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"L-cysteine/hydrogen sulfide pathway: pharmacological approaches in urinary bladder and cardiovascular diseases"

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1. PATHOPHYSIOLOGY OF HUMAN LOWER URINARY TRACT

1.1 Morphology of lower urinary tract

The urinary tract is composed by kidneys, ureters, bladder and urethra. Kidneys and ureters are generally retained to be the upper part of the urinary system, while the remaining organs are considered to make up the lower urinary tract (LUT), that is located in the lower pelvis and supported by muscles and ligaments.

The bladder can be divided into two main components: the bladder body, which is located above the urethral orifices, and the base, consisting of the trigone, uretero-vesical junction, detrusor, and the anterior bladder wall (***Figure 1***).

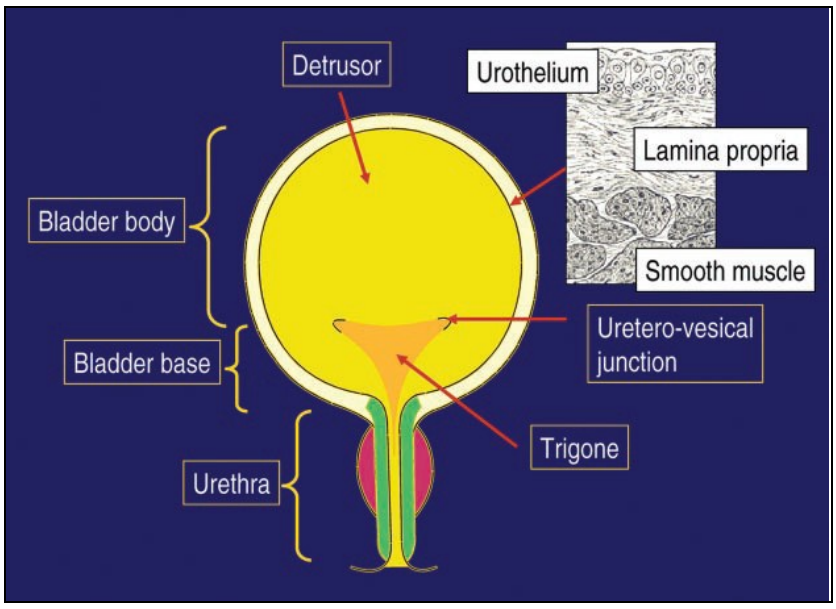

Figure 1. Schematic morphology of urinary bladder (*Urinary bladder contraction and relaxation: physiology and pathophysiology. Andersson KE, 2004*).

The bladder is essentially an elastic smooth muscle organ made up by three different layers: a mucous membrane, defined as uro-epithelium or urothelium; a sub-urothelial

layer and a muscular wall, known as detrusor muscle, formed by smooth muscle cells arranged in outer longitudinal, middle circular and inner longitudinal layers. This muscle organization confers the ability on bladder to empty during the micturition, generating the pressure necessary to expel the urine. Indeed, detrusor muscle has to hold the changes in urine volume and consequently it must be able to reorganize itself during the filling and the emptying of the bladder and maintain the physiologic shape of the bladder. The normal mechanism of micturition in male is a very complicated process, that requires simultaneously a very strict regulation of contraction and relaxation of the urinary musculature and involves bladder, prostate, urethra and vascular system as well as interaction/communication between these anatomic structures and the neuro-hormonal system. Indeed, each part of the urinary tract muscle is subjected to a neuronal and hormonal control system and expresses specific receptors for the transmitters/modulators, generated locally or released from nerves. During filling phase of the urinary bladder, the smooth muscle cells, composing the urinary wall, have to relax, elongate and rearrange to accommodate the urine. On the other hand, during bladder emptying detrusor muscle cells are required to be simultaneously contracted, while urethral muscle cells uncontracted to prevent the increase in urine pressure and to expel it through the urethra. The physiological tone of the smooth detrusor muscle and bladder neck as well as the smooth and striated sphincters in the urethra, is regulated by central and peripheral autonomic and somatic neuronal system. Specifically, there are three nerve systems involved in the regulation of micturition cycle, which are the pudendal, pelvic and hypogastric nerves (***Figure 2***).

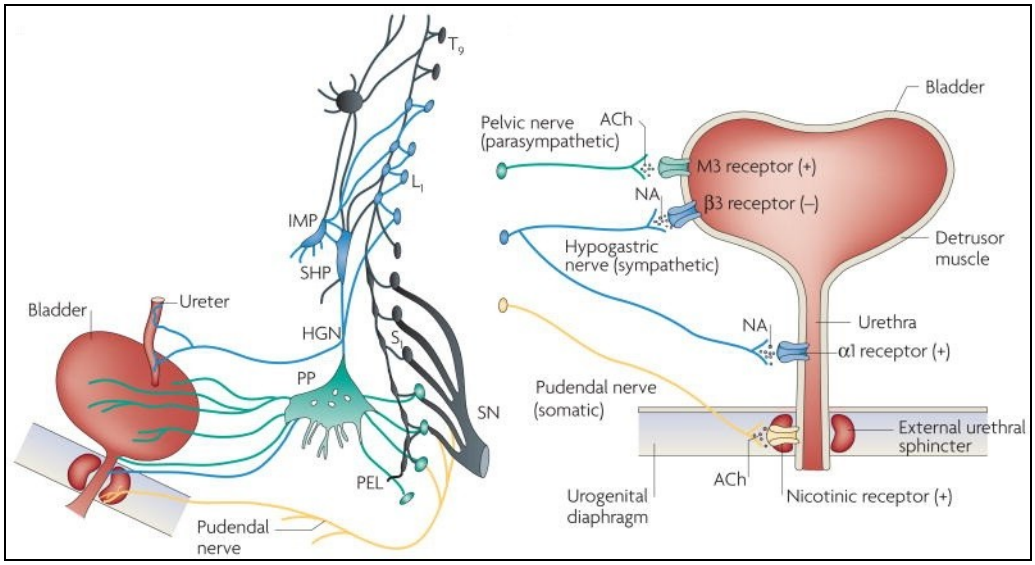

Figure 2. Central nervous system control of micturition cycle (*The neural control of micturition. Fowler JC, 2008*).

To prevent involuntary bladder emptying, during the filling phase of the bladder, the parasympathetic innervation of detrusor is inhibited, to facilitate the accommodation of urine, whereas the sympathetic system, that conveys through the hypogastric nerve fibers *plus* recruitment of pudendal motor neurons to the bladder neck and urethra, is activated to induce muscle contraction and let take place the urine storage. This reflex is activated by bladder afferents fibers, which consist of myelinated (Aδ) and unmyelinated (C) axons. The Aδ-fibers promote bladder continence responding to passive distension and active contraction, whereas the physiologically C-fibers are insensitive to bladder filling, but under pathologic conditions they may become mechanosensitive, providing nociceptive afferents to inflammation, over-distention or irritation.

Instead, during the voiding phase, the sympathetic efferent activity is inhibited with a concomitant activation of parasympathetic outflow to the bladder and urethral smooth muscle leading to the expulsion of urine[1].

1.2 Urothelium: a passive barrier?

The uro-epithelium, better known as urothelium, is a stratified epithelium and represents the interface between the lumen of urinary tract and underlying tissues; in particular it lines the inner parts of the renal pelvis, ureters, bladder and parts of the urethra. The urothelium is made up by at least three layers. The superficial or apical layer of the urothelium consists of large hexagonal flattened cells (25-250 μM), called umbrella cells, that are interconnected by tight junctions[2, 3]. The innermost layer of the urothelium consists of smaller basal cells that are separated from the suburothelial lamina propria by a basal lamina. In between the umbrella and basal cells there is an intermediate layer (***Figure 3***).

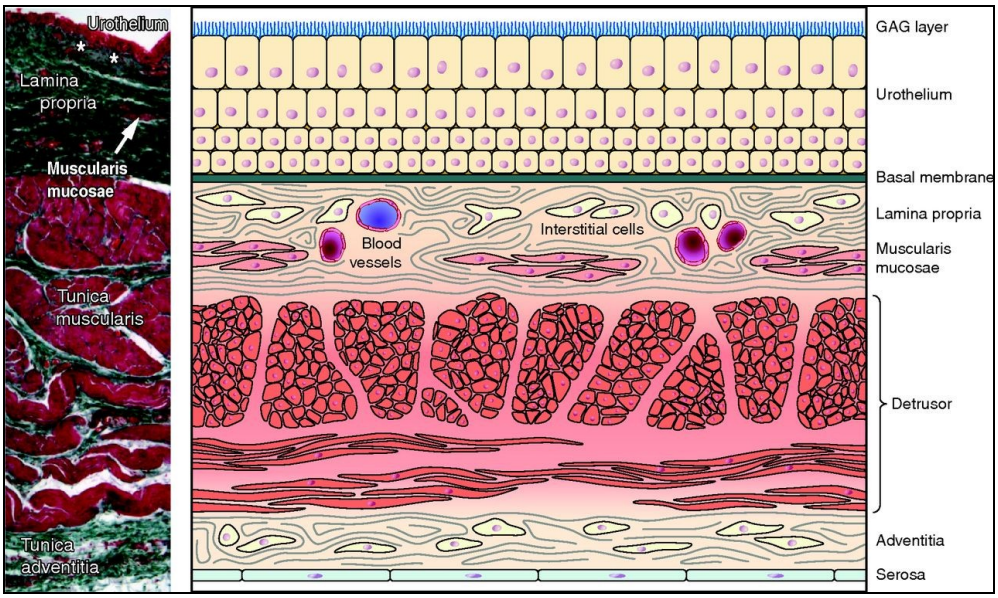

Figure 3. Components of urinary bladder wall (*Urothelial signalling. Birder L, 2013*).

The number of the intermediate layer varies in the species. Indeed, in the urinary bladder of rodents there is one intermediate layer, whereas in humans there are up to 5 intermediate layers[4, 5]. The urothelium has a pivotal role as a permeability barrier to urine and several features of the umbrella cell layer contribute in maintenance of an intact barrier, which is a prerequisite for normal afferent signalling from the bladder.

