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L-Cys/CSE/H_2S pathway modulates mouse uterus motility and sildenafil effect

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ABSTRACT

Sildenafil, a selective phosphodiesterase type 5 (PDE5) inhibitor, commonly used in the oral treatment for erectile dysfunction, relaxes smooth muscle of human bladder through the activation of hydrogen sulfide (H_2S) signaling. H_2S is an endogenous gaseous transmitter with myorelaxant properties predominantly formed from L-cysteine (L-Cys) by cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE). Sildenafil also relaxes rat and human myometrium during preterm labor but the underlying mechanism is still unclear. In the present study we investigated the possible involvement of H_2S as a mediator of sildenafil-induced effect in uterine mouse contractility. We firstly demonstrated that both enzymes, CBS and CSE were expressed, and able to convert L-Cys into H_2S in mouse uterus. Thereafter, sildenafil significantly increased H_2S production in mouse uterus and this effect was abrogated by CBS or CSE inhibition. In parallel, L-Cys, sodium hydrogen sulfide or sildenafil but not D-Cys reduced spontaneous uterus contractility in a functional study. The blockage of CBS and CSE reduced this latter effect even if a major role for CSE than CBS was observed. This data was strongly confirmed by using $CSE^{-/-}$ mice. Indeed, the increase in H_2S production mediated by L-Cys or by sildenafil was not found in $CSE^{-/-}$ mice. Besides, the effect of H_2S or sildenafil on spontaneous contractility was reduced in $CSE^{-/-}$ mice. A decisive proof for the involvement of H_2S signaling in sildenafil effect in mice uterus was given by the measurement of cGMP. Sildenafil increased cGMP level that was significantly reduced by CSE inhibition. In conclusion, L-Cys/CSE/H_2S signaling modulates the mouse uterus motility and the sildenafil effect. Therefore the study may open different therapeutical approaches for the management of the uterus abnormal contractility disorders.

1. Introduction

The uterus is a myogenic organ, able to produce regular spontaneous contraction without nervous or hormonal input [1]. Spontaneous contractions of the uterus must be strictly controlled and coordinated for the success of various reproductive functions, i.e. to facilitate the journey of sperms to the fallopian tubes or to expel the shed inner lining of the uterus during menstruation [2]. The non pregnant uterus activity is at the highest level during estrus [3]. The abnormal contractility represents the most common symptom of disorders such as dysmenorrheal and endometriosis. Although these conditions may not contribute to mortality, they are debilitating clinical disorders that can significantly affect patients' quality of life and reproductive health. Therefore, new tocolytic agents could represent an important approach for the treatment of these disorders. Despite considerable advances in the knowledge of myometrial physiology, the mechanisms by which the non-pregnant uterus autonomously initiates spontaneous contractions remain speculative and poorly understood. Nonetheless, it is well recognized that nitric oxide (NO) participates to the regulation of uterine homeostasis. Soluble guanylyl cyclase is an NO-specific target in the myometrium, generating 3′5′cyclic guanosine monophosphate (cGMP) [4,5]. Several evidence report the importance of NO/cGMP pathway in the modulation of uterine motility [6,7]. Phosphodiesterases (PDEs) are responsible for the

Abbreviations: PDE5, phosphodiesterase type 5; H_2S, hydrogen sulfide; L-Cys, L-Cysteine; CBS, cystathionine-β-synthase; CSE, cystathionine-γ-lyase; PAG, DL-propargilglycine; AOAA, aminooxiacetic acid; E_{max}, maximum effect.

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breakdown of cGMP in both males and females and offer potential selective therapeutic value [8–10]. Sildenafil is a selective PDE5 inhibitor that prevents cGMP degradation and promotes smooth muscle relaxation [11,12]. Sildenafil, is commonly used in the oral treatment for erectile dysfunction [13,14] but a potential role of sildenafil as tocolytic agent in treatment of preterm labor has been suggested [6,15]. It has been recently demonstrated that sildenafil relaxes human bladder through the contribute of hydrogen sulfide (H_2S) pathway [16]. H_2S, proposed as the third endogenous gaseous transmitter along with NO and carbon monoxide (CO), is endogenously produced from the aminoacid L-cysteine (L-Cys) principally through the activation of two pyridoxal-5-phosphate-dependent enzymes i.e. cystathionine-β-synthase (CBS) and cysthationine-γ-lyase (CSE) [17,18]. Both CBS and CSE are expressed in rat and human myometrium tissues and rat uterus homogenate is able to produce H_2S [19]. Here we have investigated the effect of sildenafil and the role of L-Cys/H_2S pathway in spontaneous uterus contractility in mice.

