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Phosphodiesterases S-sulfhydration contributes to human skeletal muscle function.

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4-Chloro-7-nitrobenzofurazan (NBF-Cl PubChem CID: 25043)

ABSTRACT

The increase in intracellular calcium is influenced by cyclic nucleotides (cAMP and cGMP) content, which rating is governed by phosphodiesterases (PDEs) activity.Despite it has been demonstrated a beneficial effect of PDEs inhibitors in different pathological conditions involving SKM, not much is known on the role exerted by cAMP-cGMP/PDEs axis in human SKM contractility. Here, we show that Ssulfhydration of PDEs modulates human SKM contractility in physiological and pathological conditions. Having previously demonstrated that, in the rare human syndrome Malignant Hyperthermia (MH), there is an overproduction of hydrogen sulfide (H_2S) within SKM contributing to hyper-contractility, here we have used MH negative diagnosed biopsies (MHN) as healthy SKM, and MH susceptible diagnosed biopsies (MHS) as a pathological model of SKM hypercontractility. The study has been performed on MHS and MHN human biopsies after diagnosis has been made and on primary SKM cells derived from both MHN and MHS biopsies. Our data demonstrate that in normal conditions PDEs are S-sulfhydrated in both quadriceps' biopsies and primary SKM cells. This post translational modification (PTM) negatively regulates PDEs activity with consequent increase of both cAMP and cGMP levels. In hypercontractile biopsies, due to an excessive H_2S content, there is an enhanced Ssulfhydration of PDEs that further increases cyclic nucleotides levels contributing to SKM hyper-contractility. Thus, the identification of a new endogenous PTM modulating PDEs activity represents an advancement in SKM physiopathology understanding.

1. Introduction

The main mechanism responsible for skeletal muscle (SKM) contraction is the excitation-contraction (EC) coupling that controls the endogenous calcium release from the sarcoplasmic reticulum (SR). The calcium release involves the "calcium release unit", a macromolecular complex formed by type 1 ryanodine receptor (RyR1) and skeletal isoform of dihydropyridine receptor L-type Calcium channel [1–3]. Active reuptake of calcium into SR, by the Ca^{2+} ATPase pump, terminates the contraction and promotes muscle relaxation. The increase in intracellular Ca^{2+} can be influenced by cyclic nucleotides (cAMP and cGMP) content, which rating is governed by phosphodiesterases (PDEs), a class of enzymes that degrades cyclic nucleotides [3]. In the past 40 years, the literature on the role played by the cAMP-cGMP/PDEs axis in

Abbreviations: PDE, phosphodiesterases; MH, malignant hyperthermia; MHN, malignant hyperthermia negative; MHS, malignant hyperthermia susceptible; IVCT, In Vitro Contracture Test; SKM, skeletal muscle; PTM, post-translational modification.

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SKM pathophysiology was erratic and not fully conclusive [4–11]. Even though experimental and clinical studies have demonstrated a beneficial effect of PDEs inhibitors in different pathological conditions involving SKM, such as ageing [12]; atrophy [13] and muscular dystrophy [14–16], few pieces of information are available on the role exerted by cAMP-cGMP/PDEs axis in human SKM contractility. This is mostly due to the intrinsic complexity of the molecular mechanisms involved in skeletal muscle contraction, that makes difficult a reliable translation to humans.

Hydrogen sulfide (H_2S) is a gaseous signaling molecule enzymatically produced in several tissues and organ systems [17,18]. Human skeletal muscle expresses the three constitutive enzymes responsible for H_2S biosynthesis, i.e cystathionine β synthase (CBS), cystathionine γ lyase (CSE, CGL or CTH) and 3-mercaptopyruvate sulfurtransferase (3-MST), all belonging to the transsulfuration pathway. Recently, we have shown that the skeletal muscle of Malignant Hyperthermia Susceptible (MHS) subjects expresses CBS at above average levels, which leads to an increased production of H_2S contributing to the hyper-contractility observed in MHS subjects [19]. MH is a rare pharmacogenetic disorder of SKM triggered by volatile anesthetics and depolarizing muscle relaxants. It is characterized by early signs like tachycardia, masseter spasm and generalized muscle rigidity, followed by late signs such as arrhythmias, high body temperature and rhabdomyolysis [20,21]. The prolonged muscle contraction is so severe that it increases whole body oxygen consumption and heat production bringing the body temperature up to 43–44 °C. All these events lead to cyanosis, hypermetabolic crisis and disseminated intravascular coagulation. In MH crises the triggering agent induces an anomalous and prolonged opening of RyR1 channels resulting in the uncontrolled release of calcium and, consequently, in sustained muscle rigidity. Therefore, we can consider MH as a paradigm of hyperactivation of EC coupling. Despite mutations of RyR1 gene have been associated with MH susceptibility, less than 50% of susceptible subjects carry RyR1 mutations [22–25]. For this reason MH diagnosis relies on a bioassay called -In vitro contracture test- IVCT, an invasive diagnostic procedure that requires a biopsy of vastus group of quadriceps muscle which is mounted in an tissue bath to assess the skeletal muscle contractility in response to two different triggers: the volatile anesthetic halothane and the non-selective phosphodiesterase (PDE) inhibitor, caffeine. The diagnosis depends on the extent of the response observed (for details, see [26]).

A recent discovered biological effects elicited by H_2S is via the post-translational modification (PTM) of proteins, a modification referred to as S-sulfhydration (or persulfidation) [27,28]. Similarly to the other PTM, S-sulfhydration targets L-cysteine thiol side chains to affect a wide range of physiological and pathological processes, such as cell proliferation/survival, mitochondrial bioenergetics, vascular reactivity and atherogenesis, as well as oxidative stress and inflammation [29–31]. In this context, we have recently shown that the potassium voltage-gated channel 7.4 (Kv7.4) in the skeletal muscle from MH subjects is S-sulfhydrated and that this modification contributes to the anomalous contractility observed in these specimens [32]. However, it is known that H_2S displays its biological activities through different of molecular mechanisms that involve the interaction with channels, enzymes and transcription factors [33]. Therefore, alternative /additive molecular mechanism, over S-sulfhydration, cannot be excluded.

The inhibition of phosphodiesterases (PDEs) is one of the recognized mechanism of action of H_2S [34–36]. The latter effect results in a slower rate of cyclic nucleotide degradation, which has profound implications for downstream signal transduction pathways that are regulated by cAMP and cGMP. Therefore, this study aims to evaluate whether the redox regulation of PDE activity involves its S-sulfhydration in skeletal muscle biopsies, and whether this PTM contributes to SKM hypercontractility.

Taking advantage of human bundles of vastus muscle not required for diagnostic use, we have assessed the role of the PDEs/H_2S interaction in human SKM contractility by using MH negative diagnosed biopsies (MHN) as normal SKM control, and MH susceptible diagnosed biopsies (MHS) as pathological model of SKM hypercontractility.