These features include the presence of tight-junction proteins, such as occludins and claudins, in addition to multiple specialized lipids and uroplakin proteins. The uroplakin proteins, along with occludins and claudins, preserve the integrity of the urothelial barrier by preventing proteins as well as ionic and non ionic substances from gaining access[2, 6]. In addition, a layer of glycosaminoglycan (GAG) or mucin-layer covers the umbrella cells and which is an antiadhesive structure against pathogens, carcinogens, and toxic substances in the urine[7]. GAG layer is retained to be vital for maintaining barrier integrity of the bladder wall. Interestingly, distension of the bladder wall during storage phase of micturition is concurrent to a change in shape of the urothelium. Furthermore, exocytosis or endocytosis mechanisms add membrane to the apical cell surface, thereby permitting an increase in bladder volume without loss of barrier function[8-10]. Several studies have demonstrated that the stretching induced by bladder filling activates exocytosis mechanisms in urothelium, involving a number of signalling molecules such as epidermal growth factor receptor[8, 11]. Moreover, it has been also reported that during storage of urine, urothelial cells exhibit a lower level of endocytotic activity, representing a protective mechanism against internalization of toxic compounds in the urine[12]. However, besides all these features appointed to this epithelial layer, the urothelium is more than a barrier against the bladder urine content. One significant function of the urothelium is to act as a mechanosensory conductor[13-15]. It has specialized sensory and signalling properties to engage in chemical communication with nerves in the bladder wall and smooth muscle. Indeed, several studies have revealed that during bladder filling, distension of the bladder wall stretches the urothelium[16] inducing the release of a number of signalling molecules such as adenosine-5'-triphosphate (ATP), acetylcholine (Ach)[17], and nitric oxide (NO)[18]. These molecules act on different receptor subtypes and interact within the urothelium modulating afferent neuronal activity and detrusor smooth muscle function. Although the urothelium maintains a tight barrier, several factors, like chemical or mechanical trauma and infection, can compromise the barrier function and damage the interface between bladder and urine. The lack of urothelium integrity can induce changes in function of underlying cells

within the bladder wall and sensory symptoms of urgency, frequency and pain during bladder filling and voiding.

1.3 Lower Urinary Tract Symptoms (LUTS)

The urinary bladder has two important functions: storage of urine and emptying. Storage of urine occurs at low pressure, which implies that the bladder relaxes during the filling phase, while emptying requires a coordinated contraction of the bladder and relaxation of the urethra. Disturbances of the storage or emptying function may result in lower urinary tract symptoms (LUTS).

The European Association of Urology and American Urological Association guidelines define LUTS as common age-related condition in both male and female patients characterized by: i) storage or irritative symptoms such as daytime urinary frequency, urgency, nocturia incontinence and bladder pain or dysuria; ii) voiding or obstructive symptoms like straining, urinary hesitancy, weak stream, intermittent stream, and incomplete emptying; iii) post-micturition symptoms, such as post-micturition dribbling, that affect the lower urinary tract[19-21]. Focusing on male population, the prevalence of these symptoms may be higher than 50% in men aged ≥ 50 years[22] with an expected increasing of percentage with aging[23]. For several years, in elderly men, LUTS have been traditionally considered to be secondary to the enlarging of prostatic gland, in particular to histological benign prostatic hyperplasia (BPH), benign prostatic obstruction (BPO) or benign prostatic enlargement (BPE). However, during the last decade the causal connection between the pathogenesis of LUTS and the prostate has come into question[24]. Indeed, recent evidence suggest that LUTS may be a non-sex and non-organ specific symptoms[19] and other factors should be retained of same importance, despite the enlargement of prostate gland can contribute to the onset of LUTS. Recent evidence indicate that LUTS may be linked to the impairment of the prostate, bladder, kidneys or erectile function[24]. Furthermore, in any single person affected with LUTS it is common to recognize more than one co-existing cause (***Figure 4***). This multi-factorial etiology of LUTS has led to develop

the viewpoint that regards the whole genito-urinary tract as a single functional unit.

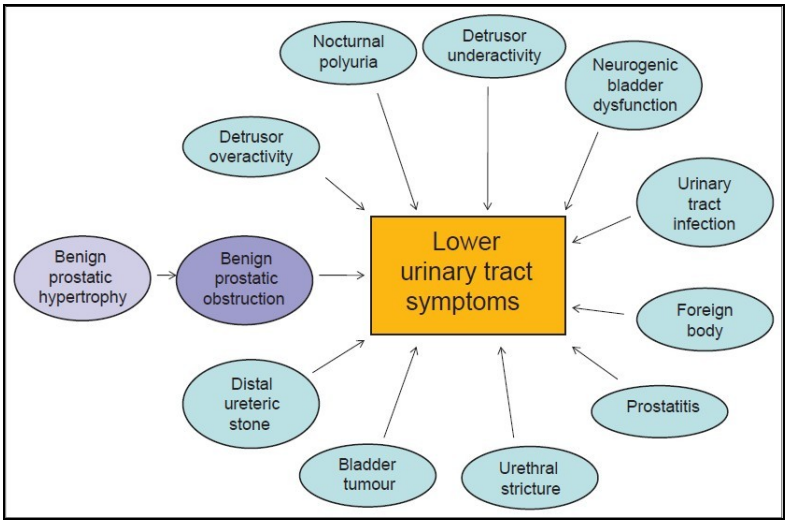

Figure 4. Multifactorial etiology of lower urinary tract symptoms (*Guidelines on the management of male lower urinary tract symptoms, incl. Benign prostatic obstruction. Oelke M, 2012*).

1.4 LUTS secondary to BPH

BPH is a common disease characterized by smooth muscle and epithelial cell proliferation within prostate transition zone, leading to a non malignant prostate gland enlargement[25, 26]. In male population, LUTS are often concurrent with BPH. It occurs with aging and the prevalence increases from 25% among men aged between 40 to 49 years to more than 80% among 70 to 79 years[27]. Although many patients affected with BPH are asymptomatic, more than 50% of men in their 60s to as many as 90% of persons aged between 80 and 89 present lower urinary disorders[28]. In addition, although prostate enlargement due to BPH has been long associated with LUTS, it is widely recognized that it is not the exclusive cause[19, 29], thus the pathophysiology of LUTS and its underlying mechanisms remain still not fully understood. Detrusor overactivity with its vascular supply and bladder outlet obstruction have been considered a key factors for the development of LUTS secondary to BPH[24, 30]. The classification of LUTS is partly based on the assumption that the enlarging transition zone of the prostate produces pressure on the urethra. This pressure represents the

static component of the bladder outlet obstruction and leads to a variety of voiding symptoms. Conversely, because of the sympathetic innervation and the high density of α_1-adrenergic receptors in prostate, prostatic capsule, and bladder neck, the increased smooth muscle tone and resistance accounts for the dynamic obstruction and the associated storage symptoms. The development of the histological features of BPH is dependent on bioavailability of testosterone and its metabolite, dihydrotestosterone[31]. Moreover, other physiological markers i.e. dehydroepiandrosterone and estradiol[32], insulin-like growth factors[33] and inflammatory markers such as C-reactive protein[34-36], as well as black race[37], obesity[38], diabetes[39], high levels of alcohol consumption[40] and physical inactivity[41] are associated with an increased risk of BPH. Nevertheless to date the mechanisms underlying these associations remain poorly understood.

1.5 BPH/LUTS and erectile dysfunction (ED)

Erectile dysfunction (ED), a common multifactorial sexual disorder associated with aging and a range of organic and psychogenic conditions, is characterized by the inability to attain and/or maintain a sufficient erection for a satisfactory sexual performance[42]. Epidemiological studies report that ED incidence increases with age and the prevalence is of 52% in men aged between 40 and 70 years[43]. Furthermore, several studies have documented a strong relationship between male sexual dysfunction and LUTS[44]. Indeed, LUTS co-exist with ED and vice versa in many patients, approximately 70%[45]. Moreover, there is a strong correlation independent from the age between the severity of LUTS and the degree of ED suggesting a causal relationship or, more possibly, the presence of common pathogenetic pathways[46]. These evidence should be taken in account managing patients with LUTS or ED. In this regard, the phosphodiesterase (PDE) type 5 inhibitors (PDE5-Is), the first line therapy for ED, are also effective in the treatment of LUTS with a synergic effect on both LUTS and ED, when associated with α-blockers.

In addition to the age, the coexistence of ED with LUTS/BPH has been identified with several other co-morbidities such as hypertension, high cholesterol, high fasting

plasma glucose levels, diabetes, indicating that metabolic syndrome might play a key role in the pathogenesis of both ED and LUTS. Other co-morbidities are represented by androgen deficiency, digestive tract disorder, arthritis, heart disease/heart failure, depression/anxiety/sleep disorder and inflammation[47]. Moreover, potential risk factors for LUTS and ED include age, sedentary lifestyle, smoking and excessive alcohol intake, depression, cardiovascular disease, hyperlipidemia, type 2 diabetes mellitus, obesity, hypogonadism, prostate disorder, inflammation and genetic predisposition[37, 45, 48]. Currently, there are 4 pathophysiological pathways, not mutually exclusive, that support the relationship between LUTS and ED[29, 46, 49, 50]: i) impairment of NO- 3', 5'-cyclic guanosine monophosphate (cGMP) signalling in penis and prostate; ii) hyper-activation of RhoA-Rho-kinase (ROCK) signalling and endothelin-1 pathway; iii) autonomic hyperactivity; iv) pelvic atherosclerosis. These mechanisms can lead to reduced function of nerves and endothelium, alterations in smooth muscle tone, arterial insufficiency, reduced blood flow and hypoxia-related tissue damage, increased smooth muscle cell proliferation in prostate and bladder hypertrophy.

The NO/cGMP pathway is believed to be the main signalling mechanism involved in the regulation of penile smooth muscle relaxation and penile erection and, moreover, a large number of evidence demonstrates the role of NO in the regulation of smooth muscle tone of bladder, prostate, and urethra. Released by nerve and endothelial cells in the corpora cavernosa of the penis, NO activates soluble guanylyl cyclase (sGC), which increases cGMP levels. Acting as a second messenger molecule, cGMP regulates the activity of Ca^{2+} channels as well as intracellular contractile proteins that affect the relaxation of corpus cavernosum smooth muscle[29, 51]. PDEs, expressed in the prostate and bladder, metabolize cGMP into linear 5'-GMP limiting NO signalling on smooth muscle. On these basis, it is possible to hypothesize the mechanism through PDE5-Is exert beneficial effects on LUTS during ED therapy [52]. Smooth muscle tone is also regulated by ROCK pathway. Rho-kinase is activated by a G-protein, RhoA, thought to be coupled to excitatory α_1-adrenoceptors. This pathway modulates the level of phosphorylation of myosin light chain II, mainly

through inhibition of myosin phosphatase, and contributes to a calcium-independent mechanism of smooth muscle contraction. An abnormal up-regulation of ROCK signalling increases the smooth muscle tone leading to the impairment of erectile function and bladder homeostasis, finally resulting in ED and LUTS[29, 51-53].