2. Materials and methods

2.1. Tissue preparation

All animal care and experimental procedures in this study followed specific guidelines of the Italian and the European Council law for animal care. These procedures were also approved by the Animal Ethics Committee of the University of Naples "Federico II" (Italy). Virgin female mice, (Charles River, Italy, 22–25 g) and CSE-ablated mice ($CSE^{-/-}$) were used. Animals were kept at temperatures of 23 ± 2 °C, humidity range 40–70% and 12 h light/dark cycles. Food and water were provided *ad libitum*. During the estrus period, mice were anesthetized with isoflurane and euthanized. Uteri were cleaned of fat and connective tissue and placed in dish containing Krebs' solution with the following composition (mM): 115.3 NaCl; 4.9 KCl; 1.46 $CaCl_2$; 1.2 $MgSO_4$; 1.2 KH_2PO_4; 25.0 $NaHCO_3$; 11.1 glucose (Carlo Erba, Milan, Italy).

2.2. Western blot analysis

Western blot was performed as previously described [20]. Briefly, uteri harvested from CTR mice or $CSE^{-/-}$ mice were homogenized in modified RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM EDTA, 1% Igepal) (Roche Applied Science, Italy) and protease inhibitor cocktail (Sigma-Aldrich, USA). Protein concentration was determined by Bradford assay using albumin (BSA) as standard (Sigma-Aldrich, USA). Denatured proteins (50 μg) were separated on 8% sodium dodecyl sulfate polyacrylamide gels and transferred to a polyvinylidene fluoride membrane. The membranes were blocked by incubation in PBS containing 0.1% v/v Tween 20 and 5% non-fat dried milk for 1 h at room temperature and then incubated with mouse monoclonal antibody for CSE (1:1000; Abnova, Milan, Italy) or rabbit polyclonal for CBS (1:1000; Santa Cruz Biotechnology, Inc.) or rabbit polyclonal for PDE5 (Santa Cruz Biotechnology, Inc.), overnight at 4 °C. Membranes were extensively washed in PBS containing 0.1% v/v Tween-20 prior to incubation with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. Following incubation, membranes were washed and developed using ImageQuant-400 (GE Healthcare, USA). The target protein band intensity was normalized over the intensity of the housekeeping protein ß-actin (1:5000, Sigma-Aldrich, Milan, Italy). Densitometric evaluations were expressed as mean ± standard error of the mean (SEM) (n = 3) and analyzed by one-way ANOVA followed by Bonferroni post-test. A p value < 0.05 was considered significant.

2.3. H_2S determination

Uteri were incubated with vehicle (saline, 0.9% sodium chloride), L-Cys (10 μM) or sildenafil (0.1 μM–10 μM) for 15 min. In order to evaluate the involvement of H_2S pathway the experiments were repeated in presence of DL-propargilglycine (PAG, 10 mM) or aminooxiacetic acid (AOAA, 1 mM) (both inhibitors were added 30 min before challenge) and in tissues harvested from $CSE^{-/-}$ mice. H_2S production was measured in mouse uteri according to d'Emmanuele di Villa Bianca et al. [21]. Briefly, samples were lysed in an appropriate buffer (potassium phosphate buffer 100 mM, pH 7.4, sodium orthovanadate 10 mM, and proteases inhibitors). Protein concentration was determined by using Bradford assay (Bio-Rad Laboratories). Homogenates were added to a reaction mixture containing pyridoxal-5′-phosphate (2 mM), L-Cys (10 mM). The reaction was performed in sealed Eppendorf tubes and initiated by transferring tubes from ice to a water bath at 37 °C for 30 min. Next, trichloroacetic acid solution (TCA, 10% wt/vol) was added to each sample followed by zinc acetate (1% wt/vol). Subsequently, *N*,*N*-dimethyl-*p*-phenylendiamine sulfate (DPD; 20 mM) in HCl (7.2 M) and $FeCl_3$ (30 mM) in HCl (1.2 M) were added, and optical absorbance of the solutions was measured after 20 min at a wavelength of 668 nm. All samples were assayed in duplicate, and H_2S concentrations were calculated against a calibration curve of NaHS (3–250 μM). Data was expressed as mean ± SEM (n = 6) and analyzed by one-way ANOVA followed by Bonferroni post-test. A p value < 0.05 was considered significant.

2.4. Organ bath studies

The uterus was divided into two horns. Each horn was cross cut into two pieces and mounted in organ bath containing oxygenated (95% O_2 and 5% CO_2) Krebs' solution at 37 °C. Tissues were connected to isometric transducers (7006, Ugo Basile, Comerio, Italy) and changes in tension were continuously recorded with a computerized system (DataCapsule-17400, Ugo Basile, Comerio, Italy). Tissues were preloaded with 0.3 g of tension and allowed to equilibrate for 30 min, to reach an homogeneous spontaneous contractility. After equilibration, a concentration-response curve of L-Cys, NaHS or D-Cys (100 nM–300 μM) as well as sildenafil (0.1 nM–3 μM) was obtained. To assess the involvement of H_2S pathway, we performed L-Cys and sildenafil curve in presence of both PAG (10 mM) or AOAA (1 mM) as inhibitors of CSE and CBS, respectively. L-Cys and sildenafil induced effect was also evaluated on uterine horns harvested form $CSE^{-/-}$ mice. Data was calculated as frequency (%) of spontaneous motility and expressed as the mean ± SEM (n = 8–10). The results were analyzed by using analysis of variance (ANOVA) followed by Bonferroni post hoc test. A p value < 0.05 was considered significant.

