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Evaluation of the Effect of Inhibition of Prostaglandinergic and Nitrergic Pathways on Hydrogen Sulfide Responses in Rat Bladder Smooth Muscles

Evaluación del Efecto de la Inhibición de las Vías Prostaglandinérgicas y Nitrérgicas en las Respuestas del Sulfuro de Hidrógeno en los Músculos Lisos de la Vejiga de la Rata

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SUMMARY: It is known that mediators including prostaglandins, nitric oxide (NO), and hydrogen sulfide (H_2S) have a role in contraction and relaxation in a variety of tissues, including the bladder. This study aimed to evaluate the contribution of nitrergic and prostaglandinergic pathways to the possible effect of H_2S and the interaction between these pathways on bladder. The study was performed on male Wistar rat's bladder strips. The effects of NaHS and L-cysteine on carbachol-mediated or EFS-evoked contractile responses were measured. The responses were also measured with cyclooxygenase (COX) inhibitors incubation and NOS inhibitor incubation. Additionally, COX inhibitors and L-NAME were incubated in combination in EFS-mediated responses of NaHS. The highest concentrations of NaHS and L-cysteine caused inhibition on carbachol-mediated responses. No statistical significance was detected in the responses in COX inhibitors (FBP, ASA, NFA) or L-NAME incubations. Upon EFS-evoked contractile responses, when NaHS was applied, it caused inhibition at the highest concentration. FBP and NFA significantly increased the effect of NaHS. While L-cysteine alone did not have a relaxant effect but statistically significant inhibition with ASA and FBP incubation. COX inhibitors and L-NAME co-incubation reversed NaHS effect at only certain concentrations. Our study shows that nitric oxide, prostaglandins, and hydrogen sulfide are mediators that interact in the contractile or relaxation function of the bladder. However, how this interaction occurs has not yet been clearly revealed. With further studies on this subject, it seems possible that they can become a new treatment target in bladder dysfunction.

KEY WORDS: Hydrogen sulfide; Nitric oxide; Cycloocxygenase inhibitors; Electrical field stimulation; Isolated organ bath.

INTRODUCTION

The urine bladder has the function of storing and outflowing urine. Bladder dysfunction is an abnormality of detrusor muscle function that affects patients' quality of life. This may occur usually as a result of detrusor muscle contraction and relaxation abnormalities (D'Ancona *et al*., 2019). In order to provide proper bladder function, a healthy muscle activity with neurological control is necessary. Urine storage occurs with the detrusor relaxation and external sphincter contraction; meanwhile, for the urine outflow, detrusor contraction and sphincter relaxation are necessary. These muscle activities are regulated via parasympathetic, sympathetic, and somatic neuronal systems with various neurotransmitters (de Groat & Yoshimura, 2001). It is critical to comprehend the potential causes of this malfunction in order to create novel therapeutic approaches.

Hydrogen sulfide (H_2S) is one of three gasotransmitter molecules besides nitric oxide (NO) and carbon monoxide. For centuries, gasotransmitters have been regarded as toxic and potentially lethal gases, and now they are known as endogenous physiological regulators / modulators in the cardiovascular, nervous, immune, and other systems in the body (Wang, 2010; Gadalla & Snyder, 2010; Huang *et al*., 2021).

Cystathionine--lyase (CSE), cystathionine--synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (3-MST or MPST) are typically considered the three major H_2S-producing enzymes (Cirino *et al*., 2023). These three enzymes are widely distributed in mammalian tissues. Only the presence of CSE has been demonstrated in the mouse

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bladder (Matsunami *et al*., 2012), but all three enzymes have been detected in human and rat detrusor muscles (Gai *et al*., 2013). It is known that H2S causes relaxation in the bladder of pigs, rats, and humans. While a study conducted on pig bladder supports this information (Gai *et al.*, 2013; Fernandes *et al*., 2013) on the contrary, Patacchini *et al*. (2004), showed that a contraction response occurred in bladder tissue isolated from rats.