A deregulation of parasympathetic and sympathetic tone leads to autonomic hyperactivity, a component of metabolic syndrome. Various α_1-adrenergic receptor subtypes have been identified in penile tissue, bladder and prostate, where they mediate smooth muscle and vascular contraction. α_1-adrenergic receptor up-regulation is known to play a crucial role in pathogenesis of LUTS by increasing bladder neck and prostate tone[52]. Indeed, concordant evidence coming from animal models of autonomic hyperactivity, reveal that sympathetic overactivity determines prostate hyperplasia, ED, and increase voiding frequency and detrusor overactivity[54]. Atherosclerosis-induced pelvic ischemia involving prostate, penis and bladder, is likely to be compatible with all theories mentioned above, given that it may induce autonomic nervous system hyperactivity, reduce NO/cGMP signalling, and up-regulate ROCK pathway[55]. Animal models mimicking pelvic ischemia and hypercholesterolemia show smooth muscle alterations of the detrusor muscle and corpus cavernosum, stromal fibrosis, glandular cystic atrophy and increase in smooth muscle contractility of the prostate[56, 57] and fibrosis in the corpora tissue. There are several potential mechanisms to explain these findings. Chronic ischemia is associated with an increased production of TGF-β1 that correlates with the severity of fibrosis[58]. It also impairs neurogenic relaxation in the prostate, which appears to involve the NO pathway, and may result in a loss of elasticity and increase in smooth muscle tone of the prostate[57]. The long-term presence of unfavourable vascular risk factor is known to have a significant impact on the genesis of ED and may also cause structural and functional impairment of LUT[29]. The contribution of pelvic arteriosclerosis as a causative factor of LUTS and ED accounts for the evidence that men with one or more risk factors of atherosclerosis (diabetes mellitus, hypertension, hyperlipidemia) are more likely to develop ED and LUTS than subjects with no risk factors[29, 51, 52]. More recent finding suggests the involvement of additional

contributing factors in the onset of LUTS, such as chronic inflammation and sex steroid imbalance[29]. In particular, it has been reported that testosterone deficiency may be a pathophysiological mechanism connecting LUTS and ED with the metabolic syndrome in men[51]. Decreased testosterone levels can cause LUTS through an impairment of smooth muscle relaxation of the prostatic urethra and bladder and the down-regulation of pro-erectile molecular mechanisms, such as NO/cGMP signalling[51, 59].

1.6 Pharmacological therapy of LUTS associated to BPH and ED

In men affected with BPH, LUTS are mainly considered to be a consequence of bladder outlet obstruction, overactive bladder, or a combination of both, and the therapy is aimed to reduce both static and dynamic pathogenetic components[22, 24]. Furthermore, the common pathophysiology between LUTS and ED might potentially imply that drugs effective in either conditions may also have an effect on the other. Recent treatment guidelines for LUTS report the use, in combination or alone, of α-blockers, 5α-reductase inhibitors (5ARIs), antimuscarinics, β_3-adrenergic agonists, and PDE5-Is[21], whose beneficial effects are provided through different, albeit not clearly understood, mechanisms of action[21].

1.6.1 α-blockers

It is well known that α_1-antagonists are the first choice of medication in men for treating moderate to severe BPH/LUTS. They are often considered the first-line drug treatment of male LUTS because of their rapid onset of action, good efficacy, as well as the low rate and severity of adverse events. Following the early use of phenoxybenzamine and prazosin in BPH/LUTS treatment, five types of α_1-blockers are currently approved by Food and Drug Administration (FDA) for the treatment of LUTS: alfuzosin, doxazosin, tamsulosin, terazosin and silodosin. Stimulation of α_1-adrenoceptors, a class of G protein-coupled receptors located in bladder, bladder neck, prostate and corpus cavernosum, leads to the activation of phospholipase C (PLC), resulting in increased formation of inositol triphosphate (IP_3) and

diacylglycerol (DAG). IP_3 subsequently induces Ca^{2+} release from the sarcoplasmic reticulum, causing the formation of Ca^{2+}/calmodulin (CaM) complex. This complex activates myosin light chain kinase (MLCK) that in turn phosphorylates MLC enabling the myosin cross-bridge to bind the actin filament and allows the contraction. On the basis of the above mentioned mechanism, α_1-blockers act by inhibiting sympathetic adrenergic receptor mediated contraction of the prostate smooth muscle cells, bladder, bladder neck and penile smooth muscle, thereby reducing prostate and penile tone and bladder outlet obstruction[24]. However, α_2-blockers have been studied also for ED and lower urinary disorders. Indeed, yohimbine, is an antagonist of pre- and post-synaptic α_2-receptors. The blockade of post-synaptic receptors by yohimbine causes a decrease of intracellular Ca^{2+} levels resulting in relaxation of penile smooth muscle. On the other hand, the inhibition of pre-synaptic α_2-receptors facilitates the release of NO from non-noradrenergic, non-cholinergic transmitter nerves, which stimulates sGC to synthesize cGMP which in turn decreases intracellular Ca^{2+} and relaxes penile smooth muscles[60]. Moreover, some evidence show that combination of α_1-blockers with PDE5-Is (alfuzosin or doxazosin *plus* sildenafil) markedly improves both ED and LUTS associated with BPH[61, 62] and the drug association is superior to the monotherapy. Furthermore, it has been reported that yohimbine, in association with sildenafil, enhances and prolongs the effect of sildenafil on erectile function without any additional hypotensive effects[63]. Available α_1-antagonists have a similar efficacy, independently from prostate size and the aging, in patients with mild, moderate, or severe LUTS[64]. The efficacy is commonly reported as a percentage improvement in International Prostate Symptom Score (IPSS)[64]. α_1-blockers are also able to improve both storage and voiding related symptoms. In addition, the wide distribution of α_1-adrenoceptors in blood vessel, non-prostatic smooth muscle cells and central nervous system explains the common side-effects, such as orthostatic hypotension, dizziness and asthenia during α_1-antagonists treatment.

1.6.2 5α-reductase inhibitors (5-ARIs)

5α-reductase inhibitors (5-ARIs) are a class of drugs with antiandrogen effects, suggested primarily in the treatment of BPH and for moderate to severe LUTS[21]. They prevent the progression of prostate growth or reduce the volume of prostate by inhibiting the production of the hormone dihydrotestosterone[24]. In particular, 5-ARIs block the conversion of testosterone, the major androgen sex hormone, to the more potent dihydrotestosterone by blocking the enzyme 5α-reductase in prostatic stroma cells. For clinical use, there are two FDA-approved 5α-reductase inhibitors: finasteride and dutasteride. Finasteride inhibits the 5α-reductase type 2 isoenzyme, decreasing the serum dihydrotestosterone levels of 70 to 90%, whereas dutasteride blocks both 5α-reductase type 1 and type 2 isoforms, leading to decreases in dihydrotestosterone to levels that approach zero. Furthermore, they induce apoptosis of prostate epithelial cells leading to a reduction of 18–28% of prostate size and about 50% of circulating prostate-specific antigen (PSA) levels after 6-12 months of treatment[65, 66]. The most relevant adverse effects displayed by these drugs are related to sexual function and include reduced libido, gynecomastia, ED, and less frequently, ejaculation disorders[67, 68].

1.6.3 β_3-adrenergic agonists

During the filling phase of the bladder, β_3-adrenergic receptors are stimulated by endogenous noradrenaline to promote smooth muscle relaxation and facilitate storage of urine. They are highly expressed on urinary tissues such as the urothelium, interstitial cells, and detrusor smooth muscle, including also human corpus cavernosum (HCC)[69-71]. β_3-adrenergic receptors belong to the family of G protein-coupled receptors which relax detrusor smooth muscle by activation of calcium-activated potassium channel (Maxi-K), and by the stimulation of the adenylyl cyclase (AC) pathway increasing 3'-5'-cyclic adenosine monophosphate (cAMP) levels which in turn activates protein kinase A (PKA) and inhibits intracellular Ca^{2+} release promoting miorelaxation. According to these evidence, mirabegron, a β_3-adrenergic receptor agonist, has been recently approved by FDA for the therapy of overactive

bladder symptoms and may constitute a new therapeutic option for BPH treatment. Furthermore, in HCC, β_3-adrenergic receptor activation elicits a relaxation of smooth muscle independently from NO/cGMP pathway, while their activity is related to the inhibition of the ROCK pathway. These observations indicate that β_3-receptors may play a physiological role in penile erection and, therefore, could represent a therapeutic target for treatment of ED[69].

1.6.4 Muscarinic antagonists

Antimuscarinic drugs are mainly suggested for the treatment of moderate to severe storage symptoms consequent to BPH[21]. The muscarinic receptor antagonists licensed for treating of overactive bladder and storage symptoms are darifenacin, fesoterodine, oxybutynin, propiverine, solifenacin, tolterodine, and trospium chloride. Muscarinic receptors are densely expressed on detrusor smooth muscle cells and other cell types, such as epithelial cells of the salivary and prostatic glands, urothelial cells, and nerve cells of the peripheral or central nervous system. Ach acting on muscarinic receptors type 3 (M_3 receptors) distributed on detrusor muscle represents the main stimulus for the induction of urinary bladder voiding. In particular, M_3 receptor activation causes PLC to generate IP_3 which binds to and opens IP_3 receptor located on endoplasmatic reticulum with a subsequent Ca^{2+} release. Ca^{2+}/CaM complex is enabled and activates MLCK-dependent pathway inducing contraction[72]. Thus, the inhibition of these receptors reduces smooth muscle cell contractions and the sensory threshold of the bladder. Randomized controlled trials have been demonstrated that antimuscarinic drugs can significantly reduce urgency incontinence, daytime frequency, and urgency-related voiding. Moreover, low treatment compliance in patients taking anticholinergic drugs has been issue with reports ranging from 35% to 44% of patients not continuing treatment, possibly due to inadequate drug efficacy or intolerable side effects such as dry mouth, constipation, micturition difficulties, nasopharyngitis, and dizziness[73] .

1.6.5 Phosphodiesterase type 5 inhibitors (PDE5-Is)

PDE is any enzyme that breaks a phosphodiester bond. To date, 11 families of PDEs have been identified in mammalian tissues. Their physiological role is to hydrolize specifically cAMP and cGMP to their respective linear 5'-nucleoside monophosphates AMP and GMP. Indeed, these enzymes vary in their substrate specificity for cAMP and cGMP. In detail, PDE5, PDE6 and PDE9 are specific for cGMP; PDE4, PDE7 and PDE8 are selective for cAMP while PDE1, PDE2, PDE3, PDE10 and PDE11 have mixed specificity for cAMP/cGMP[74-76] (***Figure 5***).

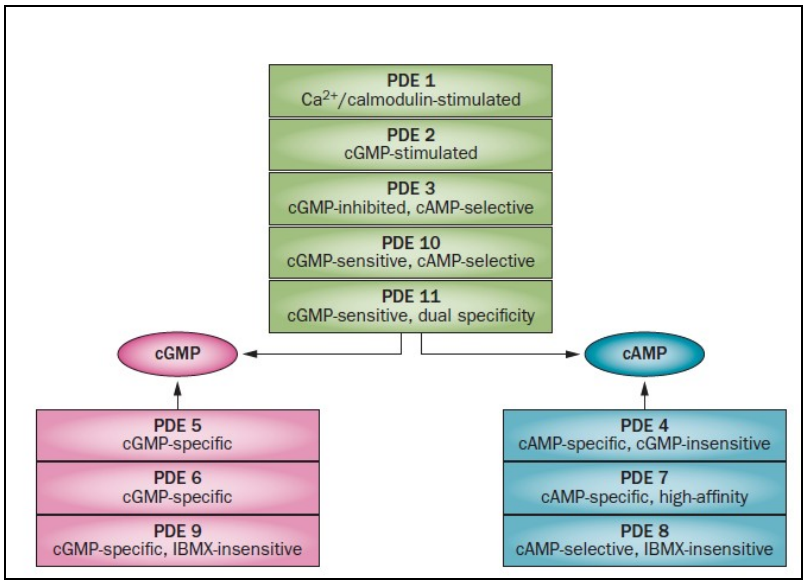

Figure 5. Different PDE isoenzymes with their affinity for cGMP and cAMP (*The role of phosphodiesterases in bladder pathophysiology. Rahnama'i M, 2013*).