2.5. cGMP measurement

In order to measure cGMP content, uteri were incubated with vehicle or sildenafil (1 μM) for 15 min in CTR mice. To assess the involvement of H_2S pathway the samples harvested from CTR mice were pre-treated for 30 min with PAG (10 mM) and then challenged with sildenafil. The uteri were dropped into 5–10 vol (ml of buffer/g of tissue) of TCA (5%) and homogenized by using a polytron-type homogenizer. Samples were centrifuged at 1500*g* for 10 min and cGMP was measured in supernatants as described in the manufactures protocol of cGMP EIA Kit (Cayman, Vinci Biochem, Vinci, Italy) [22,23]. All samples were assayed in duplicate and cGMP concentrations were calculated against a calibration curve of standard cGMP. Data was expressed as mean ± SEM (n = 6) and analyzed by

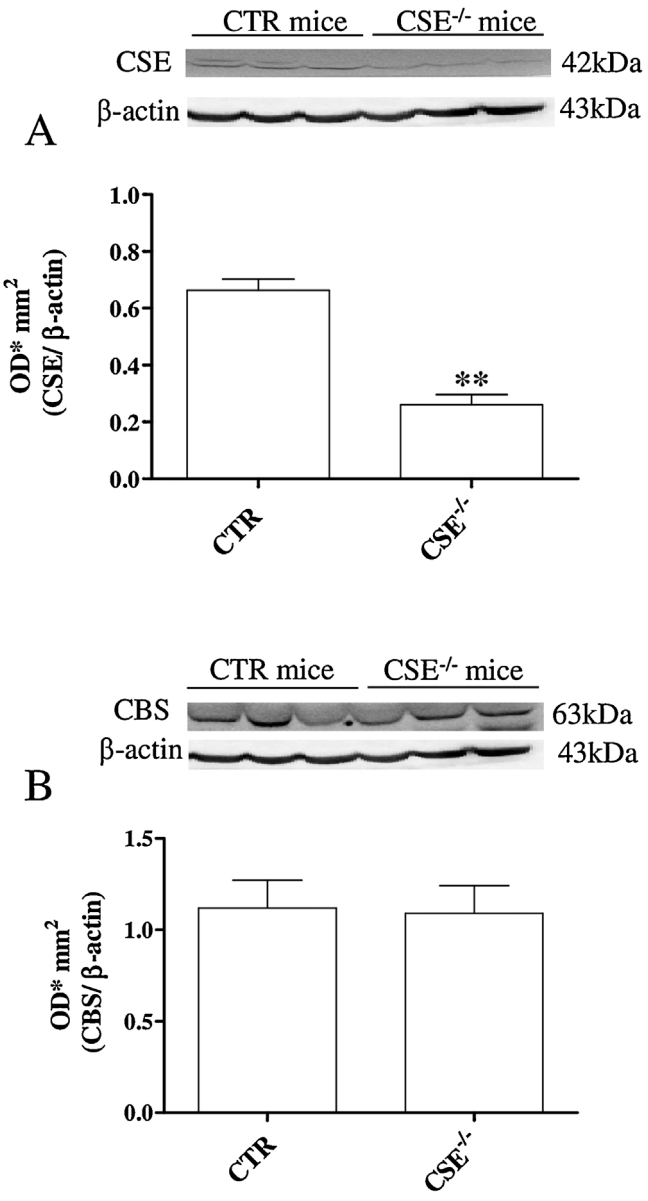

Fig. 1. Representative western blot and optical densitometric (OD) analysis for CSE and CBS in mouse uterus. (A) CSE expression is markedly reduced in CSE$^{-/-}$ mice compared to CTR mice (**p < 0.01). (B) CBS is similarly expressed in CTR and in CSE$^{-/-}$ mice. Results are normalized against β-actin as a housekeeping protein, expressed as mean ± SEM (n = 3) and analyzed by Student's *t*-test.

one-way ANOVA followed by Bonferroni post-test. A p value < 0.05 was considered significant.

3. Results

3.1. Expression of CBS and CSE in CTR and CSE$^{-/-}$ mouse uterus

Western blot analysis clearly shows that mouse uterus expresses both CSE and CBS (Fig. 1A, B). As expected, CSE expression is significantly reduced in CSE$^{-/-}$ mice compared to CTR mice (**p < 0.01; Fig. 1A). The expression of CBS is similar in both CTR and in CSE$^{-/-}$ mice (Fig. 1B).

