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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