PDEs distribution and functional significance vary among different tissues and cell type[77]. It is known that isoenzymes 1, 2, 3, 4, 5, 7, 8, 9, and 10 are expressed in the human prostate[78], whereas isoenzymes 1, 3, 4, and 5 are present in the human detrusor[79]. Like most other PDEs, PDE5 is involved in many physiological and pathological processes. However, it is best known its role in catabolism of cGMP in smooth muscle of various organs, particularly, the penis. Since the well known role of PDE5 in erectile function/dysfunction, there has been an increase of interest in

expanding its research into other tissues, in the hope that PDE5-Is can also treat these tissues' associated diseases. In particular, it seems fitting for urological researchers to shift their attention to diseases such as overactive bladder, urinary incontinence and BPH. It has been demonstrated that PDE5 is the most abundant cGMP-specific PDE isoform expressed in the LUT specifically in the prostate[78, 80-83], urethra[80, 82, 84] and bladder[80-82] (***Figure 6***).

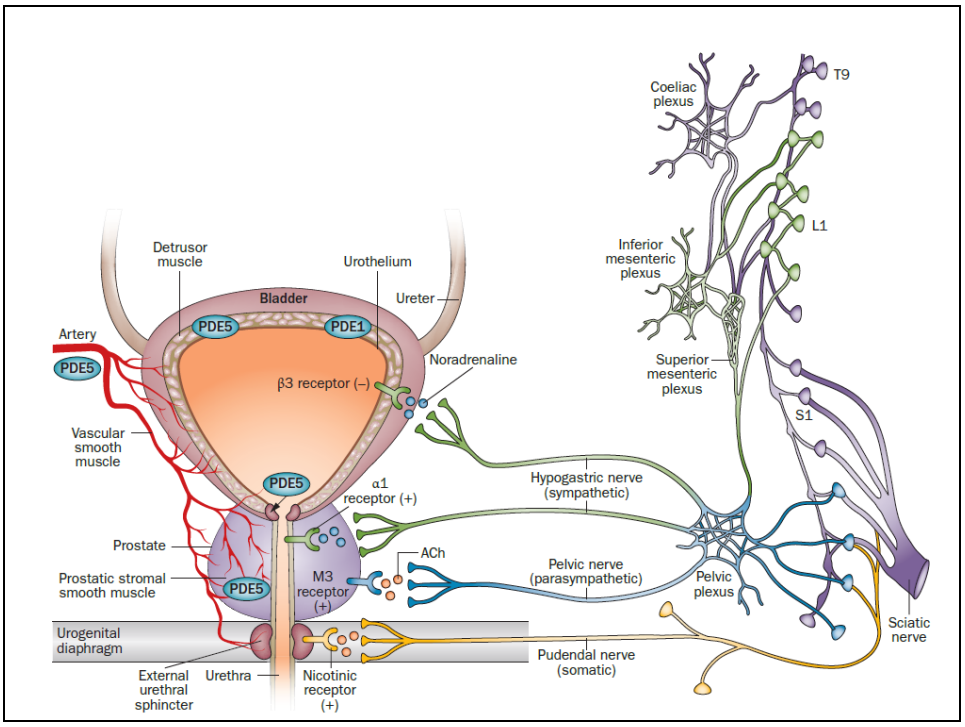

Figure 6. Phosphodiesterases distribution in prostate, urethra and bladder with innervation in human male lower urinary tract (*The role of phosphodiesterases in bladder pathophysiology. Rahnama'i M, 2013*).

In this way, several studies show that PDE5-Is, widely used as first-line oral treatment of ED, are effective in the treatment of LUTS. Since the beneficial effects of PDE5-Is seem not to be totally dependent on NO/cGMP signalling, their mechanism of action is still unclear and under investigation[85]. Actually, three selective oral PDE5-Is, i.e. sildenafil, tadalafil, and vardenafil, have been licensed in Europe for the treatment of ED and used in clinical trials in male patients with LUTS.

Nevertheless, only tadalafil (5 mg once daily) has been approved for the treatment of BPH-associated LUTS in men with or without ED. In LUT, NO is an important non-adrenergic, non-cholinergic neurotransmitter that acts alongside the classical adrenergic and cholinergic systems to regulate micturition[18, 86]. NO is thought to inhibit efferent neurotransmission in the urethra and to modulate afferent neurotransmission in the bladder, as well as related reflex pathways in the spinal cord[86, 87]. NO released from nerve endings and from urothelium diffuses into smooth muscle cells, where it induces relaxation through cGMP synthesis[88]. cGMP levels are depending on the balance between its synthesis mediated by sGC and its catabolism by PDE.

Moreover, it is well known that NO, cAMP and cGMP have a role in control of the micturition process and hence, are suggested to be involved in the pathophysiology of storage and voiding disorders. Therefore, the inhibition of PDEs increases intracellular cGMP and cAMP levels leading to urinary bladder smooth muscle relaxation. To date, although many preclinical studies have been conducted, only PDE1 and PDE5 inhibitors have been tested clinically for the management of LUTS. Treatment with PDE1 inhibitors, such as vinpocetine, the only agent approved for clinical use, might improve micturition frequency in patients with overactive bladder, whereas inhibition of PDE5 improves LUTS in men, either with or without BPH and ED. Increased cGMP production mediates most of non-lytic physiologic effects of NO. Therefore, modulating intracellular cGMP levels has been targeted for pharmacologic intervention in disorders directly influenced by vascular smooth muscle. PDE5-Is are structurally similar to cGMP and compete with cGMP at the catalytic site of PDE5, causing a reduction of cGMP degradation and an increase of its levels (***Figure 7***).

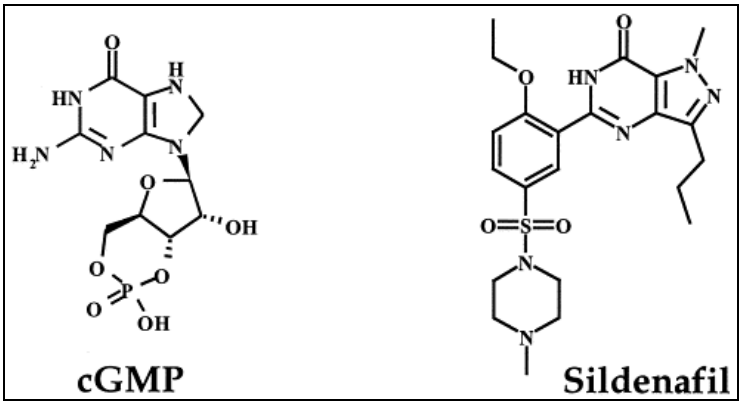

Figure 7. Comparison between cGMP, the native substrate of PDE type 5 and a competitive inhibitor, sildenafil (*Phosphodiesterase type 5 as a pharmacologic target in erectile dysfunction. Corbin JD, 2002*).

Therefore, it has been hypothesized that PDE5-Is, by increasing intracellular cGMP levels in lower urinary tract, can potentially modulate sensory signals and microvasculature dilation, reduce smooth muscle tone of the prostate, urethra, and bladder[19]. Furthermore, improvements in both storage and voiding urinary symptoms observed with PDE5-Is may be due to the regulation of the afferent nerve activity in the bladder neck, prostate, and urethra. In addition, to the relaxation of smooth muscle cells, PDE-Is may increase the LUT perfusion, resulting in increased tissue oxygenation, which is impaired during LUTS/BPH disorder[19].

1.6.6 Comparison of beneficial effects among PDE5-Is

Sildenafil. It has been reported that sildenafil monotherapy markedly improves quality of life (QoL) scores in men affected with both ED and LUTS[89] and IPSS of at least four points in 35% of 60% of patients[89]. Moreover, LUTS scores are significantly improved in all 189 men affected with ED and moderate to severe LUTS associated to BPH[90] under sildenafil therapy. Interestingly this improvement is independent from baseline body mass index (BMI) and is greater in men with severe LUTS than in men with moderate symptoms. Finally, men with both LUTS and ED, following a chronic treatment of 12 weeks with alfuzosin, α_1-blocker, and/or

sildenafil have a significant improvement in IPSS and the most pronounced beneficial effect is the association of the both drugs[91].

Vardenafil. In a clinical study, in which a cohort of 222 men with ED and LUTS secondary to BPH have been enrolled, vardenafil significantly improves these disorders and subsequently QoL, and these improvements occurs as early as 8 weeks after initiation of therapy[92]. The efficacy of a single dose of vardenafil has been also assessed in a trial of 25 male patients with micturition disorders secondary to spinal cord injury, who have been also taking the anticholinergic agent oxybutynin. Vardenafil significantly decreases maximum detrusor pressure, improves maximum cystometric capacity and increased bladder volume at first detection of detrusor overactivity[91]. The effect of the association of vardenafil and the α_1-blocker tamsulosin is superior to that showed by tamsulosin in monotherapy[93].

Tadalafil. It has been reported that a single oral dose of tadalafil exerts marked beneficial effects on urodynamic parameters in patients affected with neurogenic bladder disorder caused by soprasacral spinal cord injury[94]. A clinical study conducted on 281 men affected with ED and LUTS provides evidence that tadalafil is also affective in the therapy of LUTS secondary to BPH, even if the treatment does not show any significant changes in uroflowmetry findings[95]. Moreover, few trials have evaluated the effectiveness of PDE5-Is in combination or alone with α-blockers or antimuscarinic drugs. Patients affected with ED and urinary storage symptoms show an improvement of IPSS following a chronic treatment of 12 weeks with 5 mg/daily of tadalafil. This benefit is equivalent to that induced by the treatment with the antimuscarinic agent solifenacin[96]. Furthermore, another clinical trial, in which 30 men aged > 50 years with LUTS secondary to BPH, demonstrates that the beneficial effect exerted by the association of tadalafil with the α_1-blocker tamsulosin is superior to tamsulosin treatment alone[97]. Moreover, the long-term benefits of tadalafil treatment have been investigated in a 1-year open-label study, which shows that the drug is well tolerated, and that the efficacy of this treatment is maintained[98]. On the basis of these overall evidence, FDA has approved the use of tadalafil 5 mg once daily for the treatment of LUTS secondary to BPH in men with or without ED.