3.2. H_2S production in CTR and CSE$^{-/-}$ mouse uterus

Uterus produces a similar detectable amount of H_2S in CTR and CSE$^{-/-}$ mice, in basal condition (Fig. 2A). The biosynthesis of H_2S

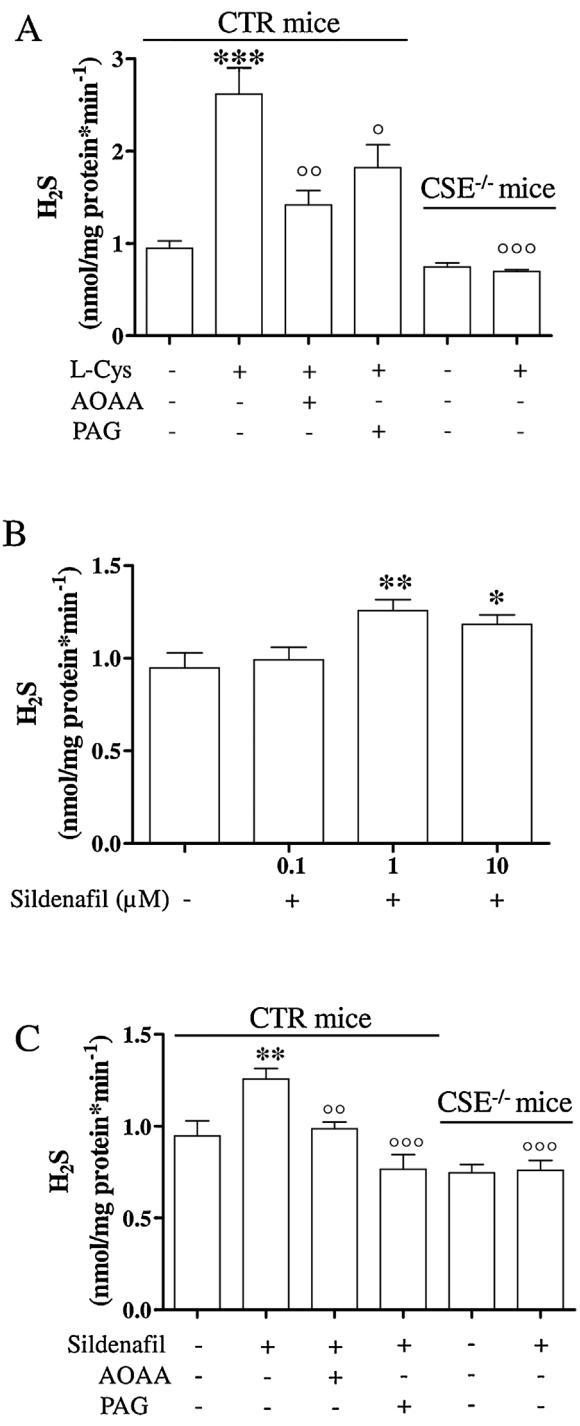

Fig. 2. H_2S production in uterus in CTR and CSE$^{-/-}$ mice. (A) L-Cys markedly enhances H_2S production in CTR mice (***p < 0.001). Both PAG (10 mM) and AOAA (1 mM) inhibit the increase in H_2S production induced by L-Cys (°p < 0.05 and °°p < 0.01). L-Cys does not affect H_2S production in CSE$^{-/-}$ mice compared to basal value. (B) Sildenafil incubation significantly increases H_2S production compared to vehicle in CTR mice (*p < 0.05 and **p < 0.01 vs vehicle). (C) CBS or CSE inhibitors (AOAA 1 mM or PAG 10 mM, respectively) notably reduces sildenafil-induced H_2S production (°°p < 0.05 and °°°p < 0.001 vs sildenafil 1 μM; **p < 0.01 vs vehicle). Results, calculated as nanomoles per milligram of protein per minute, are expressed as mean ± SEM (n = 6) and analyzed by one-way ANOVA followed by Bonferroni post-test.