Nitric oxide (NO) was first described as an endothelium-derived relaxing factor. It was later understood that it is a second messenger and neurotransmitter produced by neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) nitric oxide synthase (NOS) enzymes.

Although it is generally known to cause relaxation in smooth muscles (Andersson & Wein, 2004), a study conducted on the human detrusor muscle, showed that it may also have complex effects such as relaxation, contraction, or temporary relaxation followed by contraction (Moon, 2002).

In a study investigating the interaction of H2S and NO, it was observed that they had synergistic effects on the relaxation responses in the thoracic aortic smooth muscle (Hosoki *et al*., 1997). The evidence that they increase each other's effects has also been seen in studies conducted on rat (Yilmaz-Oral *et al*., 2022) and human (Fusco *et al*., 2012) bladders. Another study has shown that they increase each other's 'synthesis through their enzyme activations (Zhao *et al*., 2001).

Prostaglandins (PGs) are another endogenous molecule produced from arachidonic acid by COX-1 and COX-2 enzymes. PGs have different subtypes (PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2, TXA_2) and have various functions depending on their subtype and tissue. PGI2 is the major PG in rats (Jeremy *et al*., 1984) and humans (Jeremy *et al*., 1987) and PGE2 in rabbit bladder (Leslie *et al*., 1984). PGE_2 and $PGF_{2\alpha}$ provide regulation of bladder tone (Stromberga *et al*., 2020a). Contractile responses have been recorded when prostaglandins ($PGF_{2\alpha}$, PGE_1, and PGE_2) were applied to smooth muscles isolated from animals (Andersson & Forman, 1978; Khanna *et al*., 1978; Klarskov *et al*., 1983; Maggi *et al*., 1988).

In the literature, studies showed that NO and PG have a synergistic effect on inflammation, NO can regulate PGs synthesis and there are various molecular interactions between each other (Salvemini *et al*., 1993). Another study demonstrated that H_2S increases lung inflammation in sepsis via PGE2 and COX-2, which shows that prostaglandins also interact with H_2S (Ang *et al*., 2011). Although there is evidence in various tissues that H_2S interacts with NO and PGs, there are not yet sufficient studies in the literature to show its effects on contraction-relaxation responses in the bladder. In this study, we aimed to evaluate the contribution of nitrergic and prostaglandinergic pathways to the possible effect of H_2S on the rat bladder and also, we aimed to evaluate the interaction between these pathways. The study results may contribute to explaining these complex mechanisms that are underlying control of the bladder.

MATERIAL AND METHOD

Animals and Tissue Preparation: This study was approved by the Local Ethics Committee for Animal Experiments of the Gazi University (G.U.ET-23.083) and conducted on bladder strips isolated from 22 Wistar rats weighing 250-300 g. Rats were kept in standard laboratory conditions [20 ± 2 °C, 12 hours light-dark cycle], allowed to drink water, and fed with standard laboratory animal feed *ad libitum*.

The rats were euthanized under deep anesthesia with ketamine/xylazine (45 mg/kg / 5 mg/kg, intramuscular, i.m.). The bladder was excised with a midline incision and placed in a petri dish containing Krebs-Henseleit solution (composition in mmol/l: NaCl, 118; KCl, 4.7; Ca- Cl_2• 2 H_2O, 1.3; $MgCl_2$• 6 H_2O, 0.5; Na_2HPO_4• 2 H_2O, 0.9; $NaHCO_3$ 24.9; and glucose monohydrate, 11) The connective tissue and adipose tissue around the bladder were cleaned in the Petri dish and prepared in the form of 2-4 bladder strips of 2 x 10 mm.

Bladder strips were kept in a 20 ml organ bath containing Krebs-Henseleit solution aerated to 5 % CO_2 and 95 % O_2 and heated to 37 during the entire experiment, and the solution was changed every 15 min for 60 min to equilibrate. The resting tension of the strips was set to 1 g.