1.7 Phosphodiesterase type 5 localization in human prostatic gland and urinary bladder

1.7.1 Prostate. Several experimental studies have demonstrated that NO/cGMP signalling and related proteins, such as cGMP-degrading PDE5, play a key role in the regulation of the physiological function of the prostate, including the contractile activity of the smooth muscle, glandular secretion, glandular epithelial cells proliferation[99, 100]. In 1970, for the first time the activity of PDEs was isolated from human prostate gland[101], and the expression of mRNA encrypting for PDE5 was confirmed by RT-qPCR[80]. However, these findings did not convey any evidence about the localization of this enzymatic isoform in the prostate tissue. Just later by immunochemistry, this cGMP specific PDE was localized in glandular areas[102], in the smooth muscle of the prostatic stroma, and blood vessels[102, 103], with a prominent localization in vascular tissue of human prostate, including both endothelium and smooth muscle cells. Furthermore, it has been shown that PDE5 is localized in close conjunction with its main substrate cGMP and that cGMP-dependent kinase G (PKG) is abundant in the musculature. Interestingly, cAMP-binding protein kinase A (PKA) was also found in the tissue, given that the rich innervation containing the neuropeptide, vasoactive intestinal polypeptide, that promotes the formation of the intracellular messenger cAMP[104], involved in the regulation of muscle tension in the transition zone too.

1.7.2 Urinary bladder. It is already well known that NO/cGMP pathway has a pivotal role in the regulation of micturition and changes in intracellular cGMP levels could be considered as a promising possibility to achieve a selective modulation of smooth musculature tone of the bladder. The first evidence concerning the presence of PDE5 in human detrusor dates back to 1996[79]. PDE5 was localized in smooth muscle wall of the bladder and also in the bladder vascular system, in particular in endothelium and smooth muscle layer of the vesicular-deferential arteries, involved in blood perfusion of the detrusor muscle. In the matter of PDE5 expression in the urothelium, it was found only sparse[81]. Moreover, interesting to report is the

comparison of PDE5 expression among detrusor, corpus cavernosum and prostate tissue. It has been demonstrated that the expression of mRNA encoding for PDE5 was much higher in penile tissue and in detrusor muscle than in prostatic gland[80, 81].

1.8 Experimental evidence on smooth muscle relaxation induced by PDE-Is: prostate and urinary bladder

The enhancement of intracellular cGMP levels by blocking its catabolism, for example with PDE5-Is, leads to a relaxation of lower urinary smooth musculature; it has been investigated the mechanism of this enhancement in prostate and urinary bladder by using *in vitro* tissue bath studies and applying agent such as muscarinic (carbachol) or adrenergic agonists (norepinephrine) to induce contraction, as well as NO donor (sodium nitroprusside, SNP) or PDE-Is, to stimulate the cGMP signalling.

1.8.1 Prostate. Tissue bath studies conducted on smooth muscle of non diseased human prostate revealed that sildenafil and zaprinast (1 nM-10 μM) induced a dose-dependent relaxation of the pre-contracted tissue with norepinephrine[78, 105]. However, the percentage of relaxation of prostatic smooth muscle induced by PDE5-Is did not exceed the 30%. Another experimental study performed in the same condition, showed that the tissue contraction was antagonized by vardenafil and tadalafil respectively of 35% and 52%[105]. Furthermore, it has been reported that PDE-Is are more effective to induce relaxation in presence of SNP, because of the synergistic effect coming from the enhanced tissue production of cGMP by SNP and the inhibition of its catabolism by PDE5-Is. In addition, PDE5-Is may also induce prostatic miorelaxation interfering with endothelin-1 pathway[106].

1.8.2 Urinary bladder. To investigate the effect of PDE5-Is on human urinary bladder, it is really necessary to make a difference between bladder dome and bladder neck musculature, because the NO/cGMP signalling pathway regulates the human bladder smooth musculature in different way according to the region of bladder considered. Indeed, different studies on square-shaped strips of human detrusor without urothelium did not show any significant evidence supporting a role of

PDE5-Is in the regulation of human bladder, given that the relaxant effect induced by a cumulative dose-response of zaprinast (0.01-200 μM) was less than 20%[79]. Contrarily, when the tissue considered was the bladder muscle dome, sildenafil exerted a direct relaxant effect, even if high concentration of PDE5-Is were required. The mechanism of sildenafil induced relaxation involves both cGMP pathway and K^+ channels. Besides the presence of SNP did not alter the maximum effect induced by sildenafil, suggesting that nitrergic pathway makes a minor contribution in sildenafil induced relaxation[107].

2. HYDROGEN SULFIDE

2.1 Hydrogen sulfide: a new gasotransmitter

Hydrogen sulfide (H_2S), along with NO and carbon monoxide (CO), is increasingly recognized as a member of a growing family of gasotransmitters. NO received much attention over the last three decades for its pivotal role in vascular homeostasis[108] and extensively considered as a central endothelial-derived relaxing factor (EDRF) and a key regulator of cardiovascular pathophysiological responses. However, the importance of this signalling molecule is being re-evaluated with the most recently discovered gasotransmitter H_2S. Once considered to simply be a environmental toxic gas, H_2S is now believed to be an important biological mediator. It plays many important regulatory roles in several physiological systems; it is involved in regulation of vascular homeostasis, cytoprotection, neurological function, anti-inflammation, along with modulation of cell survival responses. Here, I describe recent advances in the understanding of the biosynthesis, catabolism, and cell biology of this gas. Specifically, I focus on the effect of H_2S on cell signalling processes involved in the human genito-urinary tract system.

2.2 Physical and chemical features of H_2S

H_2S is a colourless gas with a strong odour of rotten eggs. Chemically, H_2S is the sulfur analogue of water molecule and can be oxidized into sulfur dioxide, sulphate and elemental sulfur[109]. H_2S is a weak diprotic acid, characterized by pK_{a1} and pK_{a2} values of 6.76 and >12 respectively[110]. In aqueous solution, it dissociates as follows:

$$H_2S \leftrightarrow HS^- + H^+ \leftrightarrow S^{2-} + 2H^+ \ ^{111}$$

However, under physiological condition, at pH 7.4 and at mammalian body temperature of 37°C, one third of H_2S remains not dissociated (18.5%) and two thirds dissociated in hydrosulfide anion (HS^-) and H^+ (81.5%), as predicted by the

Henderson-Hasselbach equation[110]. HS^- may subsequently decompose to H^+ and sulfide ion (S^{2-}), but this reaction occurs only at high pH, thus S^{2-} does not occur *in vivo* at significant amounts[112]. Given that all these three species of sulfide are always present in aqueous solution, even if in different percentage, it has not been possible to determine which of these species is biologically active. Thus, the terminology "H_2S" usually refers to the sum of H_2S, HS^-, and S^{2-}. Similarly to NO and CO, H_2S is characterized by high lipophilicity (its solubility in lipophilic solvents is approximately fivefold greater than in water), therefore it freely permeates the hydrophobic core of the cell plasma membranes, and rapidly diffuses into or out of cells[113].

2.3 Endogenous H_2S biosynthesis

H_2S is constitutively produced in mammalian tissue *via* both enzymatic and non-enzymatic pathways[114]. The latter pathway, less important endogenous source of H_2S, consists of reduction of thiols, thiol-containing molecules, such as thiosulfate or thiocysteine, or elementar sulfur using reducing equivalents obtained from the oxidation of glucose[115] (***Figure 8***).

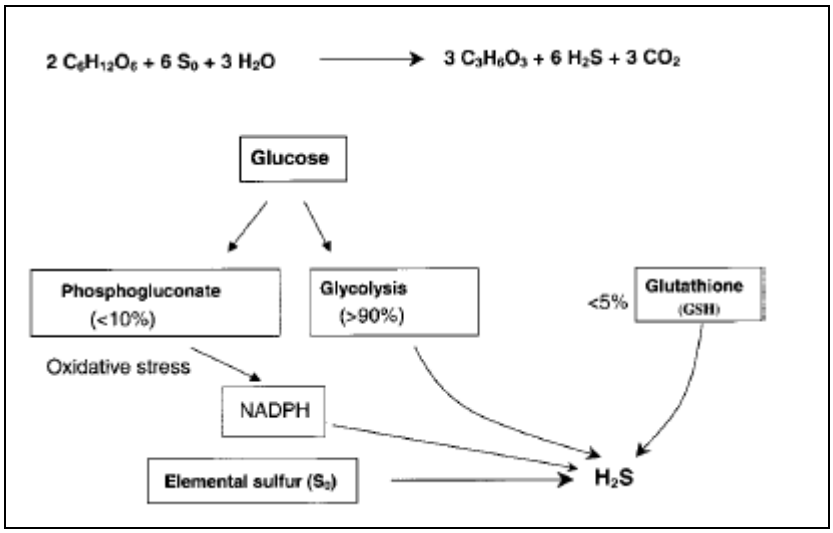

Figure 8. Non enzymatic production of endogenous H_2S (*Two's company, three's crowd: can H_2S be the third endogenous gaseous transmitter?. Wang R, 2002*).

Furthermore, H_2S can be derived under reducing conditions from sulfane sulfur, an intracellular sulfur storage[116], that occurs when H_2S interacts with cysteine thiols to form stable persulfides. This mechanism takes place at pH of 8.4, for this reason its physiological relevance is not fully understood. However, the non-enzymatic pathway contributes just partially to endogenous H_2S production[115]. Indeed, most of the interest has been placed on the enzymatic biosynthesis of H_2S (***Figure 9***).

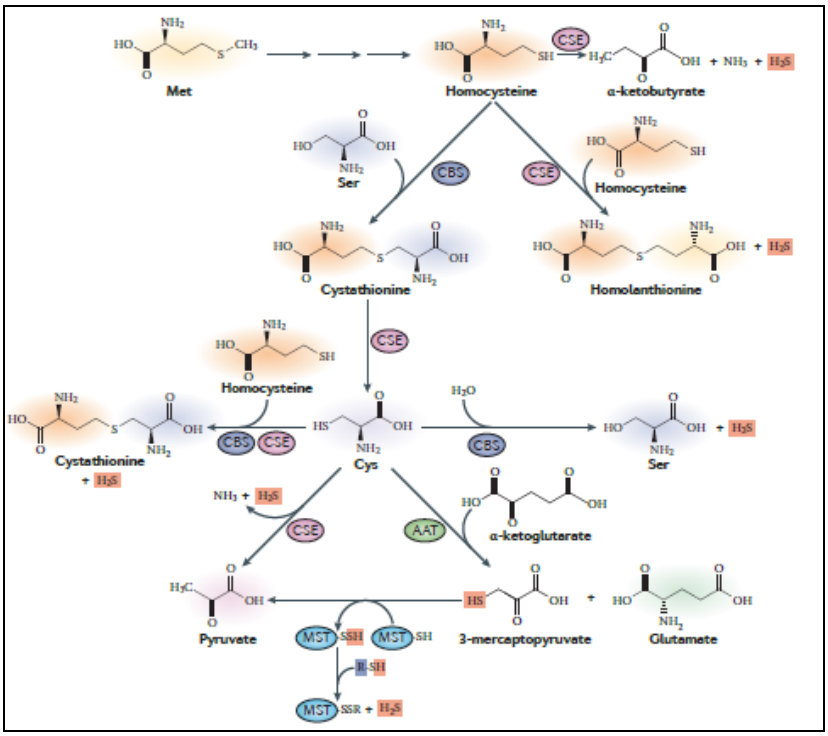

Figure 9. Enzymatic biosynthesis of endogenous H_2S (*H_2S signaling through protein sulfhydration and beyond. Bindu DP, 2012*).

