 6$ for each group. The bands represent three different experiments. (**D**) Western blotting reveals a significant reduced 3-MST expression in MHS samples compared with MHN samples. $^{*}P < 0.05$, $n = 6$ for each group. The bands represent three different experiments.

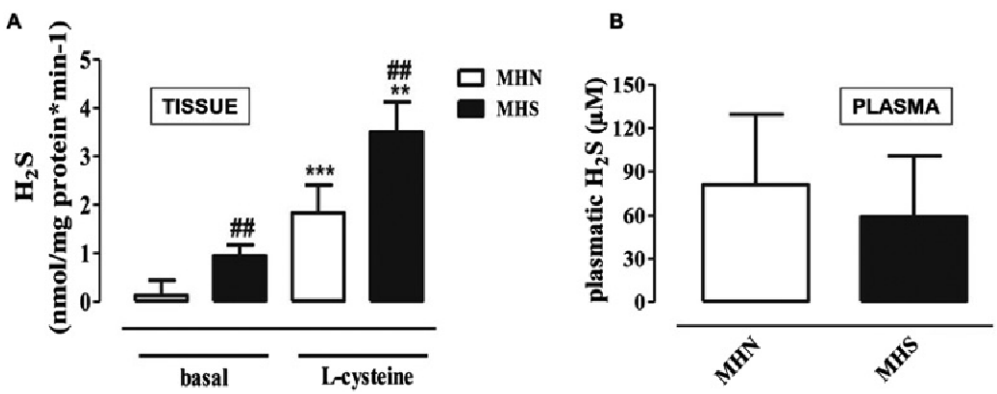

Figure 2 **Hydrogen sulfide content is enhanced in MHS muscle biopsies**

(**A**) Evaluation of H_2S levels in tissue homogenates reveals a significant increase in H_2S production in MHS samples, under both basal and stimulated (incubated with L-cysteine) conditions, compared with MHN samples. $^{***}P < 0.001$ MHN stimulated with L-cysteine compared with MHN basal; $^{**}P < 0.01$ MHS stimulated with L-cysteine compared with MHS basal; $^{\#\#}P < 0.01$ MHS compared with MHN, $n = 5$ for each group. (**B**) Evaluation of H_2S plasma levels shows no significant difference between MHN and MHS patients; $n = 9$ for each group.

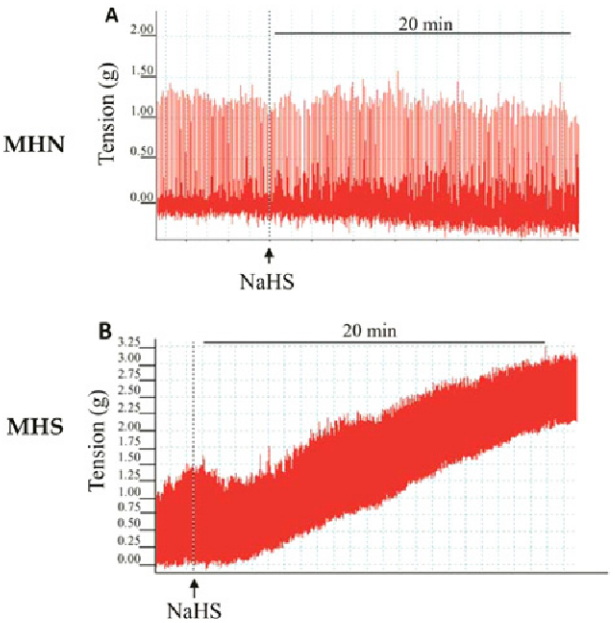

Figure 3 Hydrogen sulfide triggers a contractile response in MHS but not in MHN samples

(**A**) Representative trace of muscle samples obtained from MHN patients incubated with NaHS (3 mM) for 20 min. (**B**) Representative trace of muscle obtained from MHS patients incubated with NaHS (3 mM) for 20 min. NaHS increases the contracture in MHS biopsies but not in MHN samples; $n = 3$ for each group.

in baseline tension that mimics the IVCT response elicited by either caffeine or halothane in positive patients (Figure 3B).