In addition, a 60 min wash period was applied between consecutive experimental protocols, and solutions in the baths were changed every 15 min. Before the protocol, the effects of all incubation drugs were identified with the EFS (Electrical Field Stimulation), and the responses were recorded. The incubation period was 20 min for all drugs during the experiments.

Experimental protocol: After equilibration, to identify bladder responses, isometric contractions were evoked by EFS using platinum electrodes with a stimulation frequency of 60 Volt, 16 Hz and a 10 s duration administered every 2 min. The responses were recorded and evaluated on the MP35 system (BIOPAC, MP35 System Inc., COMMAT Ltd.). When EFS-evoked contractile responses were stabilized, the effects of NaHS (Sodium hydrogen sulfide-

H_2S donor) and L-cysteine (H_2S synthesis precursor) at increasing doses were evaluated (Cumulative doses are 10^{-5} M - 3 x 10^{-3} M for NaHS, 10^{-5} M - 3x10^{-3} M for L-Cysteine).

A washing period was performed and then the tissues were incubated with COX inhibitor Acetylsalicylic acid (ASA: 10^{-5} M) (Bas *et al*., 2011), Niflumic acid (NFA: 10^{-5} M) (Chakrabarty *et al*., 2019), Flurbiprofen (FBP: 10^{-5} M) (Nangle *et al*., 2003) or NOS inhibitor N(gamma)-nitro-L-arginine methyl ester (L-NAME:10^{-5} M) (Isli *et al*., 2019) for 20 min. After an incubation period, cumulative responses of NaHS and L-cysteine were obtained again on EFS-evoked contractile responses.

For investigating the joint contribution and interactions of prostaglandinergic and nitrergic pathways, experiments were performed again with the incubation of a PG inhibitor and L-NAME together. (as ASA + L-NAME; FBP + L-NAME; NFA + L-NAME).

In a different series of experiments; tissues were pre-contracted with carbachol (Cch) 10^{-5} M (Zou *et al*., 2018). After the contraction responses stabilized, NaHS (10^{-5} - 3x10-3 M) and L-cysteine (10^{-5} - 3x10^{-3}M) were added in cumulative doses, and the effect was evaluated. In order to investigate the contribution of prostaglandinergic and nitrergic pathways, different strips were incubated with selective and non-selective COX inhibitors that are ASA 10-5M, NFA 10-5M, FBP 10-5M or NOS inhibitor (L-NAME 10-4 M). After incubation, the strips were pre-contracted with carbachol the cumulative responses of NaHS and L-cysteine were again measured.

Ethanol used as a solvent for niflumic acid and flurbiprofen had no significant effect on EFS or carbachol-mediated contractile responses.

Drugs: Niflumic acid (NFA), Acetylsalicylic acid (ASA), L-NAME, Carbachol (Cch), NaHS and L-Cysteine used in the study were obtained from Sigma-Aldrich (St. Louis, MO, USA). Flurbiprofen was obtained from Cayman Chemical (Ann Arbor, MI, USA). ASA, L-NAME, Carbachol, NaHS and L-Cysteine were dissolved in distilled water. The other drugs used, FBP and NFA, were dissolved in ethanol.

Statistical Analysis: The control value for the EFS-evoked contractions was chosen as the last contraction before the administration of the chosen drugs. For the evaluation of the drug's effect, the last EFS-evoked contraction value after the administration of the drug was compared to the control value. While the impact of L-cysteine and NaHS on control responses was expressed, the degree of relaxation was shown as a percentage of the response. The percentage of the carbachol-induced contraction responses was used to express the effects of L-cysteine and NaHS on these responses. The normal distribution of the data was evaluated. Pre- and post-incubation evaluations were made with the Mann Whitney U test by SPSS software. Kruskal Wallis statistical analysis was used to compare three or more experimental groups.

Results were expressed as ± standard error of the mean (S.E.M.) and p 0.05 indicated statistical significance for differences.

RESULTS

NaHS inhibited EFS-evoked contractile responses by cumulatively (36.13 ± 7.37 % inhibition at the highest concentration). L-cysteine did not have any effect on EFS-evoked contractile responses (Fig. 1a -1b).