2. Material and methods

2.1. Human skeletal muscle biopsies

The Cardarelli Hospital Centre for the Study of Malignant Hyperthermia operates since December 1989. In these 32 years, 997 IVCT have been performed, and during the timeframe 2018–2020, 77 IVCT tests have been performed resulting in 61.0% MHN diagnosed (47 subjects) and 20.8% MHS diagnosed (16 subjects), 13.0% MH equivocal muscle reacting to halothane only diagnosed (MHSh, 10 subjects) and 5.2% MH equivocal muscle reacting to caffeine only diagnosed (MHSc, 4 subjects) (for diagnosis details see [26]). A previous accurate anamnesis has been carried out regarding potential muscular disorders, problems occurred during previous general anesthesia exposures, familiar sudden neonatal death. Assessment revealing muscle skeletal alterations (cifoscoliosis, club foot, flying scapulas) has been also performed coupled to clinical evaluation of squint, cryptorchidism, palpebral ptosis, inguinal and/or dischernia.The study has been performed on MHS and MHN biopsies not required for IVCT i.e. after diagnosis has been made (male 70% and female 30% in a range between 43 and 63 years old) in a timeframe of 26 months (October 2018-December 2020). The total number of biopsies available for the study has been 97, of which 69 MHN and 28 MHS diagnosed. From each patient one specimen has been used. In details, 52 fresh obtained biopsies are used for all pharmacological procedures (45 and 7 for bioassays and primary cell colture procedures, respectively), while 45 frozen biopsies have been taken from MH-diagnosis bank tissue of Cardarelli Hospital and used for molecular studies. All the surgical procedures have been performed at the Cardarelli Hospital -Centre for the Study of Malignant Hyperthermia-. The research has been carried out following the Code of Ethics of the World Medical Association (Declaration of Helsinki). The Ethical Committee of the Institution (Cardarelli Hospital Centre for the Study of Malignant Hyperthermia) in which the study has been performed, has approved it (4/13 prot. 358). The subjects have given written informed consent to the work.

2.2. In Vitro Contracture Test (IVCT)

The procedure has been performed by using either caffeine or halothane according to the guidelines of the “European Group protocol for the investigation of Malignant Hyperthermia Susceptibility” [26] and validate in our previous studies [19,32]. Briefly, under regional anesthesia, a 3–5 cm incision on the *vastus* group of the quadriceps muscle has been performed and 4–5 muscular bundles of 15–25 mm length and 2–3 mm thickness has been harvested and dissected within 15 min and quickly preserved in Krebs-Ringer solution with a following composition: NaCl 118.1 mmol/l; KCl 3.4 mmol/l; MgSO4 0.8 mmol/l; KH2PO4 1.2 mmol/l; glucose 11.1 mmol/l NaHCO3 25.0 mmol/l; CaCl2 2.5 mmol/l; pH = 7.4. Tissus have been transported from the surgery room to the laboratory within 30 min and oxygenated with carbogen, (95% oxygen and 5% carbon dioxide mixture) which continues for the duration of the whole experiment. The timeframe from biopsy to the end of the tests must not exceed 5 h. Each bundle have been tied on both ends and mounted vertically in the experimental tissue bath (15 ml volume) at 37 °C so that the lower end has been fixed and the upper end has been connected to an isometric transducer (Isometric Transducer 1–10 g.Ugo Basile) with a resting tension of 0.2 g (2 milliNewton, mN). The length between sutures has been measured and defined initial length. After 5 min in initial length, the electrical stimulus has been applied to the bundle (Stimulator LI 12006, Ugo Basile) with a 1–2 ms supramaximal stimulus at a frequency of 0.2 Hz. During electrical stimulation the muscle has been stretched slowly to 150 ± 10% of initial length, this new length has been considered to be the *optimal length*. The muscle has been allowed to stabilize at the *optimal length* for at least 15 min and

until baseline force does not vary more than 0.2 g (2 mN) within 10 min period. The signal has been amplified, continuously recorded and analyzed by PowerLab System. After a 20 min equilibration, two different protocols have been applied. One requires the addition of progressive concentration of caffeine (0.5, 1.0, 1.5, 2.0, 3.0, 4.0 mM) in the isolated organ bath; the other one requires the addition of progressive concentration of halothane (0.5%, 1%, 2% and 3%) in the isolated organ bath. An increase in resting tension of at least 0.2 g (2 mN) has been considered significant and allows a positive diagnosis to be made. The diagnosis of MH susceptibility relies on the increased resting tension of the tested muscle specimen: the threshold value for susceptibility is established as an increase of $\geq$ 0.2 g above the lowest resting tensions (Supplemental Fig. 1).

- MHShc (formerly MHS, Malignant Hyperthermia susceptible muscle) A caffeine threshold at caffeine concentration of 2.0 mmol/l or less, and a halothane threshold concentration at 0.44 mmol/l or less.
- MHN (Malignant Hyperthermia non-susceptible muscle) A caffeine threshold at a caffeine concentration of 3 mmol/l or more and halothane threshold concentration above 0.44 mmol/l.
- MHSh (formerly MHE, Malignant Hyperthermia equivocal muscle) The suffix has been added to indicate an abnormal response to halothane only.
- MHSc (formerly MHE, Malignant Hyperthermia equivocal muscle) The suffix has been added to indicate an abnormal response to caffeine only.

The signal has been amplified, continuously recorded, and analyzed by PowerLab System (ADInstruments Lab Chart 8.0, Dunedin, New Zealand). In a separate set of experiments, after standardization, MHN bundles have been incubated with PDEs non-selective inhibitor 3-Isobutyl-1-methylxanthine (IBMX) at different concentrations (2.5, 5 and 10 mmol/L). Following 5 min, caffeine (2 mmol/L) has been added, and the bundles resting tension has been observed for 15 min thoroughly.

2.2.1. *In vitro study on MHN samples pre-treated with H_2S donor*

Since MH is a rare syndrome, MHS diagnosed biopsies are very infrequent. Therefore, our study mainly relies on MHN on which a pharmacological approach has been applied. In particular, MHN bundles have been incubated with H_2S donor (NaHS) to mimic the high levels of H_2S detected in MHS biospies [19,32]. To evaluate the involvement of PDE4 and PDE5 in muscle contractility, bundles obtained from MHN patients are exposed to NaHS (3 mmol/L) or vehicle (H_2O) for 5 min, then the selective PDE4 inhibitor rolipram (100 μmol/L) have been added. Similarly, following 5 min of NaHS incubation (3 mmol/L), the selective PDE5 inhibitor sildenafil (10 μmol/L) has been added. The resting tension trend of the bundles has been observed for 20 min. The concentrations of rolipram and sildenafil has been selected taking into account the respective IC_{50} values (rolipram IC_{50} = 750 nmol/L, sildenafil IC_{50} = 3.5 nmol/L) and considering that in isolated skeletal muscle bioassay, drugs concentration used is at least 100 fold higher than IC_{50} value obtained in a cell-free assay.

2.2.2. *In vitro study on MHS biopsies*

MHS biopsies not required for IVCT, i.e. after diagnosis has been made, have been mounted in an isolated organ bath connected to an isometric transducer (Isometric Transducer 1–10 g. Ugo Basile) with a resting tension of 0.2 g. The muscle has allowed to stabilize for at least 15 min. The signal has been amplified, continuously recorded and analyzed by PowerLab System (ADInstruments Lab Chart 8.0). To confirm the possible involvement of PDEs in muscle hypercontractility, MHS bundles has been exposed to sildenafil (10 μmol/L) or rolipram (100 μmol/L) and the resting tension trend has been thoroughly observed for 20 min.