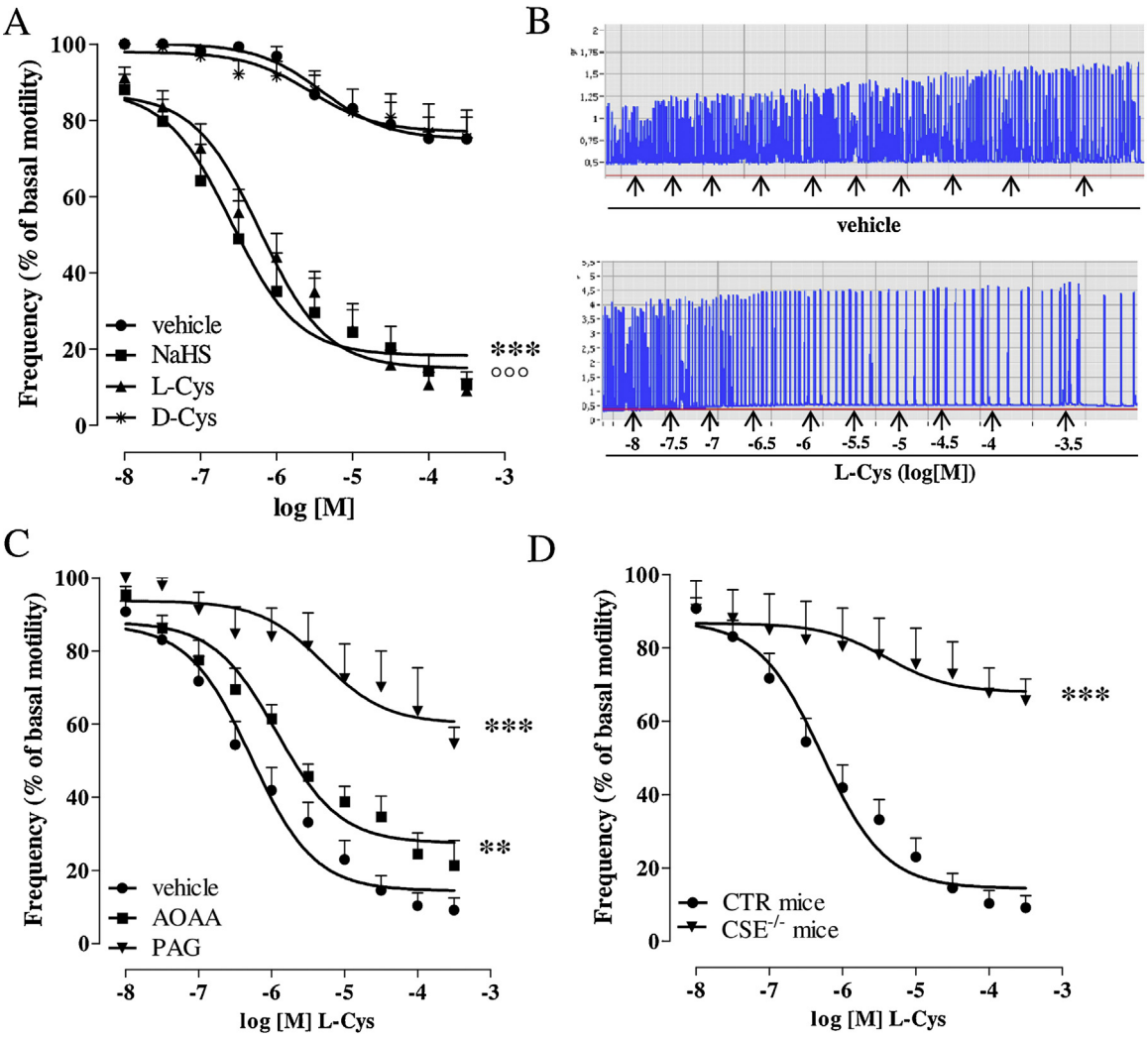

Fig. 3. Effect of H_2S on uterine spontaneous contractility in CTR and $CSE^{-/-}$ mice. (A) Both NaHS and L-Cys significantly reduce uterine spontaneous contractility in CTR mice (***,°°°$p < 0.001$). D-Cys does not affect uterine motility. (B) A typical trace of uterine spontaneous contractility, in presence of vehicle or of L-Cys in CTR mice. (C) Both AOAA (1 mM) and PAG (10 mM) significantly inhibit L-Cys-induced effect (**$p < 0.01$; ***$p < 0.001$). (D) The effect of L-Cys is notably reduced in $CSE^{-/-}$ mice compared to CTR mice (***$p < 0.001$). Results are expressed as frequency (% of the basal motility) and calculated as mean ± SEM (n = 8–10). Results were analyzed using analysis of variance (ANOVA) followed by Bonferroni post hoc test.

is significantly increased by 3-fold over basal values after incubation with L-Cys in CTR mice uterus (***$p < 0.001$; Fig. 1A). AOAA (1 mM) or PAG (10 mM) significantly inhibits the increase in H_2S production induced by L-Cys in CTR mice (°$p < 0.05$ and °°$p < 0.01$, respectively; Fig. 2A). Of note, L-Cys does not affect H_2S production in $CSE^{-/-}$ mice and this value is notably lower compared to CTR mice (°°°$p < 0.01$: Fig. 2A). Sildenafil incubation considerably increases H_2S production in CTR mice (*$p < 0.05$ and **$p < 0.01$; Fig. 2B), reaching the maximum effect at 1 μM. Interestingly, the inhibition of either CSE or CBS significantly reduces sildenafil-induced H_2S production (°°$p < 0.01$ and °°°$p < 0.001$, respectively; Fig. 2C). Remarkably, sildenafil does not affect H_2S levels in $CSE^{-/-}$ mice that are significantly lower compared to CTR tissues (°°°$p < 0.001$; Fig. 2C).