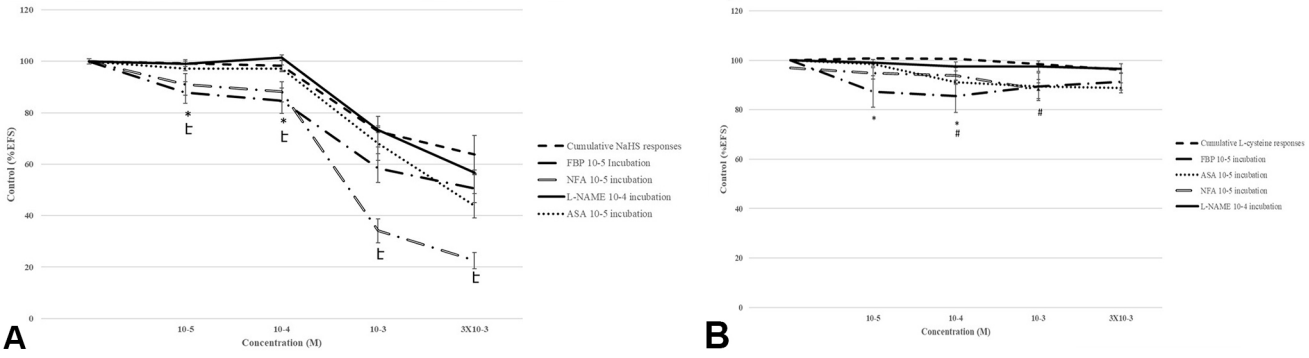

Fig. 1. Cumulative responses of NaHS (10-5 – 3x10-3) (Figure 1a) or L-cysteine (10-5 – 3x10-3) (Figure 1b) on EFS-evoked contractile responses both alone and with FBP (10-5), NFA(10-5), L-NAME(10-4), ASA(10-5) incubations in rat bladder strips. In every experiment step, the response prior to the first consentration application served as the control response. (NFA incubation), * (FBP incubation) or # (ASA incubation) significant difference compared to no incubation group. The percentage of the control responses was used to display the results.($p < 0.05$).

NaHS and L-Cysteine at the highest concentration caused relaxation responses on precontracted tissues with carbachol 55.86 % ±1.47 and 29.33 % ± 3.57, respectively (Fig. 2a-2b).

Effect of various COX inhibitors on NaHS and L-Cystein responses: FBP, a non-selective COX inhibitor, increased the inhibitor effect of NaHS on EFS-evoked contractile responses. NaHS effects at 10^{-5} M and 10^{-4} M concentration were significantly increased 12.21 % ± 4.16 and 15.38 % ± 4.95 respectively by FBP. Niflumic acid incubation significantly increased the effect of each concentration of NaHS, meanwhile ASA, and L-NAME did not cause a statistically significant change in the NaHS responses on EFS-evoked contractile responses (Fig. 1a).

While L-cysteine alone does not have a decreasing effect on EFS-evoked contraction responses, with the incubation of FBP, decreased EFS-evoked contractile responses at 10^{-5} M and 10^{-4} M concentrations, 12.70 % ± 6.35 and 14.56 % ± 6.48 respectively.

Similarly, with the ASA incubation, L-cysteine at concentrations of 10^{-4} M and 10^{-3} M effect potentiated 8.95 % ± 0.72 and 10.65 % ± 1.54 on EFS evoked contractile responses (Fig. 1b).

None of the COX inhibitors alter NaHS and L-cysteine-induced relaxation responses on precontracted tissues with carbachol (Fig. 2a-2b).

Effect of L-NAME on NaHS and L-Cysteine: L-NAME incubation did not alter NaHS and L-Cycteine effects on EFS-evoked contractile responses (Fig. 1).

L-NAME incubation did not affect NaHS and L-Cysteine-induced relaxation responses on precontracted tissues with carbachol (Fig. 2).