2.3. *Western blotting*

Muscle biopsies obtained from MHN (n = 5) and MHS (n = 5) patients have been homogenized in RIPA buffer as previously described [37]. Briefly, denatured proteins (40 μg) have been separated on 10% sodium dodecylsulphate polyacrylamide gels and transferred to polyvinylidene fluoride membrane (PVDF). Membranes have been blocked in phosphate-buffered saline containing 0.1% v/v Tween-20 (PBST) and 3% w/v non-fat dry milk for 45 min, followed by overnight incubation at 4 °C with rabbit polyclonal PDE4D (1:500; Santa Cruz Biotechnology) and PDE5 antibody (1:500; Elabscience). Membranes have beenextensively washed in PBST prior to incubation with horseradish-peroxidase conjugated secondary antibody for 2 h. Following incubation, membranes have been washed and developed using a ChemiDoc imaging system (BioRad, Italy). The target protein band intensity have been normalized over the intensity of the housekeeping protein Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:5000, Sigma-Aldrich, Milan, Italy).

2.4. *Cyclic nucleotides determination*

Biopsies obtained from MHN (n = 11–12) and MHS (n = 11–12) patients have been homogenized in 8 volumes of buffer containing 5% trichloroacetic acid per gram of tissue. cGMP and cAMP have been extracted and measured using a commercially available enzyme immunoassay kit (Cayman Chemical, Michigan, USA) following the manufacturer's instructions (5) [34].

2.5. *Primary human skeletal muscle cell culture*

Primary human skeletal muscle cells (PHSKMC) have been obtained by MHN biopsies according to Vellecco et al. [32]. In brief, PHSKMC have been cultured and grown in medium (DMEM with 4,5 g/L glucose) supplemented with 2 mmol/L L-glutamine, 20% heat-inactivated fetal bovine serum, 1% (v/v) antibiotics (penicillin/streptomycin and gentamycin), 7 mmol/L HEPES, 4 μg/ml insulin. For the experiments, the cells have been incubated for 72 hr with the differentiation medium, DMEM supplemented with 10% horse serum to promote the fusion of myogenic satellite cells into myotubes. Staining for myogenic markers i.e. myogenin, desmin and skeletal muscle-specific alpha-actin have been performed routinely to confirm population homogeneity. Following 72 hr, the cells have been treated with an excess of a well-known H_2S donor, NaHS 3 mmol/L, or its vehicle for 15 min, and then the pellets have been collected and used for cyclic nucleotide determination and S-Sulfhydration antibody array.

2.6. *Assay of PDE S-sulfhydration by antibody array*

Antibodies at a concentration of 1 μg/ml have been added to a 96 well plate (3D-NHS Surface, PolyAn, Berlin) at a final volume of 50 μl in PBS buffer (150 mmol/L Na_2HPO_4 / $NaHPO_4$ and 50 mmol/L NaCl, pH 8.5). As a negative control, 50 μl of 5% BSA in TBST (137 mmol/L NaCl and 20 mmol/L Tris base, pH 7.4, supplemented with 0.1% Tween) and containing 0.002% NaN_3 has been used. The plate has been covered and incubated at 4 °C overnight with agitation. The solutions have been discarded, and the wells have been washed 5 times with 1.5 x PBS buffer supplemented with 0.01% Tween. All wells then have been blocked with 50 mmol/L ethanolamine in 100 mmol/L Tris at pH 9 for 2 h at RT with agitation. The blocking solution has been discarded, and wells have been re-blocked with 5% BSA in TBS with 0.01% Tween-20 for a further 60 min at RT with agitation. Then samples have been lysed with RIPA buffer and incubated with Daz2:Cy5 click mix (1 mmol/L Daz2 Cayman Chemical, Michigan, USA), 1 mmol/L Cyanine5 alkyne (Lumiprobe GmBH, Hannover, Germany), 2 mmol/L copper(II)-TBTA complex (Lumiprobe) and 4 mmol/L ascorbic acid (Merck, Darmstadt, Germany) made in situ, have been added sequentially in 1.5 x PBS buffer mixed

with 30% (vol/vol) acetonitrile. The solution has been mixed at RT overnight and then quenched with 20 mmol/L ethylenediaminetetraacetic acid (EDTA) and mixed at RT for 2 h in PBS for 1 h at 37 °C. Samples (0.5 μg protein/ml in a total volume of 100 μl) have been added and incubated at 4 °C overnight with agitation in the 96 well plates. After washing, the plate has been recorded on a fluorescent microplate reader (Perkin Elmer, Rodgau, Germany) at 488 nm and 633 nm.

2.7. Reagents

All reagents for Krebs solution preparation have been purchased from Carlo Erba Reagents (Milan, Italy). Caffeine, Tris-HCl, Triton-X, sodium deoxycholate, sodium chloride, EDTA, protease inhibitor cocktail, sodium fluoride, dimethyl sulfoxide (DMSO), hydrogen sulfide (NaHS), Sildenafil and 3-Isobutyl-1-methylxanthine (IBMX) have been purchased from Sigma-Aldrich (Milan, Italy). Rolipram has been obtained from Tocris (Bristol, UK). Rolipram and IBMX have been dissolved in DMSO, all the other drugs in dH_2O.

2.8. Statistical analysis

Data are expressed as mean ± SEM and analyzed using Student's t-test (two groups), one-way ANOVA followed by Dunnet's for multiple comparison test (more than two groups). GraphPad Prism 8.0 software (San Diego, CA, USA) has been used for analysis. A P value < 0.05 has been considered significant.

3. Results

3.1. PDE4 and PDE5 activity is reduced in hyper-contractile (MHS) biopsies

PDE4 (subtypes A-B-C-D), PDE5, PDE7, and PDE11 are the most prominent PDE isoforms detected in human and murine skeletal muscle [16,38]. Given that selective inhibitors of PDE7 and PDE11 are not currently available, this study focused on PDE4 and PDE5, for which well-characterized inhibitors are usable. To evaluate whether MH was linked to an alteration in PDEs expression, the levels of PDE4 and PDE5 were determined by Western blotting analysis. As shown in Fig. 1A,B, no difference were found in enzymes expression between MHN (normal) and MHS (hyper-contractile) biopsies. To acquire further information on the possible altered activity of PDE4 and PDE5 between MHN and MHS, cyclic nucleotides content was determined. Both cAMP (as an index of PDE4 activity) and cGMP (as an index of PDE5 activity) levels were significantly higher in MHS compared to MHN samples. These observations imply that in hyper-contractile samples the catabolic activity of both PDE4 and PDE5 is decreased resulting in basal increased levels of cyclic nucleotides (Fig. 1C,D).