H_2S triggers an MHS-like response in MHN samples

After incubation with NaHS (5 min), the tissues were challenged with either 2 mM caffeine (Figure 4C) or 2% halothane (Figure 5C), using the same procedure as in the IVCT. Under these experimental conditions, NaHS exposure leads to an increase in muscle contracture with either caffeine (Figure 4C) or halothane (Figure 5C) that is similar in onset and shape to a typical MHS response. The effect driven by NaHS is concentration-dependent (Table 1). When MHN samples are exposed to equimolar concentrations of NaCl, as a control, and challenged with either caffeine or halothane, no change in baseline tension was observed, thereby indicating that an equimolar concentration of Na^+ to NaHS does not modify skeletal muscle reactivity (see Supplementary Figure S1).

Selective K_{ATP} channel blockade reduces halothane-induced contraction in susceptible tissues (MHS)

As CBS-selective inhibitors are not available [37], we investigated whether interference with a molecular target of H_2S leads to a reduction of signalling in the IVCT through the use of MHS samples. One of the most characterized molecular mechanisms through which H_2S acts is the opening of K_{ATP} channels [30,31]. As volatile anaesthetics, among several mechanisms of action, facilitate K_{ATP} channel opening [24–28], we evaluated whether K_{ATP} channel activation could be involved in halothane-induced contracture in MHS samples. To do so, MHS bundles were presented with a selective K_{ATP} channel inhibitor glibenclamide before challenge with halothane. Glibenclamide (Figure 6) significantly inhibited halothane-induced contracture ($58.5 \pm 27\%$ of inhibition; $P < 0.05$, $n = 4$). The addition of glibenclamide itself did not modify the baseline tension of MHS samples (results not shown).

DISCUSSION

In the present study, we have investigated the possible involvement of the L-cysteine/H_2S pathway in MH susceptibility. The whole study was performed using negative and susceptible human biopsies of the vastus muscle. The three main enzymes responsible for H_2S biosynthesis, namely CBS, CSE and 3-MST, are all expressed in human skeletal muscle as determined by the qPCR study performed on MHN biopsies. When the MHS samples were analysed, we found that CBS mRNA levels were almost doubled in MHS biopsies. Accordingly, CBS protein expression was almost tripled. In contrast, CSE and 3-MST protein expression resulted in down-regulation even though the mRNA levels of both enzymes were unchanged (Figure 1). This discrepancy could be associated with post-transcriptional mechanisms. Indeed, the down-regulation of CSE and 3-MST protein in MHS may involve the endogenous production of miRNAs which, in turn, could lead to an adaptive response, thereby limiting H_2S generation. These data suggest that, in MHS, there is a rearrangement of the balance among these three enzymes producing H_2S, leading to an increasing role for CBS-derived H_2S. To understand whether this change in CBS expression affected H_2S biosynthesis, we measured the H_2S content in both MHN and MHS biopsies. In MHN biopsies there was a small but detectable basal amount of H_2S (without the addition of substrate L-cysteine). When MHN biopsies were incubated with L-cysteine and H_2S was then measured, there was a significant increase in its content (Figure 2A). Therefore, in normal skeletal muscle, there is a basal physiological (tonic) production of H_2S. Conversely, in MHS samples, the H_2S basal content was approximately 10-fold higher compared with MHN samples, and it was further increased by incubation with L-cysteine. As in MHN skeletal muscle of the susceptible patients (MHS), the other two enzymes, 3-MST and CSE, responsible for H_2S biosynthesis are both down-regulated, the CBS-derived H_2S is the main source of the 'pathological' H_2S.

To understand whether the CBS up-regulation in skeletal muscle could also lead to an increase in circulating levels of H_2S, we measured plasma concentration. Plasma values did not differ between MHS and MHN patients (Figure 2B). These results indicate that the increased H_2S production observed in MHS biopsies is confined within the muscle, implying that the CBS-enhanced H_2S biosynthesis is a local skeletal-muscle-confined phenomenon.