Effect of L-NAME and COX inhibitors on NaHS and L-Cysteine: We also assessed the combined impact of L-NAME and each COX inhibitor on NaHS responses in our investigation.

We mentioned before NaHS inhibitor effect statistically significant enhanced with FPB incubation on 10^{-5} M and 10^{-4} M concentrations. When we compared NaHS effect on EFS evoked contractile responses between at FBP incubation and co-incubation of FBP and L-NAME, we found that NaHS effect significantly regressed at 10^{-5} M and 10^{-3} M concentrations on EFS-evoked contractile responses. Results were displayed at Table I.

NFA incubation enhanced NaHS effect on EFS-evoked responses at each concentration, whereas NaHS effect on EFS-evoked responses decreased at 10^{-5} M, 10^{-4} M and 10^{-3} M concentrations with NFA and L-NAME co-incubation compared only NFA incubation. NFA and L-NAME co-incubation effect on NaHS results at only 310^{-3} M concentration on EFS-evoked responses significantly different compared with L-NAME incubation alone, or no incubation (Table I).

The co-incubation of ASA and L-NAME only increased NaHS effect on EFS-evoked responses at the 3^{10-3} M concentration. We did not observe significant differences between only ASA incubation or L-NAME co-incubation (Table I).

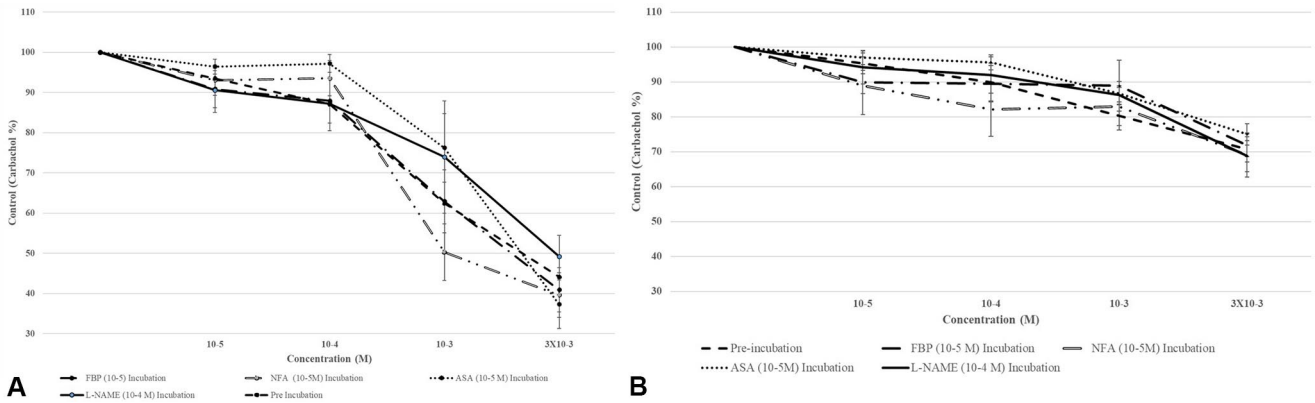

Fig. 2. Cumulative relaxation responses of NaHS (10-5 – 3x10-3) (Figure 2a) or L-cysteine (10-5 – 3x10-3) (Figure 2b) on precontracted rat bladder strips with carbachol(10-5); both alone and with FBP (10-5), NFA(10-5), L-NAME(10-4), ASA(10-5) incubations respectively. All values are given as percentage of the carbachol-induced contraction responses. (*, $p < 0.05$)

Table I. NAHS cumulative effect on EFS-evoked responses.