3.2. Endogenous S-sulfhydration of PDEs occurred in SKM and it is increased in hyper-contractile (MHS) biopsies

The *S*-sulfhydration of proteins by H_2S and related sulfane sulfur compounds impacts on protein function by affecting catalytic activity, protein folding and dimerization etc. [27,28,39]. Since it has been already shown that S-sulfhydration of PDEs, triggered by exogenous hydrogen sulfide, leads to an inhibition of the enzymatic activity [40], we have explored the possibility that PDEs S-sulfhydration could represent a regulatory mechanism of PDE activity within the SKM. By

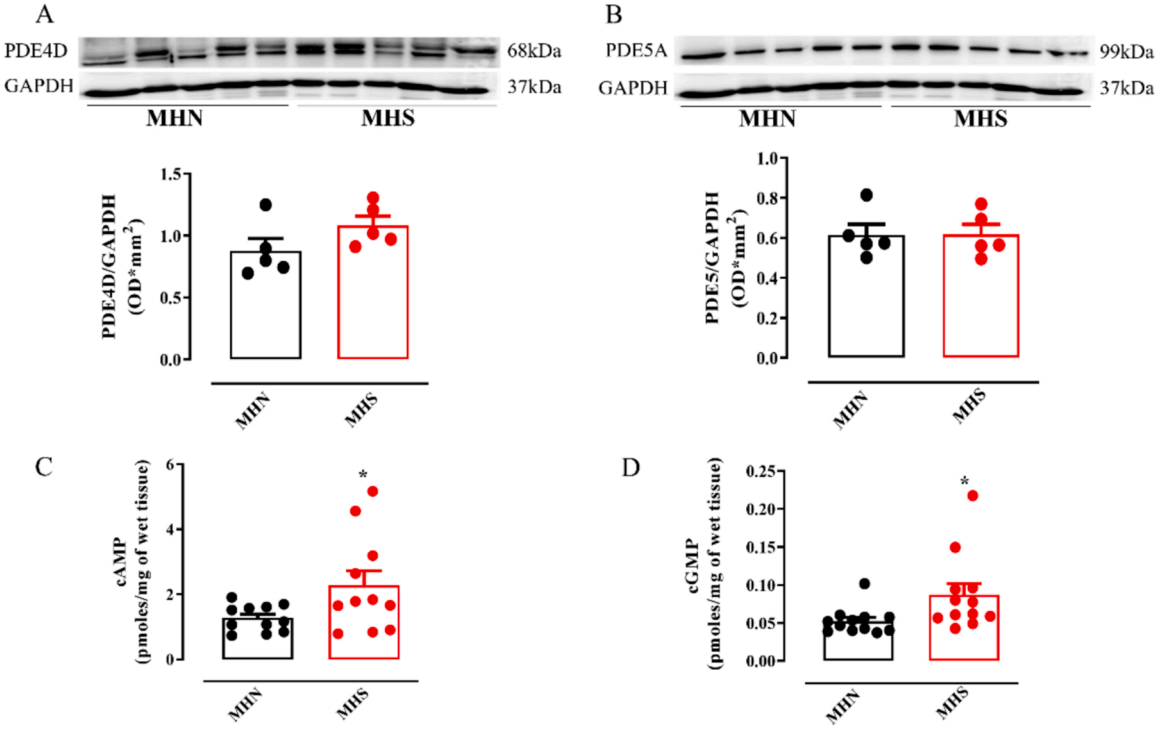

Fig. 1. In hyper-contractile skeletal muscle (MHS) cyclic nucleotides are increased without changes in PDE4 and PDE5 expressions. (A,B) Western Blot analysis reveals no significant difference in PDE4D and PDE5A expression between normal (MHN) and hyper-contractile (MHS) samples, the data refers to n = 5 biopsies for MHN and MHS groups, respectively. Values are normalized against GAPDH as a housekeeping protein, expressed as mean ± SEM and analyzed by Student's test. (C, D) Evaluation of cAMP and cGMP content in tissues homogenates shows a significant increase in hypercontractile (MHS) compared with normal (MHN) biopsies, n = 11 for cAMP and n = 12 for cGMP (*=$p < 0.05$ vs. MHN). Values are calculated as pmoles per mg of tissues, expressed as mean ± SEM and analyzed by Student's t test.

using the prediction software (Dianna 1.1), we have identified PDE1, PDE4, PDE5, PDE7, and PDE11 as containing L-cysteine residues potentially targeted by *S*-sulfhydration (Fig. 2A). To determine whether these PDEs could be *S*-sulfhydrated in SKM, both normal (MHN) and hyper-contractile (MHS) biopsies were subjected to an antibody array based on the dimedone switch method [31]. This approach revealed not only that PDE4, PDE5, PDE7 and PDE11 were S-sulfhydrated in all tissues tested, but also that the extent of modification was significantly greater in MHS samples compared to MHN (Fig. 2B). These results strongly suggest that i) H_2S-derived S-sulfhydration takes place physiologically in skeletal muscle modulating PDEs activity, ii) increased generation of H_2S resulted in enhanced S-Sulfhydration of at least four different PDE isoforms, as happens in hyper-contractile (MHS) samples.

3.3. *In primary human skeletal muscle cells (PHSKMC) derived from MHN biopsies, H_2S exposure increases PDEs S-sulfhydration coupled to an increased cyclic nucleotide content*

To further assess the role of PDE S-sulfhydration on cyclic nucleotides levels primary skeletal muscle cells obtained from MHN biopsies were exposed to the H_2S donor NaHS (3 mM for 15 min). Under these experimental conditions, the S-sulfhydration of PDE4, PDE5, PDE7, and PDE11 was enhanced compared to cells exposed to vehicle (H_2O) (Fig. 3A). Interestingly, the incubation of MHN-derived cells with NaHS led to an increase in both cAMP and cGMP levels (Fig. 3B). Taken together, these data strongly suggest that the S-sulfhydration of PDEs directly reduced their enzymatic activity.

3.4. *PDE4 and PDE5 inhibitors induce a contractile response in MHN biopsies exposed to NaHS*

To verify if PDE S-sulphydration participates to SKM contractility, we have performed the IVCT bioassay on normal muscle bundles (MHN) exposed to NaHS (3 mM for 5 min) to mimic the augmented levels of H_2S observed in hypercontractile (MHS) samples. This is an approach previously demonstrated to switches a negative MH response into a positive one in the IVCT bioassay [37] that resulted very helpful considering the difficulty to obtain human samples from individuals susceptible to this rare syndrome. Normal (MHN) muscle bundles were exposed to NaHS and then challenged, instead of the standard triggers (caffeine or halothane), with sildenafil (10 μM) or rolipram (100 μM), selective inhibitors of PDE5 and PDE4, respectively. Both drugs were used within the micromolar range considering that, in isolated skeletal muscle bioassay, the concentration used was 100-fold higher than IC50 value obtained in the cell-free assay (sildenafil IC50 = 3.5 nM, rolipram IC50 = 750 nM). As shown in Fig. 4, in this condition both sildenafil (Fig. 4A) and rolipram (Fig. 4D) led to an increase of resting tension over 0.2 g (the threshold of MH susceptibility diagnosis). The latter effects were similar in extent to those observed using hyper-contractile (MHS) biopsies. In the absence of NaHS, sildenafil and rolipram failed to increase tension (Fig. 4B, E for sildenafil and rolipram, respectively).