H_2S is endogenously produced from the substrate L-cysteine, a sulfhydryl amino acid derived from the diet or synthesized from methionine (Met) through the transsulfuration pathway[117] by the action of three enzymes: cystathionine β-synthase (CBS), cystathionine-γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3-MST). Despite three different pathways have been identified, only two enzymes, CBS

and CSE, are believed to be the most important protein involved in the endogenous H_2S generation. CBS condenses homocysteine, derived from Met, with L-serine generating cystathionine, which is converted to L-cysteine by CSE. This L-cysteine is combined with homocysteine by CBS[118-120] or CSE leading to the production of cystathionine and H_2S. CSE can also produce H_2S and pyruvate from L-cysteine[119-121]. A third potential H_2S generating pathway is mediated by aspartate aminotransferase (AAT), which catalyzes the reaction of L-cysteine with keto acids (α-ketoglutarate) to form 3-mercaptopyruvate, which is then desulfurated by 3-MST to generate H_2S. The sulfur group of 3-mercaptopyruvate is linked to the cysteine residue in the catalytic site of 3-MST (3-MST-SH) to produce a persulfide (3-MST-SSH). The persulfide releases H_2S in the presence of a reducing agent, which generates the corresponding disulfide (3-MST-SSR) and H_2S. CBS, CSE and AAT require necessarily for their enzymatic activity the cofactor pyridoxal 5'-phosphate (PLP), which is the active form of vitamin B_6, conversely to 3-MST which activity is zinc-dependent. Furthermore, these enzymes differ from each other for their intracellular compartmentalization. Indeed, AAT and 3-MST are both mitochondrial and cytosolic[122], whereas CBS and CSE appear to be exclusively cytosolic[123]. The expression of these enzymes has been found in a number of cell types, including those from the liver, kidney, heart, vasculature, brain. Moreover, it has been characterized by multiple evidence the organ-specific expression of CBS and CSE. CBS is the predominant H_2S-generating enzyme in nervous system and brain[124] and it is also highly expressed in liver and kidney, while CSE is mainly expressed in vascular and non-vascular smooth muscle[125]. Concerning 3-MST, along with AAT, it contributes to H_2S formation in both the brain[126] and the vascular endothelium[127]. In addition, it has been demonstrated that CBS and CSE are secreted by endothelial cells and hepatocytes, circulate as members of the plasma proteome, thus existing also as circulating enzymes[128].

CSE is a protein of 405 amino acid residues and is a tetramer formed by two homodimers, both contributing to the active site pocket[129]. It is selectively activated by Ca^{2+}/CaM complex in a similar way to the activation of endothelial NO synthase

(eNOS)[130]. The regulation of CSE is not totally understood, but there are evidence reporting that myeloid zinc finger 1 and specificity protein 1 have an important role in its basal transcriptional activity and that it can be up-regulated by bacterial endotoxin[131] and NO[132]. Regarding CBS, more evidence are known about its regulation and they are described in details below.

2.3.1 CBS: a modular protein organization. CBS catalyzes the condensation of L-serine and homocysteine to give cystathionine and H_2S[133]. In this reaction, the hydroxyl group of L-serine is replaced with the thiolate of homocysteine. In addition, CBS can also efficiently generate H_2S from a combination of L-cysteine and homocysteine[118, 119]. In addition to H_2S production, CBS has a prominent role in maintaining homocysteine levels *in vivo*. Patients with hyper-homocysteinaemia (HHcy) exhibit substantial cardiovascular disability[134] due to an impairment of CBS activity. The gene of human CBS is localized to chromosome 21 at 21q22.3[135]. In human and rat, CBS exists primarily as homo-tetramer consisting of 63 kDa subunits, and each one comprises 551 amino acid residues[136]. CBS displays a modular organization in which every subunit contains the binding sites for the cofactors PLP, heme[137], and S-adenosylmethionine (SAM) (***Figure 10***).

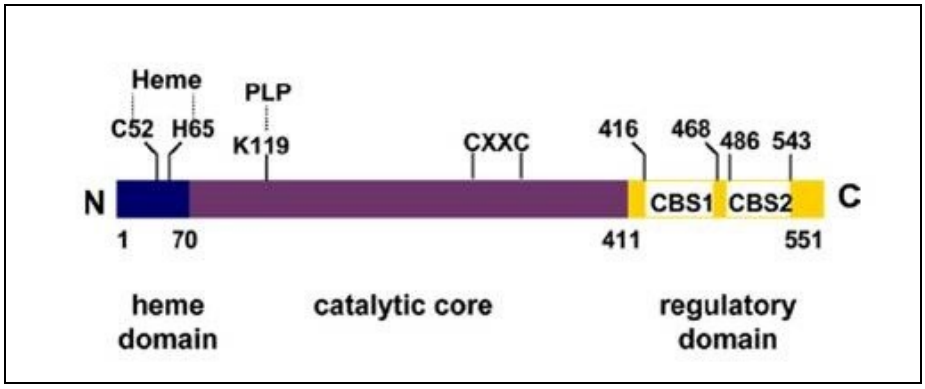

Figure 10. CBS: a modular protein organization (*Redox regulation and reaction mechanism of human cystathionine-β-synthase: a PLP-dependent hemesensor protein. Banerjee R, 2004*).

In details, the NH_2-terminal of CBS contains the binding site for heme followed by PLP binding site. The COOH-terminal of CBS contains a regulatory domain of ≈140 amino acid residues, and confers responsiveness to SAM, that is an allosteric activator of the enzyme. The heme group is a redox sensor[138] and it binds to the NH_2-terminal portion of CBS comprising about 70 amino acids, while the PLP binding domain is considered to be the catalytic pocket, and it is deep in the heme domain. The ferrous form of CBS, which forms under oxidizing conditions, is less active than its ferric form[139]. When CBS is in the ferrous state, CO and NO can bind the heme domain[140]. CO binding inhibits in a reversible way the catalytic activity of CBS[141-143] with higher affinity (K_i ≈5.6 μM), whereas NO is only about two percent as potent so that its binding probably is not physiologically relevant (K_i ≈360 μM)[140, 144]. In the presence of physiological reducing system, such as methionine synthase reductase and NADPH, the inhibition of CBS can be reverted[145]. However, the redox potential of Fe^{3+}/Fe^{2+} couple in CBS is very low (-350 mV), thus the feasibility of CBS to be in oxidized form has been in question. Human CBS is activated by the allosteric regulator SAM[124, 146], a major methyl donor and the precursor of S-adenosylhomocysteine and homocysteine. Although the binding site for SAM is not known yet, it is retained to occur by binding to the COOH-terminal domain of CBS, which increases 2-fold the H_2S production[118, 120]. Furthermore, it has been reported that the COOH-terminal portion of CBS contains tandem repeat of two "CBS domains". This part appears to be an inhibitory domain as its deletion activates CBS of 4-fold, giving a super-activated state of the enzyme[147, 148]. In addition, the CBS domains have been proposed to act as energy sensors[149]. This notion is based on findings that AMP-activated protein kinase binds the enzyme at its CBS domains[149].
Recently, it has also been reported that Ca^{2+}/CaM pathway is involved in the regulation of CBS activity[150], given that both CBS and CaM coimmunoprecipitate. The binding consensus sequence for the complex Ca^{2+}/CaM has been identified in CBS domains[150] and enzymatic activity is suppressed by specific inhibitors of this complex. In the absence of interaction between the Ca^{2+}/CaM complex and CBS, the enzymatic activity remains at basal level because the COOH-terminal may cover the

catalytic domain. The binding of the complex Ca^{2+}/CaM to the 19 amino acid binding consensus sequence induces the exposure of the catalytic domain leading to CBS activation.

2.4 Catabolism of H_2S

To date, three mechanisms have been indicated for H_2S inactivation in the biological systems: 1) oxidation, 2) methylation and 3) scavenging by reactions with metallo-proteins (***Figure 11***).

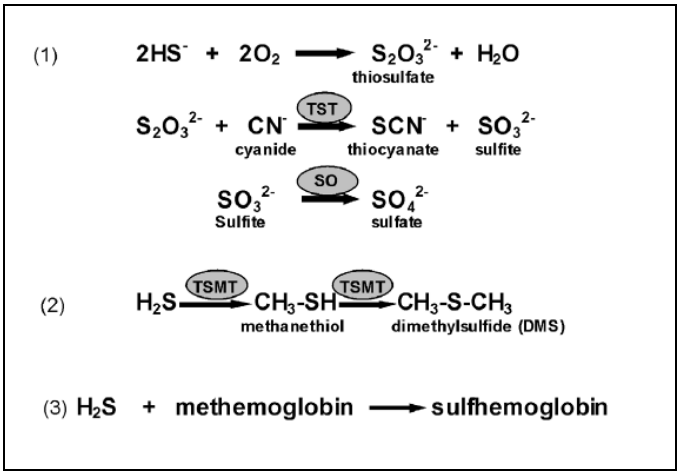

Figure 11. Catabolism of endogenous H_2S through mitochondrial oxidation, cytosolic methylation and binding to hemoglobin (*Hydrogen sulfide- the third gas of interest for pharmacologists. Lowicka E, 2007*)

However, the most important pathway identified is the oxidation, and mitochondria are very effective in this reaction. Endogenous H_2S is quickly metabolized by oxidation in mitochondria to thiosulfate ($S_2O_3^-$) which is further converted to sulfite (SO_3^{2-}) and sulfate (SO_4^{2-}). Oxidation of H_2S to $S_2O_3^-$ is probably a non enzymatic process associated with mitochondrial respiratory electron transport, although this reaction could be catalyzed by superoxide dismutase[151]. Furthermore, thiosulfate cyanide sulfurtransferase (TST) catalyzes the conversion of $S_2O_3^-$ to SO_3^{2-}, transferring a sulfur atom from $S_2O_3^-$ to cyanide or other acceptors[152]. SO_3^{2-} in turn is

rapidly oxidized to SO_4^{2-} by sulfite oxidase (SO). Both $S_2O_3^-$ and SO_4^{2-} are excreted in urine, but under physiological conditions SO_4^{2-} is retained to be a major end-product of H_2S metabolism, indeed $S_2O_3^-$ concentration in urine is less than 1% of SO_4^{2-} [153]. However, urinary $S_2O_3^-$ is considered to be a specific marker of whole-body H_2S production[154]. Nevertheless, it is important to clarify that if the amount of sulfide exceeds the capacity of the mitochondrial enzymes to oxidize it, H_2S can represent a poison for the enzymes[155]. Methylation is an additional metabolic pathway to inactivate H_2S, even though less important than oxidation. Contrarily to oxidation, the methylation takes place mainly in the cytoplasm and it consists in the conversion of endogenous H_2S by thiol S-methyltransferase (TMST) into methanethiol and dimethylsulfide, that it is also a substrate for rhodanase, leading to the generation of thiocyanate (SCN^-) and SO_4^{2-} [156]. In addition, H_2S is a powerful reducing agent and is mainly consumed by endogenous oxidant such as peroxinitrite, superoxide and hydrogen peroxide in the vascular system[157-159]. Finally, H_2S can be scavenged by methemoglobin forming sulfhemoglobin[160] or metallo- or disulfide- containing molecules such as oxidized glutathione (GSSG)[161]. The interaction of hemoglobin with H_2S requires a special attention. Hemoglobin is a common sink for CO and NO in forming carboxyhemoglobin and nitrosyl haemoglobin, respectively. If this sink is filled with one of this gas, the binding of other gases is affected and their individual ability to act on specific targets is altered.