To understand and further support this hypothesis we designed a specific experimental protocol. Due to the reduced availability of MHS biopsies, we used mainly MHN samples. In this protocol,

Table 1 **Exposure to H_2S causes a concentration-dependent increase in contraction of MHN skeletal muscle tension, triggered by both caffeine and halothane**

Treatment	Trigger	Increase in tension (mg) (mean ± S.D.) (*n*)	Statistical significance (*P*)
0.5 mM H_2S	2 mM caffeine	3.3 ± 2.9 (4)	Not significant
1 mM H_2S	2 mM caffeine	254.3 ± 118.9 (7)	<0.001
3 mM H_2S	2 mM caffeine	462.5 ± 179.7 (4)	<0.001
1 mM H_2S	2% halothane	505.4 ± 361.4 (5)	<0.05
3 mM H_2S	2% halothane	518.0 ± 301.8 (4)	<0.01

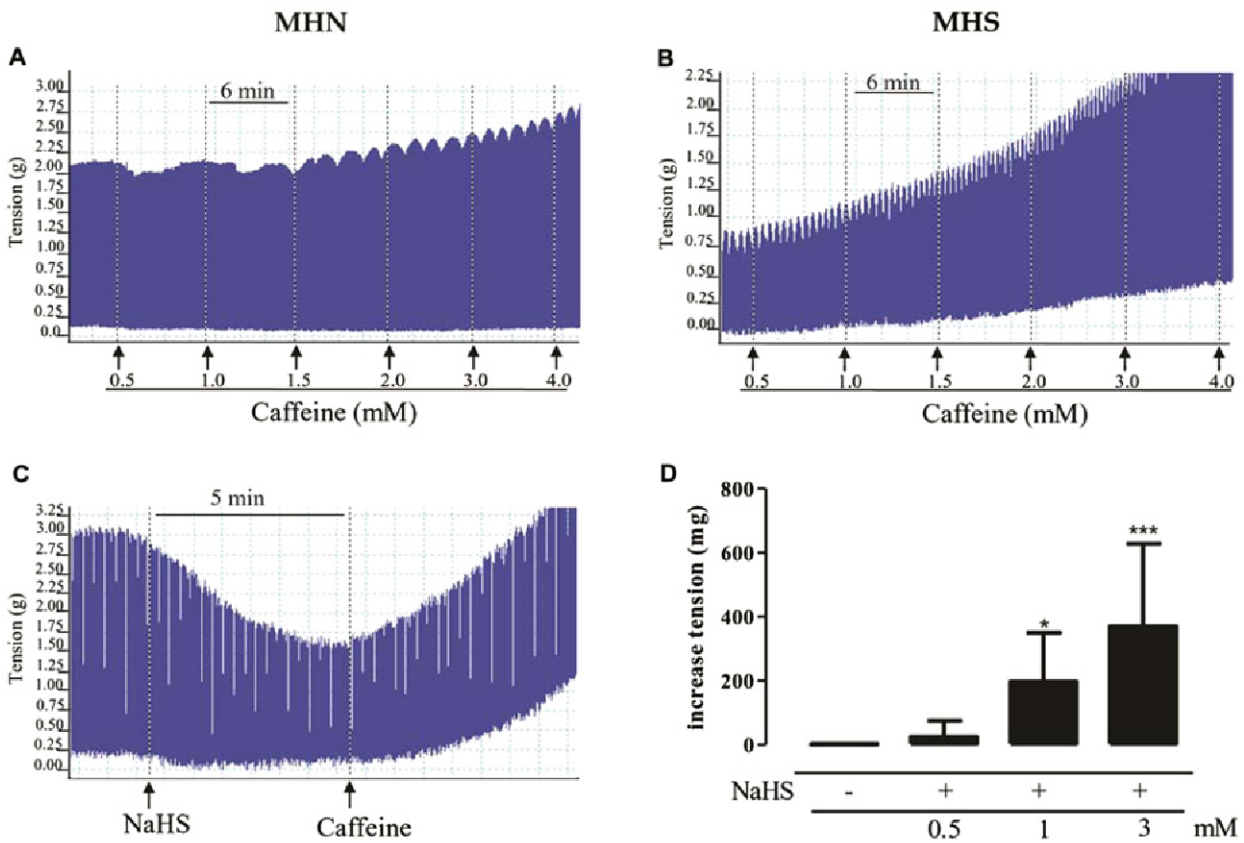

Figure 4 **H_2S triggers an MHS-like response in MHN samples after caffeine exposure**
(**A**) MHN diagnosis after caffeine exposure: after 20 min of muscle bundle stabilization, caffeine was added to the organ bath with a flow of 5 ml/min at progressive concentrations of 0.5, 1.0, 1.5, 2.0, 3.0, 4.0 and 32.0 mM (6 min of contact for each concentration). The absence of any contracture from the resting tension provides an MHN diagnosis for caffeine. (**B**) MHS diagnosis after caffeine exposure: after 20 min of muscle bundle stabilization, caffeine was added to the organ bath with a flow of 5 ml/min at progressive concentrations of 0.5, 1.0, 1.5, 2.0, 3.0, 4.0 and 32.0 mM (6 min of contact for each concentration). The progressive increase in