A

Protein	Residue
PDE1	Cys282
PDE4	Cys45, Cys46
PDE5	Cys310, Cys447
PDE7	Cys38
PDE11	Cys66

B

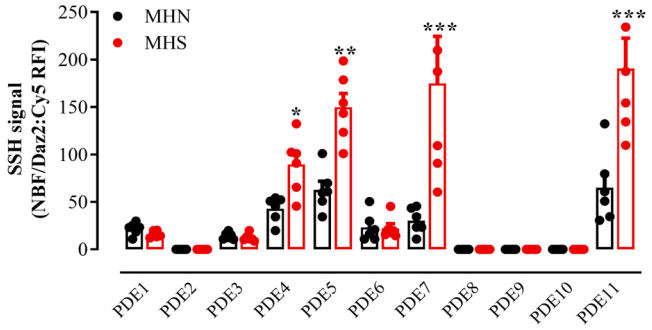

Fig. 2. S-sulfhydration of PDE4, PDE5, PDE7 and PDE11 is enhanced in hyper-contractile (MHS) SKM. (A) Predicted cysteine residues to be modified by oxidative post-translational modifications in the PDE family. (B) S-sulfhydration signal of specific PDE proteins expressed as NBF/DaZ2: Cy5 relative fluorescence signal (RFI) in normal (MHN) and hyper-contractile (MHS) biopsies. The data refers to n = 5–6 biopsies for MHN and MHS groups (*=$p < 0.05$; **=$p < 0.01$; ***=$p < 0.001$ vs. MHN). Values are expressed as mean ± SEM and analyzed by Student's t test.

3.5. *PDE4 and PDE5 selective inhibitors act as triggers for MH*

To confirm the role of PDEs/H_2S interaction in skeletal muscle function, the contraction of MHS biopsies challenged with sildenafil or rolipram was evaluated. As shown in Fig. 5, in hyper-contractile (MHS) biopsies, characterized by high levels of H_2S within SKM, both drugs elicite an increase of resting tension over 0.2 g (Fig. 5A, D for sildenafil and rolipram, respectively) mimicking IVCT response induced by either caffeine or halothane in MH susceptible patients. Conversely, no change was observed in normal (MHN) biopsies exposed to sildenafil (Fig. 5B) or rolipram (Fig. 5E). Even though the number of SKM positive biopsies obtained was limited, these results confirm the involvement of PDEs in the MH-related hypercontractility of skeletal muscle.

3.6. *PDEs inhibition switches a MHN response to a MHS one in IVCT*

To demonstrate that the negative modulation of PDEs activity is a biochemical event that actively participates in SKM contraction, normal (MHN) biopsies were incubated with the non-selective PDE3, PDE4 and PDE5 inhibitor 3-Isobutyl-1-methylxanthine (IBMX), and then challenged with caffeine, one of the two triggers used in IVCT procedure for MH diagnosis [26]. Like NaHS, IBMX per se did not exert any effect on baseline tension but it led to an increase in muscle contracture following caffeine addition (Fig. 6D). The contraction observed was similar in onset and shape to a typical hyper-contractile (MHS) response. When normal (MHN) samples were exposed to vehicle (DMSO) and challenged with caffeine, as expected from MHN biopsies, no change in baseline tension was observed (Fig. 6C). This result confirms that a condition of reduced PDEs activity is involved in the hypercontractility of SKM.

4. Discussion

Here, we have demonstrated that H_2S-derived S-sulfhydration of PDEs modulates skeletal muscle contractility. Taking advantage of the human skeletal muscle biopsies obtained from patients susceptible or negative to MH, we had an unique opportunity to evaluate the role of this PTM in presence of physiological (MHN) and pathological (MHS) levels of H_2S on human skeletal muscle contractility. Several authors have shown that in both cardiac and skeletal muscle cells, the increase of intracellular cAMP levels by β-adrenergic stimulation, leads to activation of canonical cAMP-dependent signal transduction, coupled to phosphorylation of type 1 voltage-gated L-type Ca^{2+} channel (Cav1.2/Cav1.1 α1 subunit), highly expressed on the transverse tubule (T-tubule). These events translated into an increased Ca^{2+} influx from RyR1 enhancing the force of contraction [41–44]. Similarly, it has been shown that in striated muscle fibers nNOS-derived NO, via the canonical sGC-cGMP-PKG signal transduction, phosphorylates regulatory myosin

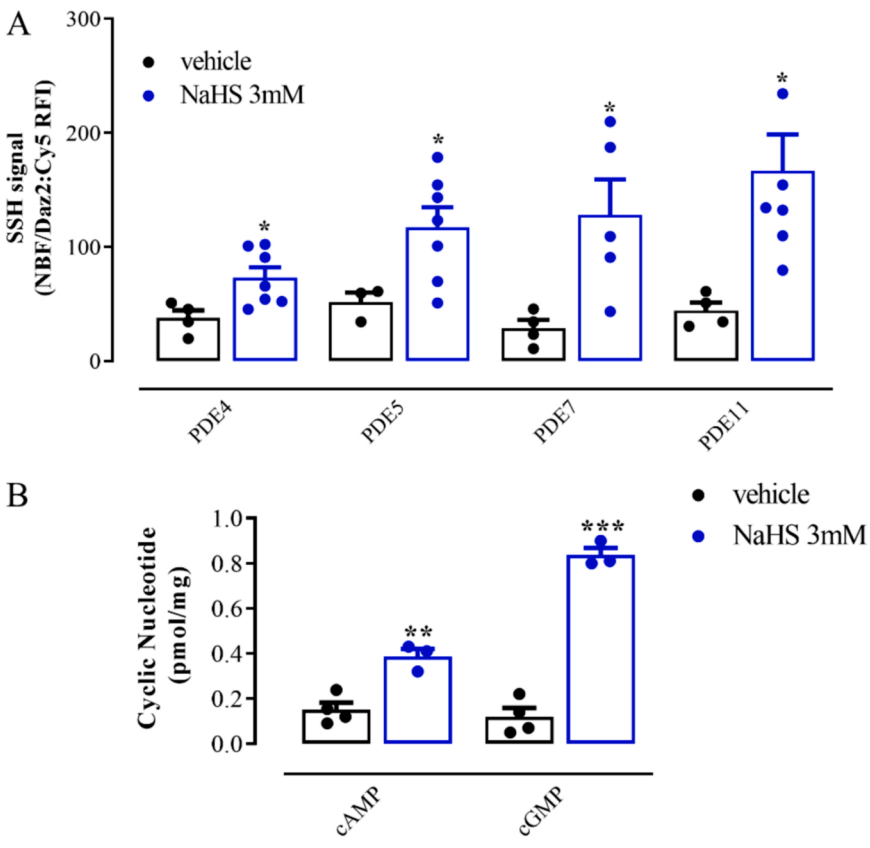

Fig. 3. In MHN -derived PHSKM cells pre-exposed to H_2S display increased S-sulfhydration of PDEs and augmented cyclic nucleotides levels. (A) S-sulfhydration signal of PDEs proteins expressed as NBF/DaZ2: Cy5 relative fluorescence signal (RFI) in MHN-derived PHSKM cells incubated with NaHS 3 mM for 15 min or vehicle (dH_2O). In MHN-derived PHSKMC, NaHS treatment S-sulphydrates PDE4, PDE5, PDE7 and PDE11 isoforms. The data refers to n = 5–7 biopsies-derived cells for NaHS and n = 3–4 biopsies-derived cells for vehicle (*=$p < 0.05$ vs. vehicle). Values are expressed as mean ± SEM and analyzed by Student's t test. (B) cAMP and cGMP assay reveals a significant increase in nucleotides content in MHN-derived PHSKM cells treated with NaHS 3 mM for 15 min, the data refers to n = 3 for NaHS and n = 4 for vehicle (**=$p < 0.01$, ***=$p < 0.001$ vs. vehicle). Values are calculated as pmol per mg of protein, expressed as mean ± SEM and analyzed by Student's t test.