2.5 Mechanisms of relaxation induced by H_2S

2.5.1 H_2S is a K_{ATP} channels opener

Endogenously produced H_2S exerts a host of biological effects on various targets and participates in the regulation of several physiological processes including cardiovascular, nervous, endocrine, reproductive, gastrointestinal and immune systems[117, 162-164]. Furthermore, deregulation of H_2S production is found in many pathological disorders like heart failure, atherosclerosis, diabetes, hypertension, inflammation, sepsis, and erectile dysfunction[117, 165, 166]. H_2S acts as a signalling molecule in physiology and disease through a variety of pathways. Early studies

focused on H_2S involvement in the central nervous system and cardiovascular system. It regulates the action of N-methyl-D-aspartate (NMDA) receptors in the brain and the smooth muscle tone in cardiovascular system. Various studies conducted on cardiovascular[167], reproductive[168], and gastrointestinal[167] smooth muscles have shown that H_2S produces both direct and indirect smooth muscle relaxant effects, inhibits spontaneous motility or prevents chemically or electrically induced contractile responses. In details, H_2S functions as a vasodilatator[130]. To cause vasodilatation, H_2S hyperpolarizes the smooth muscle cells opening the ATP-sensitive K^+ channels (K_{ATP}), as confirmed by sensitivity to glibenclamide or pinacidil, K_{ATP} channels antagonists. Recently, it has been raised the possibility that H_2S induces smooth muscle relaxation, not only *via* opening K_{ATP} channels, but also by activation of myosin-light-chain (MLC) phosphatase induced by cGMP signalling[169]. The precise mechanism by which H_2S induces the activation of K_{ATP} channels is not clear, but the hypothesis is that H_2S affects protein function by S-sulfhydration. H_2S is also known to act on a number of other ion channels. It activates the transient receptor potential channels (TRP) in both urinary tract and airway smooth muscle, while inhibits big conductance Ca^{2+}-sensitive K^+ (BK_{Ca}) channels, T-type and L- type Ca^{2+} channels. A recent report points the involvement of intracellular pH changes and the $Cl^-/HCO3^-$ exchanger in the process of H_2S induced relaxation, and that vascular effect of sulfide is also dependent on O_2 concentration. Indeed, H_2S acts as a vasoconstrictor in well-oxygenated vascular rings systems[170].

2.5.2 H_2S is an endogenous PDE inhibitor

cGMP mediates NO-stimulated vasorelaxation[171] and its levels inside the cells are dependent on the balance between cGMP production and degradation, which is produced by sGC and degraded by PDE[171, 172]. It is well established that cGMP/PKG activates MLC phosphatase leading to the dephosphorylation of MLC, and consequently to smooth muscle relaxation. Furthermore, most of the current evidence indicate that H_2S induce cGMP accumulation by either directly inhibiting the

enzymatic activity of PDE or by increasing intracellular NO bioavailability (***Figure 12***).

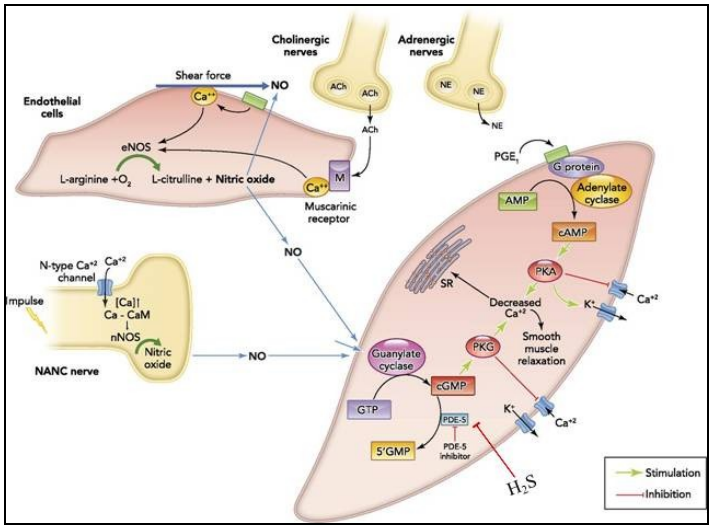

Figure 12. H_2S is an endogenous inhibitor of PDE activity (*Modulation of soluble guanylate cyclase for the treatment of erectile dysfunction. Lasker GF, 2013; with modification*).

Since H_2S affects the function of heme proteins[173], the increase of intracellular cGMP levels could be due to the activation of sGC, in a similar way to NO and CO. Nevertheless, experimental evidence do not show any activation of sGC induced by H_2S[174]. Indeed, it has been demonstrated that H_2S does not stimulate sGC, and its vasorelaxing effect is not inhibited by 1H-[1,2,4] oxadiazolo-[4,3-a] chinossalin-1-one (ODQ), a sGC inhibitor[125]. Having ruled out the possibility that increased cGMP levels in smooth muscle cells results from sGC activation, it has been evaluated the contribution of H_2S on PDE activity. It has been demonstrated by *in vitro* studies that low concentration (nM) of NaHS, an H_2S donor, inhibits transiently PDE activity, increasing both cAMP and cGMP, suggesting that H_2S is a non-selective PDE inhibitor. In addition, the incubation of cultured rat aortic smooth muscle cells with NaHS increased in a concentration dependent-manner cGMP levels[175]. Interestingly, cGMP levels were highly enhanced or attenuated following the overexpression or CSE gene silencing[175].

H_2S can inhibit the PDEs by three mechanisms which involve S-sulfhydration of PDE, binding to Zn^{2+}, a required cofactor for its catalytic activity, and formation of disulfide bonds, very important for PDE activity[175]. Actually, the possibility that H_2S affects the formation of disulfide bonds remains the only viable hypothesis to confirm. An additional mechanism of increasing cGMP levels has been observed in endothelial cells and cardiomyocytes. The exposure to NaHS activates Akt[176], leading to enhanced eNOS phosphorylation on Ser1177[177-180] and reduced phosphorylation of the inhibitory site Thr495[181]. In addition, it has been reported that H_2S induces S-sulfhydration of Cys443, preventing the formation of inactive monomer eNOS[182]. Therefore, H_2S increases NO bioavailability that in turn stimulates sGC producing cGMP.

2.6 Role of H_2S in erectile function

Penile corpus cavernosum function is depending on vasculature tone. Erection is reached when blood vessels or sinusoidal smooth muscle cells in the penis are relaxed and this relaxation is depending on local availability of NO[183]. In presence of erectile disorder, the PDE5-I, sildenafil, exerts its beneficial effect sustaining the local effect of NO by inhibiting the breakdown of cGMP. On the basis of all above mentioned evidence, H_2S may show the same functional effect as NO in dealing with erectile dysfunction. Indeed, it has been reported that H_2S induces relaxation of penile tissue[184, 185]. An earlier study shows that HCC expresses both CBS and CSE[186]. More specifically, muscular trabeculae and smooth muscle component expresses both enzymes, while peripheral nerves only CSE[187]. The blockade of CBS and CSE with the enzymatic inhibitor, AOAA and PPG respectively, enhances the muscle tension induced by electrical stimulation, suggesting the contribute of both enzymes in erectile function. Furthermore, the exposition of human penile tissue to NaHS, or L-cysteine, the endogenous precursor of H_2S, induces relaxation and triggers an erection *in vivo*[187]. H_2S induced human erection seems to involve the activation of K_{ATP} channels in penile smooth muscle cells[187]. Appointed to H_2S a physiological role in

penile function, it has come in question why CSE knocking out in mice does not affect the fertility[130]. It has been demonstrated that the lacking of CSE in mouse penis is likely compensated by the increasing of CBS expression. NaHS induce relaxation of penile tissue at high concentration between 100 μM and 1 mM, suggesting that it is less potent than NO, but on the other hand the life span of NO is much shorter than relative to H_2S. Therefore, low potency of H_2S is compensated by its long-lasting pro-erectile function.

2.7 Role of H_2S in urinary system

Actually, H_2S signalling is well characterized in many physiological processes and pathological conditions related to the cardiovascular and nervous system. To date, the role of H_2S in animal and human urinary system remains poorly understood and very less consistent, because of the availability of conflicting evidence concerning its role in bladder homeostasis. However, many interesting findings regarding H_2S and kidneys continue to be identified, suggesting a pivotal function of H_2S in renal system. Indeed, in addition to the liver, brain, heart and skeletal muscle, kidneys are identified as the major source of H_2S and all three enzymes are involved in normal renal physiology. It has been demonstrated that CBS, CSE and 3-MST are expressed in kidneys[188] and they are mainly present in proximal tubules within the renal cortex[189-191]. In particular, it has been noted that CBS is primarily localized in the proximal convoluted tubule in the outer cortex, while CSE is mostly predominant in straight tubule in the inner cortex[131]. Moreover, a quantitative analysis shows that protein levels of CBS are 20-fold lower than those related to CSE, but in presence of sufficient substrate, CBS produce much more H_2S than CSE in the kidneys[192]. Despite it has been thought for a long time that CBS and CSE were important in the metabolism of homocysteine, a role in vascular and tubular function has been appointed to H_2S. Among tubular functions, it is known that H_2S increases glomerular filtration rate and renal blood flow, augments urinary sodium and potassium excretion. These effects are mediated by induction of vasodilatation and