resting tension above 2 mN (0.2 g) provides an MHS diagnosis for caffeine. (**C**) Representative trace of muscle samples obtained from MHN patients incubated for 5 min with NaHS (3 mM) followed by a caffeine trigger (2 mM). The increase in tension achieved is similar to that for MHS samples, i.e. >2 mN (0.2 g) above the resting tension. (**D**) Effect of incubation of different concentrations of NaHS for MHN biopsies on caffeine exposure (for statistical parameters see Table 1).

MHN biopsies were incubated with different concentrations of H_2S, to assess whether H_2S could trigger an increase in baseline tension in an MHN tissue (Figure 3A). The concentration response of H_2S was performed using a concentration range similar to that used for caffeine in the IVCT. These concentrations are required for the IVCT procedure to achieve better reproducibility of the response for fulfilment of the quality criteria, with consequent patient safety [38], and worldwide they are accepted and applied by both European and North American MH groups. It must be noted that, at the concentration of H_2S used for MHN biopsies, incubation in the organ bath in itself modified neither the skeletal muscle tension nor the tissue viability, within the timeframe of the IVCT and for up to 60 min. Therefore H_2S, similar to caffeine and halothane, does not trigger any response in normal skeletal muscle (MHN) up to the maximal concentration tested, and it is not toxic as demonstrated by the