NaHS	10^{-5} M	10^{-4} M	10^{-3} M	3 x 10^{-3} M
No Incubation	99.29±1.34	98.27±1.87	72.71±5.95	63.87±7.37
L-NAME	99.05±1.27	101.40±1.2	73.33±4.54	56.80±5.67
FBP	87.79±4.16 [a]	84.62±4.95 [a]	58.43±5.59	50.53±5.45
FBP+L-NAME	98.98±1.24 [b]	99,07±2,23	74.59±4.23 [b]	48.11±3.75
NFA	90.99±4.15 [a]	88.17±3.94 [a]	34.07±4.68 [a]	22.58±3.16 [a]
NFA+L-NAME	98.66±1.16[c]	100.13±1.96[c]	70.82±6.41[c]	36.75±5.56 [a,d]
ASA	97.15±0.88	97.24±1.24	68.17±6.68	43.85±4.83
ASA+L-NAME	98.67±1.27	99.05±1.75	67.10±8.89	43.32±6.25 [a]

Results were represented as the mean of the percentage of the control responses.
[a] Statistical significance compared to no incubation group with Mann- Whitney U test p0.05
[b] Statistical significance compared to FBP incubation with Mann- Whitney U test p0.05
[c] Statistical significance compared to NFA incubation with Mann- Whitney U test p0.05
[d] Statistical significance compared to L-NAME incubation with Mann- Whitney U test p0.05

DISCUSSION

Bladder relaxation and contraction are a complex system, and this system is controlled by multiple neuronal, hormonal, and signaling systems (Andersson & Arner, 2004). In our study, the effects of H_2S, NO, and 3 different cyclooxygenase inhibitors on rat bladder contraction responses and their interaction were evaluated. While incubation of different cyclooxygenase inhibitors had no effect on carbachol-contracted tissues, the effect was observed in EFS-evoked tissues with incubation at different concentrations of some cyclooxygenase inhibitors. Bladder contraction and relaxation have a complex structure, and it is considered that H_2S, PGs and NO have a modulatory effect on these responses.

Abe & Kimura (1996) stated that H_2S modulates synaptic activity. Gai *et al*. (2013), previously reported that hydrogen sulfur is the mediator of relaxation of both rat and human bladder. 3-mercaptopyruvate sulfurtransferase [MPST], and cysteine aminotransferase [CAT] enzymes have been detected in rat bladder, and similarly to our results (Gai *et al*., 2013). It was found that, NaHS induces relaxation in the pre-contracted bladder with carbachol (Zou *et al*., 2018). It has been shown that NaHS causes a relaxation in carbachol-induced contractile responses in different tissues, for example,in porcine iridea (Monjok *et al*., 2008). Prostaglandins are also locally produced in human bladder (Abrams *et al*., 1979). It has been previously reported that PGE_2 and $PGF_{2\alpha}$, TXA_2, PGD_2, and PGI_2 induce a contractile response in porcine bladder (Stromberga *et al*., 2020b). Same authors reported that both PGE_2 and $PGF_{2\alpha}$ mediate contractile response via FP Receptor in the porcine bladder (Stromberga *et al*., 2020a). Although these studies were conducted in different tissues, these literature results are compatible with our study.

Interestingly, H_2S inhibition leads to a decreased expression of COX-2 via down-regulation of cyclooxygenase-2 messenger RNA expression in the rat gastrointestinal tract. In the same study, it was also stated that H_2S had no effect on COX-1 (Wallace *et al*., 2009). NFA, which is also a chloride channel inhibitor, has been shown to potentiate the sodium sulphide relaxant effect on spontaneously active rat uterus (Mijuskovic *et al*., 2015).

According to our results selective COX-2 inhibitor NFA incubation augmented for each dose of NaHS on EFS-evoked responses. In the potentiation effect of NFA, its effects on chloride channels, as well as COX inhibition should be taken into consideration.

The effect of H_2S-releasing nonsteroidal anti-inflammatory drugs has been studied on gastrointestinal mucosa and various cancer cell lines. H2S-releasing NSAIDs such as HS-acetyl salicylic acid are more potent inhibitors on cell proliferation or they have better protective effect on gastrointestinal mucosa in comparison to NSAIDs alone (Kashfi & Olson, 2013). Another non-selective COX inhibitor FBP, has been shown to increase the slow-release hydrogen sulfide donors GYY4137 induced relaxation on bovine ciliary arteries (Chitnis *et al*., 2013).