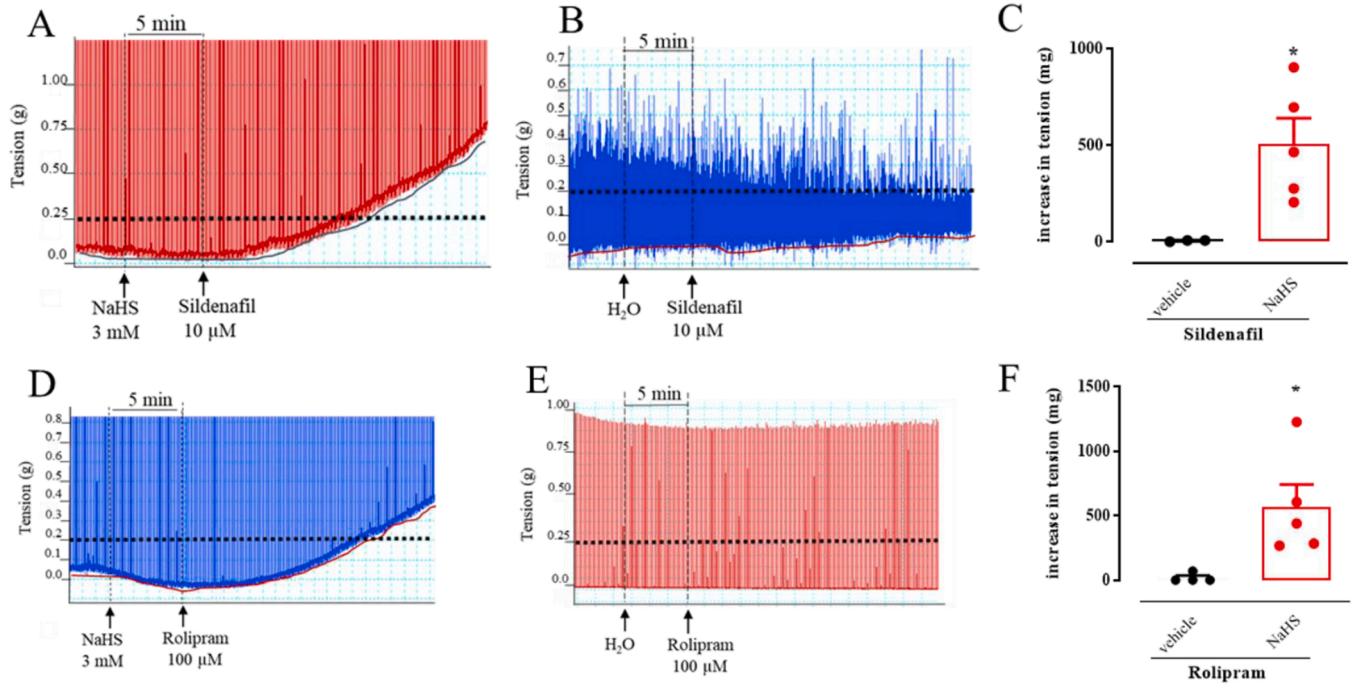

Fig. 4. Normal (MHN) SKM pre-exposed to NaHS display a hyper-contractile (MHS) like response to PDE4 and PDE5 selective inhibitors. Representative original traces of skeletal muscle bundle obtained from normal (MHN) biopsies incubated for 5 min with NaHS (3 mM) followed by (A) sildenafil (10 µM) or (D) rolipram (100 µM) stimulation. The increase in tension achieved exceeds the threshold of 0.2 g above the resting tension as observed in hyper-contractile (MHS) biopsies. Representative original traces of skeletal muscle bundle obtained from normal (MHN) biopsies incubated for 5 min with vehicle (dH_2O) followed by (B) sildenafil (10 µM) or (E) rolipram (100 µM) addition. No increase in tension has been observed. Effect of NaHS (3 mM) or vehicle (dH_2O) pre-incubation followed by (C) sildenafil or (F) rolipram addition in normal (MHN) biopsies. The data refers to n = 5 biopsies for NaHS and n = 3–4 biopsies for vehicle (*=$p < 0.05$ vs. vehicle). Values are calculated as increase in tension, expressed as mean ± SEM and analyzed by Student's t test.