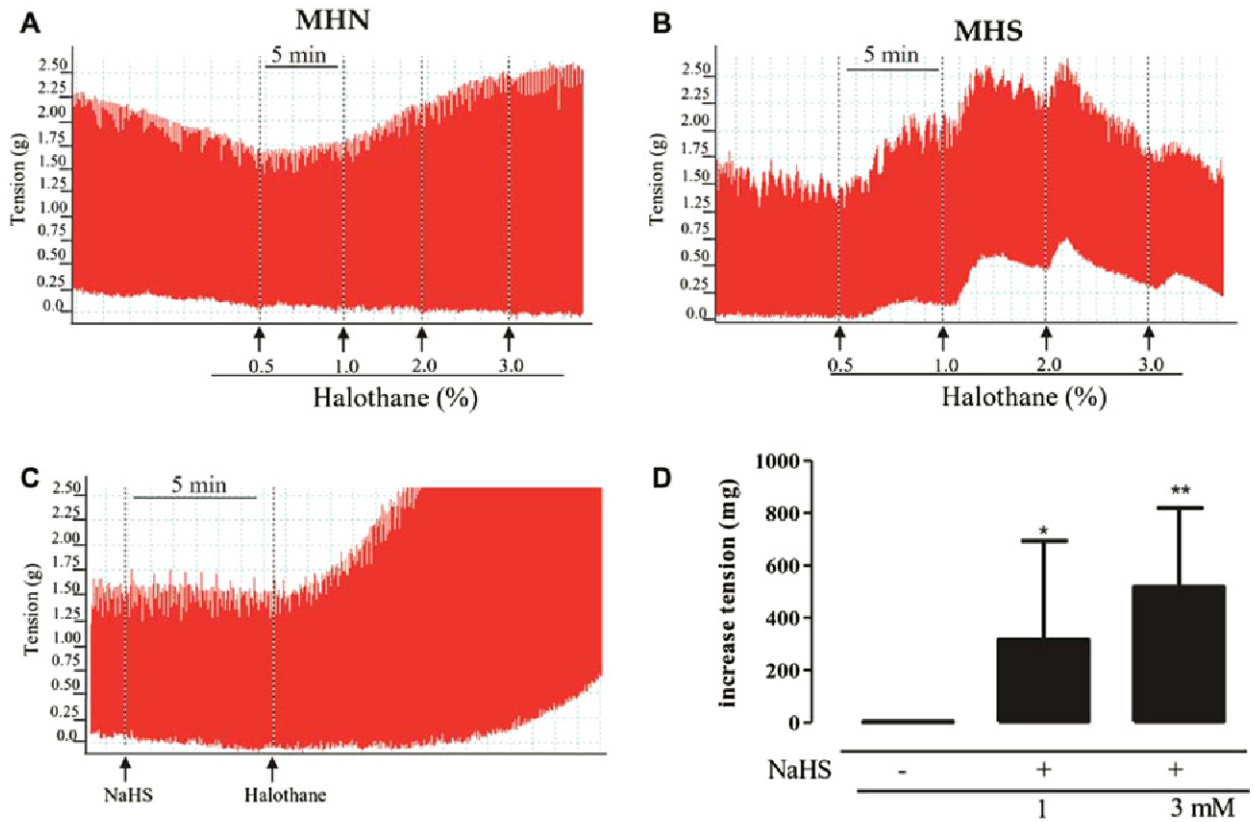

Figure 5 **H_2S triggers an MHS-like response in MHN samples after halothane exposure**

(**A**) MHN diagnosis after halothane exposure: after 20 min of stabilization the muscle bundle was exposed to progressive halothane concentrations of 0.5%, 1%, 2% and 3% (5 min of contact for each concentration). The absence of any contracture from the resting tension provides an MHN diagnosis for halothane. (**B**) MHS diagnosis after halothane exposure: after 20 min of muscle bundle stabilization, halothane concentrations of 0.5%, 1%, 2% and 3% (5 min of contact for each concentration) were added. The progressive increase in resting tension >2 mN (0.2 g) provides an MHS diagnosis for halothane. (**C**) Representative trace of muscle samples obtained from MHN patients incubated for 5 min with NaHS (3 mM) followed by halothane trigger (2%). The increase in tension achieved is similar to that for MHS samples, i.e. >2 mN (0.2 g) above the resting tension. (**D**) Effect of incubation of different concentrations of NaHS for MHN biopsies on halothane exposure (for statistical parameters see Table 1).

finding that tissue tension is not affected by H_2S exposure (see Supplementary Figure S2). However, when either halothane or caffeine was added to the MHN tissues exposed to H_2S, as described above, a concentration-dependent increase in tension was observed (Figures 4 and 5). In particular the shape and magnitude of the contracture elicited were similar to those obtained with the IVCT when an MHS diagnosis was made. Consequently, these data strongly support our hypothesis that high concentrations of CBS-derived H_2S, endogenously generated within the skeletal muscle of MHS patients, are involved in the anomalous contracture triggered by both caffeine and halothane.

For further confirmation of our hypothesis, we directly challenged skeletal muscle tissue specimens ($n = 3$) already diagnosed as MHS using the IVCT with exogenous H_2S. H_2S incubation of MHS biopsies induced an increase in baseline tension similar to that elicited by caffeine and halothane (Figure 3B). At this point it is intuitive that experiments *in vitro* with a selective inhibitor of CBS, before addition of halothane and caffeine, would be the most appropriate for confirming the involvement of CBS-derived H_2S in MH. Unfortunately, up to now, no selective CBS inhibitor has been available. As has recently been shown [37], all inhibitors of H_2S biosynthesis currently on the market are CSE-selective inhibitors. Moreover, the only non-selective CBS/CSE inhibitor, amino-oxyacetic acid, which has been widely used as a CBS inhibitor at the concentration of 1 mM, is toxic to the tissue. Therefore, to address this issue, we used an indirect approach by interfering with one of the major recognized molecular targets of H_2S, i.e. the K_{ATP} channels. To perform this experiment we incubated MHS tissue samples with glibenclamide, a selective K_{ATP} channel blocker. Glibenclamide caused a significant reduction in halothane-induced contracture in MHS samples (Figure 6), thereby implying that K_{ATP} channel activation is involved in the anomalous contraction elicited by halothane in MHS patients.