3.3. H_2S modulates uterus spontaneous contractility

In order to evaluate the contribution of H_2S pathway in spontaneous contractility of mouse uterus, a concentration–response curve of vehicle, NaHS, L-Cys or D-Cys (10 nM–300 μM) has been performed (Fig. 3A). D-Cys, similarly to vehicle, does not affect uterus motility. Conversely, NaHS as well as L-Cys challenge, significantly reduces uterus motility (***,°°°$p < 0.001$; Fig. 3A) in a concentration-dependent fashion, with a similar profile. AOAA (1 mM) and even more PAG (10 mM) markedly reduce L-Cys-induced effect (**$p < 0.01$; ***$p < 0.001$; Fig. 3C), indicating a predominant contribute of CSE in the control of uterus spontaneous motility. To confirm this point, a concentration-response curve of L-Cys (10 nM–300 μM) has been performed also on uterus harvested from $CSE^{-/-}$ mice.

After equilibration the spontaneous motility of CTR and $CSE^{-/-}$ uteri was 1.812 ± 0.182 and 1.906 ± 0.305 contraction/min, respectively, indicating no difference in frequency of uterus contraction in $CSE^{-/-}$ compared to CTR mice. Of note, the deletion of CSE significantly reduced L-Cys-induced effect observed in CTR mice (***$p < 0.001$; Fig. 3D).

3.4. Sildenafil reduces uterus spontaneous contractility through H_2S pathway activation

Sildenafil (0.1nM–3 μM) significantly reduces uterus spontaneous motility (***$p < 0.001$; Fig. 4A) in CTR mice. Interestingly, PAG (10 mM) significantly reduces sildenafil-induced effect (***$p < 0.001$; Fig. 4C). AOAA (1 mM) treatment causes a weak