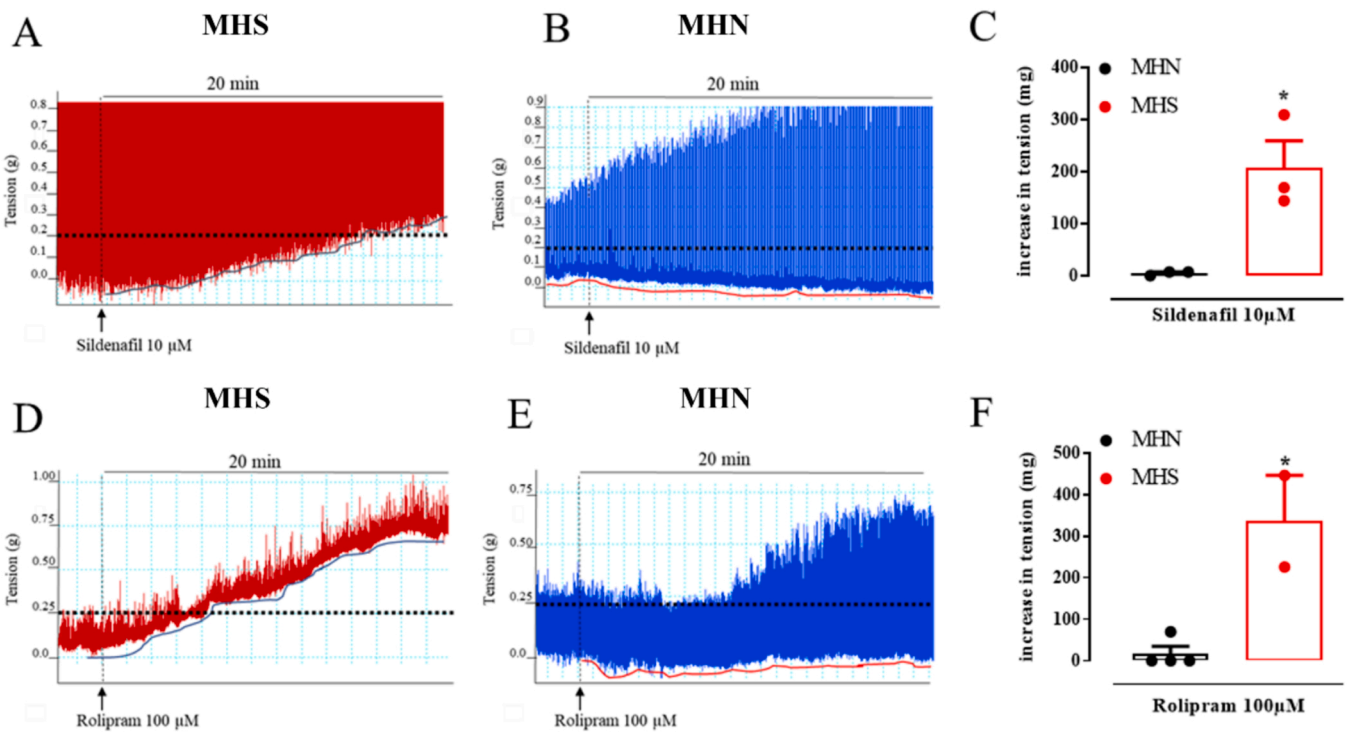

Fig. 5. PDE5 and PDE4 inhibitors trigger a contractile response in hyper-contractile (MHS) SKM. Representative original traces of (A) hypercontractile (MHS) and (B) normal (MHN) biopsies incubated with sildenafil for 20 min. (C) Sildenafil increases the contracture in hyper-contractile (MHS) bundles over the threshold of 0.2 g, the value for MH susceptibility diagnosis. The data refers to n = 3 biopsies for MHN and MHS groups (*=p < 0.05 vs. MHN). Representative original trace of (D) hyper-contractile (MHS) and (E) normal (MHN) biopsies incubated with rolipram for 20 min. (F) Rolipram increases the contracture in hyper-contractile (MHS) biopsies over the threshold of 0.2 g, the value for MH susceptibility diagnosis. The data refers to n = 4 biopsies for normal (MHN) and n = 2 biopsies for hyper-contractile (MHS) (*=p < 0.05 vs. MHN). Values are expressed as mean ± SEM and analyzed by Student's t test.

light chain increasing Ca^{2+} sensitivity leading to an enhanced muscle contractile function [45,46]. Despite it has been demonstrated a beneficial effect of PDEs inhibitors in different pathological conditions involving SKM, not much is known on the role exerted by cAMP-cGMP/PDEs axis in human SKM contractility. In the relevant literature regarding MH syndrome, there are some earlier evidence showing the presence of higher basal levels of cAMP in the skeletal muscles of MHS swine [47,48]. This observation led the authors to hypothesize that skeletal muscle hypercontractility related to MH syndrome could involve enhanced signaling related to cyclic nucleotides as second messengers, but no further investigation so far has been undertaken. Our study, on human tissue, clearly shows that cAMP and cGMP levels are almost doubled in hyper-contractile (MHS) biopsies as compared to normal (MHN) biopsies confirming the previous evidence and suggesting a role for PDEs in muscle contractility.

By hydrolyzing cAMP and cGMP, PDEs activity tightly and continuously regulates their concentration within the physiological range [3]. The anomalous high concentrations of both cyclic nucleotides found in MHS biopsies let us hypothesize a reduced activity of PDEs within SKM of MH susceptible patients. This condition leads to an increased basal level of cyclic nucleotides that puts at risk of hypercontractility when further PDEs inhibition occurs. Such hypothesis has been supported by the bioassay performed on hyper-contractile (MHS) samples. Indeed, both sildenafil and rolipram, the selective PDE5 and PDE4 inhibitors, induce an increase of tension over the physiological threshold (0.2 g) that allows diagnosis of MH susceptibility. Unfortunately, the data presented in Fig. 5D should be considered preliminary since relies on two MH-positive patients. This is due to the stop of the diagnostic IVCT procedure for the Covid-19 pandemic since March 2020.To confirm that augmented levels of H_2S, by inhibiting PDEs activity, triggers a MHS-like response when PDEs are further inhibited, normal (MHN) samples have been incubated with NaHS and then exposed to sildenafil and rolipram. In these experimental conditions, NaHS pre-treatment leads to an increase of muscle contracture following both drugs exposure similar in onset and shape to a typical MHS response, i.e. over 0.2 g increase from baseline tension. When MHN biopsies were exposed to vehicle (H_2O), used as a control for NaHS, and challenged with PDE inhibitors, no change in baseline tension was recorded. This finding not only confirms the role of PDEs/cyclic nucleotide axis in skeletal muscle contraction but also may suggest the existence of a sort of threshold in cyclic nucleotide content beyond which a paroxysmal contraction occurs. Fiege and co-authors have already proposed the involvement of PDEs in hyper-contractility of MHS skeletal muscle; indeed in two different studies, performed on skeletal muscle specimens from human and swine susceptible to MH [49,50] they demonstrated that cumulative administration in the millimolar range of PDE3 inhibitor enoximone on hyper-contractile (MHS) biopsies induced an increase in tension at the same extent of the classical triggers. The selectivity of enoximone for PDE3 vs other PDEs is within the micromolar range (enoximone IC50 = 5.9 microM; [51]). Considering the higher concentrations used (within millimolar range) also PDE4 and PDE5 are affected by enoximone inhibitory action [52].

We have previously demonstrated that MHS biopsies display increased levels of CBS-derived H_2S that contributes to hyper-contractility [19]. More recently, we have shown that in MHS biopsies S-sulfhydration of voltage-dependent potassium channels 7 (Kv7) occurs and it accounts for the anomalous contractility [32]. To address the hypothesis that high levels of H_2S detected in MHS patients could also affect PDEs activity by post-translational mechanism, we evaluated PDEs S-sulfhydration in normal (MHN) and hyper-contractile (MHS). The main PDE isoforms expressed in SKM (PDE4, PDE5, PDE7, PDE11) are all S-sulfhydrated in both specimens strongly suggesting that S-sulfhydration of PDEs is a physiological PTM contributing to SKM contractility. Interestingly, the extent of S-sulfhydration is significantly