It is important to consider that H_2S, being a gasotransmitter, could interact with many other potential molecular targets involved in the MH syndrome, e.g. it is known that the RYR1 contains up to 100 cysteine residues per subunit, which can be

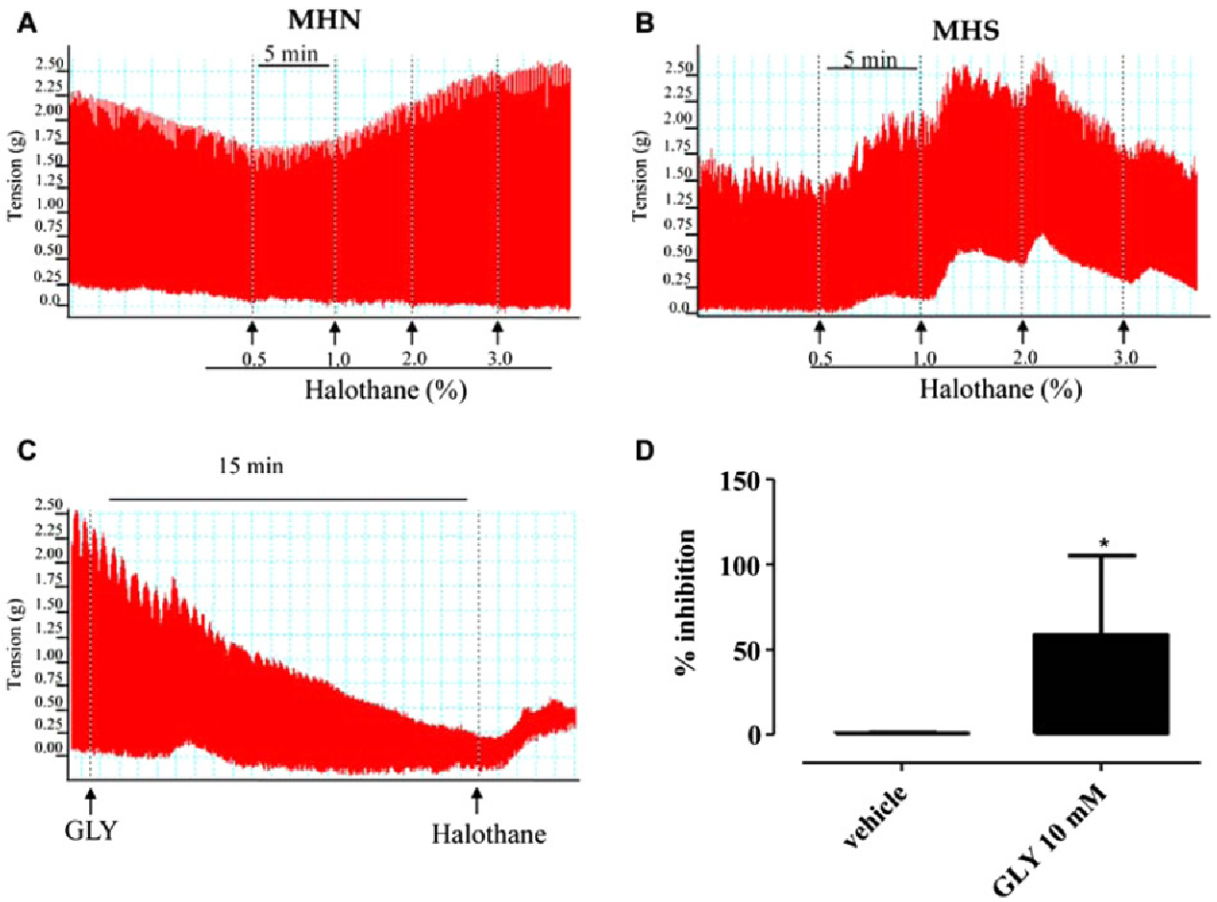

Figure 6 **Selective K_{ATP} channel blockade reduces halothane-induced contraction in susceptible tissues (MHS)**
(**A**) MHN diagnosis after halothane exposure: after 20 min of stabilization the muscle bundle was exposed to progressive halothane concentrations of 0.5%, 1%, 2% and 3% (5 min of contact for each concentration). The absence of any contracture from the resting tension provides an MHN diagnosis for halothane. (**B**) MHS diagnosis after halothane exposure: after 20 min of muscle bundle stabilization, halothane concentrations of 0.5%, 1%, 2% and 3% (5 min of contact for each concentration) were added. The progressive increase in resting tension >2 mN (0.2 g) provides an MHS diagnosis for halothane. (**C**) Representative trace of muscle samples obtained from MHS patients incubated for 15 min with the K_{ATP} channel-selective inhibitor glibenclamide (10 mM) followed by a halothane trigger (2%). (**D**) Glibenclamide induces a significant reduction of the MHS response (58.5 ± 27% of inhibition; $^{*}P < 0.05$, $n = 4$).

oxidized or reduced by different reagents modifying its activity [39,40]. In this context it has been shown that various classes of thiol-group-specific agents can react with hyperreactive thiols on ryanodine receptors, which could play a role in the regulation of normal contractile function and explain contractile dysfunction in pathological conditions [41]. Therefore it is feasible that the enhanced CBS-derived H_2S production in MHS patients may directly interact with RYR1, shifting it to a more active state.