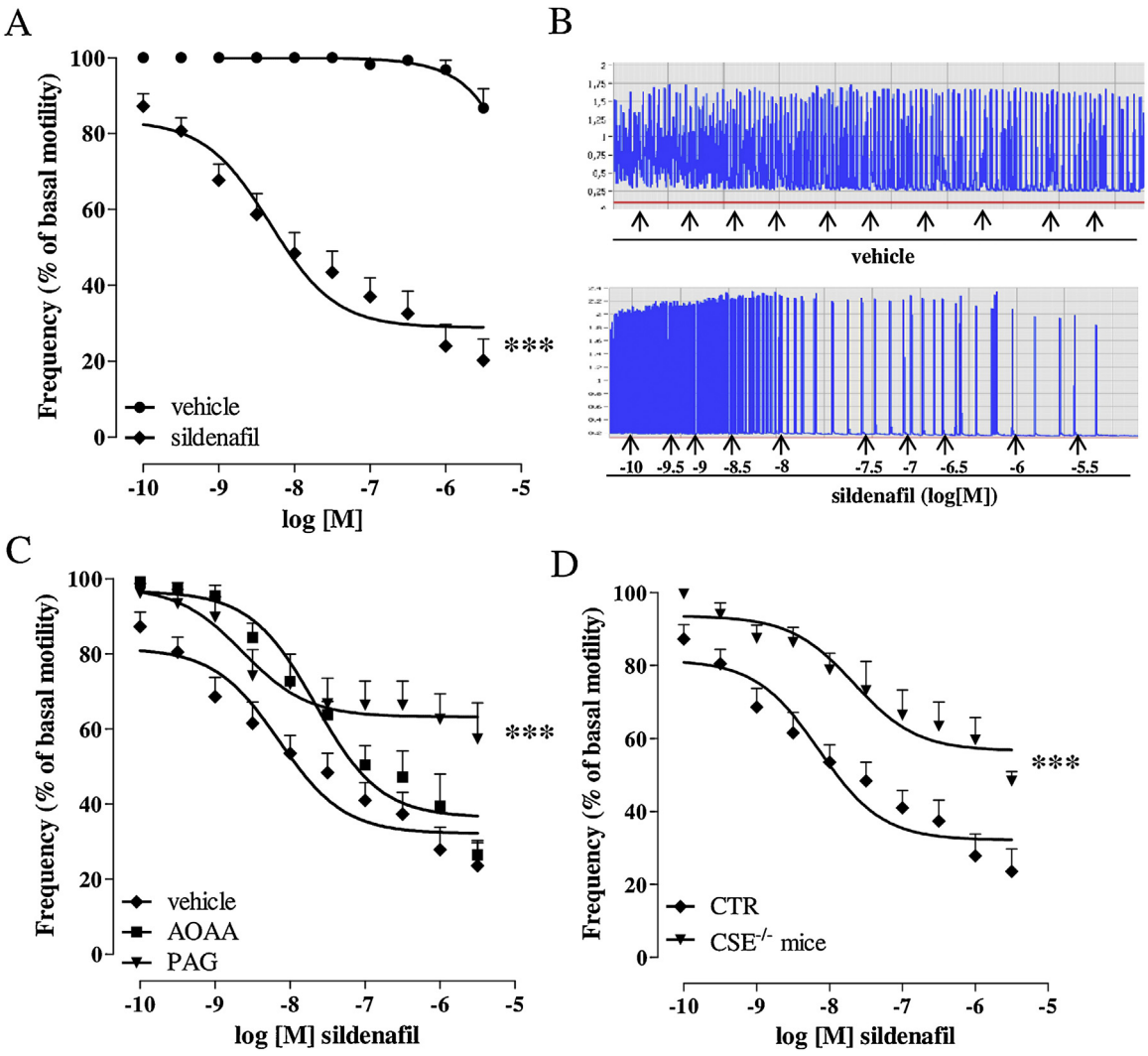

Fig. 4. Effect of sildenafil on uterine spontaneous contractility in CTR and $CSE^{-/-}$ mice. (A) Sildenafil markedly reduces uterine spontaneous contractility in CTR mice (***$p < 0.001$). (B) A typical trace of uterine spontaneous contractility, in presence of vehicle or sildenafil in CTR mice. (C) Sildenafil-induced effect is significantly inhibited by PAG (10 mM) (***$p < 0.001$). (D) The effect of sildenafil is notably reduced in $CSE^{-/-}$ mice compared to CTR mice (***$p < 0.001$). Results are expressed as frequency (% of the basal motility) and calculated as mean ± SEM (n = 8/10). Results are analyzed using analysis of variance (ANOVA) followed by Bonferroni post hoc test.

effect only at lower concentration of sildenafil (Fig. 4C). To confirm the involvement of H_2S pathway in sildenafil-induced effect on uterus motility, a concentration-response curve of sildenafil (0.1 nM–3 μM) has been executed also on uterus harvested from $CSE^{-/-}$ mice. CSE deletion markedly reduces sildenafil-induced effect compared to CTR mice (***$p < 0.001$; Fig. 4D).

3.5. *Sildenafil promotes cGMP generation through H_2S pathway activation*

Mouse uteri generate a detectable amount of cGMP (Fig. 5). As expected, sildenafil (1 μM) markedly increases cGMP production (***$p < 0.001$; Fig. 5A). Of note, the inhibition of CSE significantly reduces sildenafil-induced increase in cGMP content (°$p < 0.05$; Fig. 5A). The presence of PDE5 expression is confirmed by western blot analysis (Fig. 5B).

4. Discussion

PDE5 inhibitors, such as sildenafil, can enhance cGMP levels and are widely used in the therapy of erectile dysfunction [24,25]. On the other hand there are limited investigations on sildenafil effect in women. In 1999 sildenafil was prescribed for women affected by antidepressant-induced sexual dysfunction [26] and the use of vaginal sildenafil was shown to improve uterine artery blood flow in patients undergoing in vitro fertilization [10]. Moreover it has been reported that sildenafil increases myometrial sensitivity to nifedipine in human suggesting it as tocolytic agent [15]. Subsequent evidence showed that PDE5 is expressed in both human and rat myometrium and no changes in expression were observed during pregnancy [27]. H_2S is a signaling molecule mainly generated by two enzymes CBS and CSE and it participates in the regulation of various physiological processes also in the reproductive system [28–30]. In addition, it has been shown that the sildenafil relaxing effect on human bladder involves the L-Cys/H_2S pathway [16] and that CBS and CSE are expressed in rat and human myometrium tissue [19]. Here, we show that both CBS and CSE are expressed in mouse uterus and are able to convert L-Cys into H_2S. Indeed, incubation of mouse uterus with L-Cys leads to about a 3 fold increase in H_2S generation. This effect is reverted by either PAG or AOAA. Since the available CBS and CSE inhibitors are not highly selective to get further insight in to the relative involvement of these two enzymes, we used $CSE^{-/-}$ mice [31]. We could not use $CBS^{-/-}$ mice since they are not available, they suffer of severe growth retar-