Some studies indicate that endogenous NO or NO donors cause relaxation responses in response to EFS stimulation (Fernandes *et al*., 2016). Conversely, it has been shown that EFS-induced responses increased with L-NAME and indomethacin incubation at human detrusor strips (Kosan *et al*., 2008). In another study, it was observed that L-NAME had no effect on EFS-induced responses in adult guinea pig bladder strips; it only increased

EFS responses in the elderly experimental group bladder (Gómez-Pinilla *et al*., 2007). Nile & Gillespie (2012), previously reported a complex interaction mechanism between muscarinic receptors, NO, and PG production in the bladder wall. Furthermore, the effectiveness of the L-Arginin/NO pathway varies according to the different parts of the bladder. This effect is more prominent in the bladder outlet zone (Persson *et al*., 1992). L-NAME by itself had no effect on H2S responses in our investigation.

However, our results showed that the effect of H_2S on EFS-evoked responses that was potentiated by FBP and NFA was reversed by blocking NO with L-NAME. This effect was not observed compared with ASA incubation and coincubation of ASA and L-NAME. It is considered that molecular variations are the cause of these circumstances. Various non selective COX inhibitors have different activity on COX enzyme active site for example, ASA has rapid and reversible to COX enzyme while FPB has rapid and low affinity (Rao & Knaus, 2008). Vural *et al*. (2024) reported that COX pathway is a very complex and interacts with many pathways such as fatty acid amide hydrolase in rat vas deference tissue.

CONCLUSION

All these findings indicate that H_2S and prostaglandinergic pathways and NO have a role on bladder smooth muscle tone regulatory mechanisms. Hydrogen sulfide releasing non steroid anti-inflammatory drugs may be more effective alternatives in regulating bladder capacity. This effect needs to be evaluated with further studies.

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RESUMEN: Se sabe que los mediadores que incluyen prostaglandinas, óxido nítrico (NO) y sulfuro de hidrógeno (H_2S) tienen un efecto en la contracción y relajación en una variedad de tejidos, lo que también incluye la vejiga. Este estudio tuvo como objetivo evaluar la contribución de las vías nitrérgicas y prostaglandinérgicas al posible efecto del H_2S y la interacción entre estas vías en la vejiga. El estudio se realizó en tiras de vejiga de ratas Wistar macho. Se midieron los efectos de NaHS y L-cisteína en las respuestas contráctiles mediadas por carbacol o provocadas por EFS. Las respuestas también se midieron con la incubación con inhibidores de la ciclooxigenasa (COX) y la incubación con inhibidores de NOS. Además, los inhibidores de COX y L-NAME se incubaron en combinación en las respuestas de NaHS mediadas por EFS. Las concentraciones más altas de NaHS y L-cisteína causaron inhibición en las respuestas mediadas por carbacol. No se detectó significación estadística en las respuestas en las incubaciones con inhibidores de COX (FBP, AAS, NFA) o L-NAME. Tras las respuestas contráctiles provocadas por EFS, cuando se aplicó NaHS, causó inhibición en la concentración más alta. FBP y NFA aumentaron significativamente el efecto de NaHS. Mientras que la L-cisteína sola no tuvo un efecto relajante, pero sí una inhibición estadísticamente significativa con la incubación con AAS y FBP. La co-incubación con inhibidores de COX y L-NAME revirtió el efecto de NaHS solo en ciertas concentraciones. Nuestro estudio muestra que el óxido nítrico, las prostaglandinas y el sulfuro de hidrógeno son mediadores que interactúan en la función contráctil o de relajación de la vejiga. Sin embargo, aún no se ha revelado con claridad cómo se produce esta interacción. Con más estudios sobre este tema, parece posible que puedan convertirse en un nuevo objetivo de tratamiento en la disfunción vesical.

PALABRAS CLAVE: Sulfuro de hidrógeno; Óxido nítrico; Inhibidores de la ciclooxigenasa; Estimulación del campo eléctrico; Baño de órganos aislados.

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