In conclusion we have demonstrated that: (i) CBS is overexpressed in MHS biopsies – the opposite of 3-MST and CSE; (ii) H_2S itself elicits contracture on MHS but not on MHN biopsies in the IVCT; (iii) incubation with H_2S of MHN biopsies before caffeine or halothane challenge switches an MHN into an MHS response; (iv) the L-cysteine/CBS/H_2S pathway is the major source of H_2S within MHS skeletal muscle; and (v) blockade of K_{ATP} channels reduces the halothane-induced response in MH susceptibility.

Therefore evaluation of CBS expression and/or H_2S levels using needle biopsies together with genetic testing may help to define a new, less invasive, diagnostic procedure.

CLINICAL PERSPECTIVES

- The gold standard procedure for diagnosing MHS is the IVCT, which requires a biopsy from the quadriceps muscle. The mechanism of action of H_2S includes activation of K_{ATP} channels and PDE inhibition. As both mechanisms are potentially involved in MH, in the present study we addressed the involvement of the L-cysteine/H_2S pathway in MH.
- Human skeletal muscle biopsies obtained from MHS patients show an increase in CBS, protein expression and H_2S biosynthesis. H_2S addition to MHS samples in itself evokes a response similar to that elicited by either caffeine or halothane.

Incubation of negative biopsies with H_2S, before caffeine or halothane challenge, triggers an MHS-like response.

- The evaluation of CBS expression and/or H_2S together with genetic testing could help to define a new, less invasive, diagnostic procedure. In addition CBS may represent a feasible therapeutic target.

AUTHOR CONTRIBUTION

Valentina Vellecco, Antonio Mancini, Chiari Attanasio, Antonietta Di Martino, Gennaro Savoia and Barbara Andria performed the *in vitro* experiments and analysed the data. Anna Cantalupo, Angela Ianaro and Elisabetta Panza performed the Western blot and PCR experiments and analysed the data. Vincenzo Calderone performed the amperometric analysis and interpreted the data. The experiments were conceived and designed by Antonio Mancini, Valentina Vellecco and Mariarosaria Bucci. Mariarosaria Bucci and Giuseppe Cirino co-ordinated, wrote and revised the paper.

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