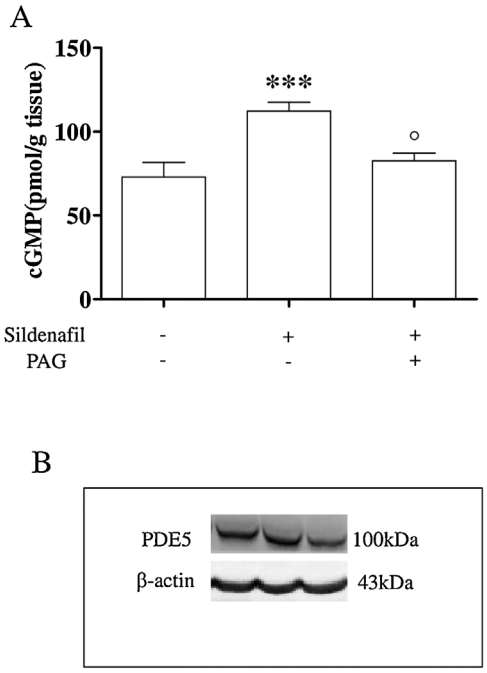

Fig. 5. cGMP production in uterus in CTR mice. (A) Sildenafil causes a significant increase in cGMP production compared with vehicle (***$p < 0.001$). PAG (10 mM) inhibits the enhancement of cGMP production induced by sildenafil (°$p < 0.05$). (B) Representative western blot analysis for PDE5 expression in uterus. Results are expressed as pmol/g of tissue and calculated as mean ± SEM ($n = 6$). Results are analyzed by one-way (ANOVA) followed by Bonferroni post-test.

dation and die within 3/4 weeks [32]. $CSE^{-/-}$ mice have normal CBS level in homogenate uterus, therefore allowing us to evaluate the relative contribute of CBS vs CSE. When in vitro experiments were performed using uterus harvested from $CSE^{-/-}$ mice, L-Cys failed to increase H_2S generation. Also sildenafil caused a significant increase in the H_2S production in uterus but it was weaker of that elicited by L-cysteine, the endogenous substrate. As for L-cysteine, sildenafil effect was reduced by CBS or CSE inhibition. Of note sildenafil-induced increase in H_2S observed in CTR mice, was lost in uterus obtained from $CSE^{-/-}$ mice. These results suggested that i) H_2S pathway could be involved in modulating uterus motility ii) CSE-rather than CBS-derived H_2S seems to give a major contribute iii) sildenafil can increase H_2S generation.

In order to further understand the role played by CSE-derived H_2S in uterus contractility and sildenafil effect, we performed functional studies by using uterus harvested by CTR or $CSE^{-/-}$ mice. The functional studies clearly demonstrated that H_2S pathway is involved in the modulation of uterus contractility. Indeed, NaHS (an exogenous source of H_2S), or L-Cys (the enzyme substrate) but not D-Cys significantly reduced the spontaneous uterus contractility. AOAA or PAG significantly reduced the L-Cys effect and PAG treatment was more significantly effective than AOAA. Therefore, the functional study indicated H_2S as signaling molecule that negatively modulates the uterus contractility and that CSE-derived H_2S plays a major role. Indeed, the L-Cys effect on uterus motility is almost abolished in $CSE^{-/-}$ mice as compared to control mice. This latter finding matches with the lack of effect of L-Cys-induced increase in H_2S production in uterus homogenates. Therefore, upon endogenous stimuli that boost uterus contractility an increase in CSE-derived H_2S can modulate this effect. Since we have previously demonstrated that sildenafil increases H_2S production in human bladder [16] we evaluated the effect of sildenafil on spontaneous uterus contractility. Firstly we assessed the presence of PDE5 in mouse uterus. Sildenafil caused a markedly significant and concentration-dependent reduction of spontaneous uterus contractility. Interestingly, sildenafil reduced of about 80% the spontaneous contractility. The pharmacological modulation study suggests that CSE-derived H_2S plays a major role in driving sildenafil effect than CBS. Indeed, PAG significantly inhibited sildenafil effect as opposite to AOAA that did not significantly modified the maximum effect (E_{max}). AOAA displayed only a weak effect at the lower sildenafil concentrations. The involvement of CSE-derived H_2S in sildenafil effect was further confirmed by the experiments performed in the isolated uterus of $CSE^{-/-}$ mice. In these experiments we found a reduced effect of sildenafil that was indistinguishable by the effect obtained with PAG in the pharmacological modulation study. Indeed. the E_{max}, expressed as frequency of percent of basal motility, for PAG was 57.4 ± 9% not significantly different from $CSE^{-/-}$ mice 48.3 ± 9%. H_2S has been shown to be an endogenous non selective inhibitor of PDE [33] and since sildenafil is a selective inhibitor of PDE5, in order to dissect the relative contribute of H_2S, we measured the cGMP levels in presence of PAG. As expected incubation of uterus with sildenafil caused an increase in cGMP levels that were almost doubled. Incubation with PAG, the CSE inhibitor, significantly inhibited the increase in cGMP caused by sildenafil indicating that the sildenafil effect is explicated in the uterus mainly through H_2S.

5. Conclusion

In conclusion, we have demonstrated that CSE-derived H_2S contributes to mouse uterus homeostasis. In addition we show that sildenafil reduces uterus contractility and that its effect involves CSE-derived H_2S. Thus these findings may explain the gap of the literature to understand the behind mechanism of sildenafil to decrease contractions in myometrium. These data suggest that H_2S-donors or drugs that can enhance H_2S production, such as sildenafil, can be developed as tocolytic agents.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phrs.2016.06.017.

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