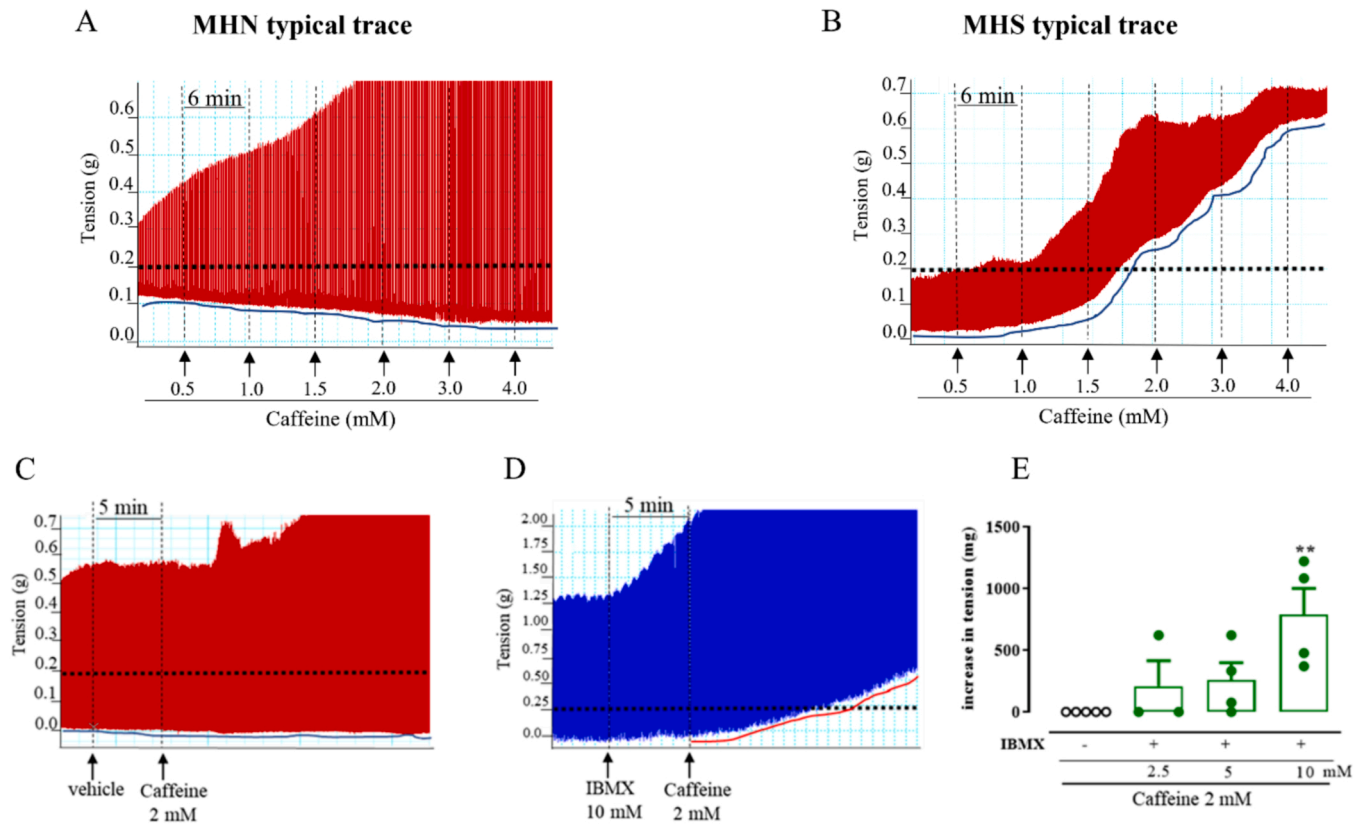

Fig. 6. Normal (MHN) SKM pre-incubated with IBMX display a hyper-contractile (MHS)-like response to caffeine. (A) Representative original trace of normal (MHN) SKM biopsy following caffeine exposure in IVCT procedure. After 20 min of muscle bundle stabilization, caffeine has been added to the organ bath with a flow of 5 ml/min at progressive concentrations of 0.5, 1.0, 1.5, 2.0, 3.0 and 4.0 mM (6 min of contact for each concentration). The absence of any contracture from the resting tension provides a MHN diagnosis for caffeine. (B) Representative original trace of bundle obtained from hyper-contractile SKM biopsy following caffeine exposure. After 20 min of muscle bundle stabilization, caffeine has been 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 and 4.0 mM (6 min of contact for each concentration). The progressive increase in resting tension above 0.2 g provides a MHS diagnosis for caffeine. Representative original trace of normal (MHN) biopsies incubated for 5 min with (C) vehicle or (D) IBMX followed by caffeine stimulation (2 mM). The increase in tension achieved exceeds the threshold of 0.2 g above the resting tension as observed in hypercontractile (MHS) biopsies. (E) Effect of IBMX (2.5, 5 and 10 mM) or vehicle (DMSO) incubation in normal (MHN) biopsies followed by caffeine stimulation, the data refers to n = 3–4 biopsies for IBMX and n = 5 biopsies for vehicle (***=$p < 0.001$ vs. vehicle). Values are calculated as increase in tension, expressed as mean ± SEM and analyzed using one-way ANOVA followed by Dunnnet's for multiple comparison test.

greater in MHS compared to MHN and this event is coupled to an increase of cyclic nucleotides levels. These findings imply that S-sulfhidration inhibits PDEs activity . The reduction of PDE enzymatic activity due to S-sulfhidration has also been hypothesized by Sun and co-authors [40]. Indeed, they show that NaHS significantly enhances the PDE5A S-sulfhydration in rat aorta leading to a significant reduction of 5′-GMP. Considering the difficulty to obtain human samples from individuals susceptible to this rare syndrome, we have developed and characterized the primary human skeletal muscle cells starting from both normal (MHN) and hyper-contractile(MHS) biopsies [32]. Here, we confirm that PDEs S-sulfhydration negatively regulates PDEs activity in primary human SKM cells derived from normal (MHN) biopsies. In fact, the exposure of these human cells to H_2S donor induces further S-sulfhydration of PDE4, PDE5, PDE7 and PDE11 isoforms, coupled to an increase of both cAMP and cGMP levels. We have previously demonstrated and clarified that the millimolar concentration of NaHS affects neither SKM biopsies nor primary cell culture-derived SKM vitality and function [32,37]. In addition, it is well known that in SKM in vitro studies the concentration of drugs used is very high, even the MH diagnostic test IVCT, widely accepted worldwide, requires drugs concentrations up to 20 mM. The final demonstration of our hypothesis comes from the finding that incubation of MHN with IBMX, a non-selective inhibitor of PDEs, switches a negative response (no contraction observed) to that elicited by MHS (contraction over 0.2 g) confirming reduced activity of PDEs as a molecular mechanism involved in hypercontractility of SKM. This result reinforces the concept that PDEs activity participates in skeletal muscle contraction by regulating cyclic nucleotide content.

In conclusion, the hypercontractility observed in MHS samples relies on a constitutively "slowed down" metabolic activity of PDEs. This condition is sustained by high levels of CBS-derived H_2S production within the SKM that in turn operates S-sulfhydration of PDEs as post-translational regulatory mechanism with consequent increase of cAMP and cGMP. In hyper-contractile (MHS) biopsies further inhibition of PDEs, by raising both cAMP and cGMP, leads to the sustained muscle contraction typical of MH. These data unveil the role of PDEs activity and its modulation by H_2S, through S-sulfhydration, in physiological skeletal muscle contractility adding a new molecular mechanism in the complex and still not fully clarified phenomenon of skeletal muscle contraction.

Founding sources

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CRediT authorship contribution statement

V.V., O.L.M., M.R.C, and R.V. conceived and performed the in vitro experiments and analyzed the data. F.M., F.R., and G.M.C. performed cell culture, western blot experiments and analyzed the data. E.P and M. S performed RT-PCR experiments and analyzed the data. S.I.B. and I.F. performed the S-sulfhydration experiments and analyzed the data. M.B., V.V. and E.P. conceived, designed the study M.B. and R.D.V.B. coordinated all the data. M.B. and G.C. wrote and revised the manuscript.

Declaration of interest

None.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phrs.2022.106108.

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