inhibition of tubular Na^+/K^+ ATPase activity and $Na^+/K^+/2Cl^-$ co-transporter[193]. H_2S is also involved in regulation of renin angiotensin system by modulating directly cAMP levels and indirectly ROS, affecting all hemodynamic parameters[194, 195]. All these findings suggest that H_2S production could be induced by changes in physiological function. Although several studies have demonstrated that H_2S therapy can attenuate glomerular injury[196, 197], CBS and CSE are lacking in glomerular epithelial cells (GECs)[188, 198]. Given that H_2S is a paracrine signalling molecule and characterized by high membrane permeability, its biosynthesis in proximal tubular epithelial cells or vascular endothelium may be enough for GECs function[196]. H_2S has been also proposed as a modulator of the renal oxidative stress response, through both up-regulation of antioxidant proteins, such as Nrf2, superoxide dismutase, catalase, and down-regulation of enzymes responsible of ROS generation[199]. Thus, it is clear that a deregulation of renal H_2S levels can be directly or indirectly a potential concurrent factor in the pathogenesis of any renal disease and represent a considerable pharmacological target. Indeed, decreased endogenous H_2S levels, consequent to CBS reduction, have been associated to chronic kidney disease and glomerulosclerosis induced by HHcy, despite the mechanisms involved in this decrease are not determined yet[200]. Anyway, supplementation of H_2S in mice affected with HHcy-associated renal damage regularizes the glomerular filtration rate and the mechanisms involved in its beneficial effect include suppression of superoxide production, increase of glutathione levels, reduction of macrophage infiltration and inflammation factors[200]. This mechanism is proved by a porcine model of kidney ischemia reperfusion in which Na_2S, an H_2S donor, exerts renal protective effect, largely through its antioxidant and anti-inflammatory actions[201]. While all above mentioned mechanism are related to CBS activity and its expression, the functionality of CSE seems to be more critical in protecting kidneys from diabetic nephropathy. Indeed, in a rat model of diabetes induced by streptozotocin, CSE expression and H_2S levels are markedly reduced in renal cortex[197]. In addition to the kidneys, H_2S also targets other organs in urinary system, such as the bladder, despite much less is known about its role in this organ and conflicting evidence are actually available,

even though coming from different animal species. Indeed, it has been showed that H_2S constricts the detrusor muscle of rat urinary bladder through stimulation of capsaicin-sensitive primary afferent neurons[202], that causes the release of tachykinins. H_2S induced contraction can be prevented by using selective antagonists of tachykinin NK1 and NK2 receptor, suggesting that tachykinins are the mediators of excitatory effect mediated by H_2S in the rat bladder. Furthermore, experimental studies performed on trout bladder show that both response to H_2S and the potential mechanism of H_2S action is unlike that observed in the rat. It has been reported that trout bladder produces H_2S, which relaxes the bladder and decreases spontaneous contractions[203]. To date, any evidence has been reported on endogenous H_2S, its biosynthesis and its cell signalling in human urinary system. Since this lacking in findings, it was very interesting to investigate the H_2S signalling pathway specifically in urinary bladder.

3. AIM I

The clinical efficacy of PDE5 inhibitors in the management of LUTS has been recently demonstrated and it has been suggested that their beneficial effects on voiding symptoms involves the NO/cGMP signalling pathway[99], while their effects on storage symptoms remain still not well understood. However, *in vitro* studies report that sildenafil induces a direct relaxation of human detrusor smooth muscle through the involvement of intracellular messenger, like cGMP and cAMP, and the K_{ATP}, BK_{Ca} and SK_{Ca} channels-dependent signalling pathways[107], while the contribution of NO seems to have a minor importance. Therefore, considering that all the above signalling pathways are also regulated by endogenous H_2S, aim of this study was *in primis* to investigate whether the L-cysteine/H_2S pathway could contribute to bladder function and then evaluate if the relaxing effect of sildenafil on human detrusor dome could involve the H_2S signalling.

4. MATERIAL AND METHODS I

4.1 Human tissue

Bladder dome samples were obtained from 25 male patients aged between 61 and 73 years and affected with severe LUTS secondary to BPH undergoing prostatectomy. All patients were aware of all procedures and gave their informed consent. All patients presented urodynamic obstruction and enlargement of prostatic gland (> 80 ml). Patients who presented bladder stones, urinary infections, detrusor areflexia, a history of urothelial cancer, or recent (< 7 days) or frequent use of PDE5-Is for erectile dysfunction were kept out from the study. Tissue harvesting and all experimental procedures were approved by the Local Ethical Committee (Faculty of Medicine and Surgery, University of Naples Federico II, Naples, Italy). The samples were cleared of blood, adherent tissue, serosal layer and adventitia. To perform the following experiments full thickness bladder (detrusor *plus* urothelium) samples were used.

4.2 Western blot analysis

Bladder dome samples from 9 patients were immediately snap frozen and then homogenized in modified RIPA buffer (50mmol/L Tris-HCl, pH 7.4, 1% v/v Triton, 0.25% w/v sodium deoxycholate, 150mmol/L sodium chloride, 1mmol/L EDTA, 1mmol/L phenylmethanesulphonylfluoride, 10mg/ml aprotinin, 20mmol/L leupeptin and 50mmol/L sodium fluoride). 40 µg of proteins were separated on 8% sodium dodecyl sulphate polyacrylamide gels and transferred to a polyvinylidene fluoride membrane (PVDF). Membranes were blocked for 30 minutes in phosphate buffered saline (PBS) containing non-fat dry milk (5% w/v), followed by overnight incubation at 4°C with rabbit polyclonal CBS (1:500; Santa Cruz) or mouse monoclonal CSE (1:500; Abnova Novus Biologicals). Membranes were extensively washed in PBST (PBS with Tween 20 0.01% v/v) prior incubation with horseradish peroxidase

conjugated secondary antibody for 2 hours at RT. After incubation, membranes were washed and developed using Image Quant 400 (GE Healthcare, US). Rabbit anti-GAPDH antibody was used as loading control (1:5000, Sigma-Aldrich, Milano, Italy). Data were expressed as the mean ± SEM.

4.3 Human bladder strips: functional study

Longitudinal human bladder strips were isolated from the sections obtained from 6 patients. Strips were suspended in 3 mL organ chambers filled with Krebs buffer (115.3 mM NaCl; 4.9 mM KCl; 1.46 mM $CaCl_2$; 1.2 mM $MgSO_4$; 1.2 mM KH_2PO_4; 25.0 mM $NaHCO_3$, 11.1 mM glucose), maintained constantly at 37° C and continuously bubbled with 95% O_2 and 5% CO_2. The strips were connected to isometric transducers (Ugo Basile, Comerio, Italy), stretched until they reached a resting tension of 0.5 g and allowed to equilibrate for 60 min. The tension changes were registered and monitored by a computerized system (DataCapsule, Ugo Basile, Comerio, Italy). In each experiment, the tissues were first challenged with carbachol (1 μM; Sigma, Milan, Italy) until the contracturant responses were reproducible. On stable carbachol tone, a dose-response curve to an H_2S donor, sodium hydrosulfide (NaHS, 0.1 μM to 1 mM; Sigma, Milan, Italy), the enzymatic substrate, L-cysteine, (0.1 μM to 1 mM; Sigma, Milan, Italy), or sildenafil (0.1 μM to 10 μM; Sigma, Milan, Italy) was performed. In another set of experiments, the strips were pre-treated for 30 min with DL-propargylglycine (PPG, 10 mM; Sigma, Milan, Italy) and/or aminooxyacetic acid (AOAA, 1 mM; Sigma, Milan, Italy) inhibitors of CSE and CBS, respectively. Data were calculated as percentage of relaxation and expressed as mean ± SEM.

4.4 H_2S measurement: methylene blue assay

H_2S production rate in human bladder tissue was measured by using Stipanuk and Beck assay with modifications[188]. Briefly, tissues obtained from 5 patients were

homogenized in a potassium phosphate buffer (PPB, 100 mM, pH 7.4) containing sodium orthovanadate (10 mM) and protease inhibitors. Protein concentration was determined by using Bradford assay (Bio-Rad Laboratories, Milano, Italy). Homogenates were added in a reaction mixture (total volume: 500 µl) containing piridoxal-5'-phosphate (2 mM, 20 µl), saline (20 µl). Distilled water (20 µL) or L-cysteine (10 mM, 20 µl) were added to measure the H_2S production in basal or stimulated condition. PPG 10 mM and/or AOAA 1 mM were added 5 min before addition of L-cysteine to inhibit respectively CSE and/or CBS activity. The reaction was performed in sealed tubes and initiated by transferring tubes from ice to a water bath at 37° C and carried out for 40 min. After incubation, zinc acetate (1% w/v, 250 µl) was added, followed by trichloroacetic acid (10% w/v, 250 µl). Subsequently, N,N-dimethylphenylendiamine sulphate (20 mM/7.2 M HCl, 133 µl) and ferric chloride (30 mM/1.2 M HCl, 133 µl) were added, and the optical solution absorbance was measured at a wavelength of 650 nm. All samples were assayed in duplicate, and H_2S concentration was calculated against a standard curve of NaHS (3.12–250 µM). Data were calculated as nanomoles per milligram of protein per minute and expressed as mean ± SEM.

4.5 Sildenafil effect on H_2S production in human bladder tissue

To investigate whether sildenafil had the ability to induce H_2S production in human bladder tissue, fresh samples obtained from 5 patients were incubated with an established sildenafil concentration (10 µM) for 15, 30, or 45 min or with different concentrations of sildenafil (1, 3, 10, and 30 µM) for 30 min, that resulted as the best incubation time. A pre-treatment with AOAA (1 mM) and/or PPG (10 mM), respectively CBS and CSE inhibitors, was carried out for 30 min before sildenafil (10 µM, 30 min) incubation. Finally, in another set of experiments, the fresh tissues were treated with stable analogues of cGMP or cAMP, respectively 8-bromo-cGMP (8-Br-cGMP, 100 µM; Tocris, UK) and dibutyryl-cAMP (d-cAMP, 100 µM; Tocris, UK) for 30 min. The concentration 100 µM was selected on the basis of the *in vitro* use

[204]. After treatment all samples were immediately frozen and then processed for the methylene blue assay. To exclude the feasibility that sildenafil acted as a substrate of CBS and CSE, sildenafil (10 μM, 100 μM, and 1 mM) was added to the homogenates in place of L-cysteine for 40 min. Data were calculated as nanomoles per milligram of protein per minute and expressed as mean ± SEM.

4.6 Statistical analysis

Data were expressed as mean ± SEM. Statistical analysis was determined by using ANOVA and Bonferroni as a post hoc test. Differences were considered statistically significant when p value was less than 0.05. GraphPad Prism software (version 4.02, GraphPad Software, San Diego, CA) was used for all the statistical analysis.

5. RESULTS I

5.1 CBS and CSE expression in human bladder and H_2S levels

CBS and CSE were both expressed in human bladder tissue as showed by Western blot analysis (***Figure 13A***). The homogenate of human tissues generated in basal condition detectable amounts of H_2S (***Figure 13B***). The biosynthesis of H_2S was markedly increased when L-cysteine was added as enzymatic substrate in tissue homogenates as compared to basal H_2S levels (***Figure 13B***; $p < 0.001$). Furthermore, this increase in H_2S level was significantly reverted by pre-treatment with CBS and/or CSE inhibitor, respectively AOAA and PPG (***Figure 13B***; $p < 0.01$ and $p < 0.001$ *versus* L-cysteine, respectively). The replacement of L-cysteine with sildenafil (10 μM, 100 μM, 1 mM) during the incubation of tissue homogenates did not show any changes in H_2S production at any concentrations, suggesting that sildenafil is not an enzymatic substrate of CBS and/or CSE (data not shown).

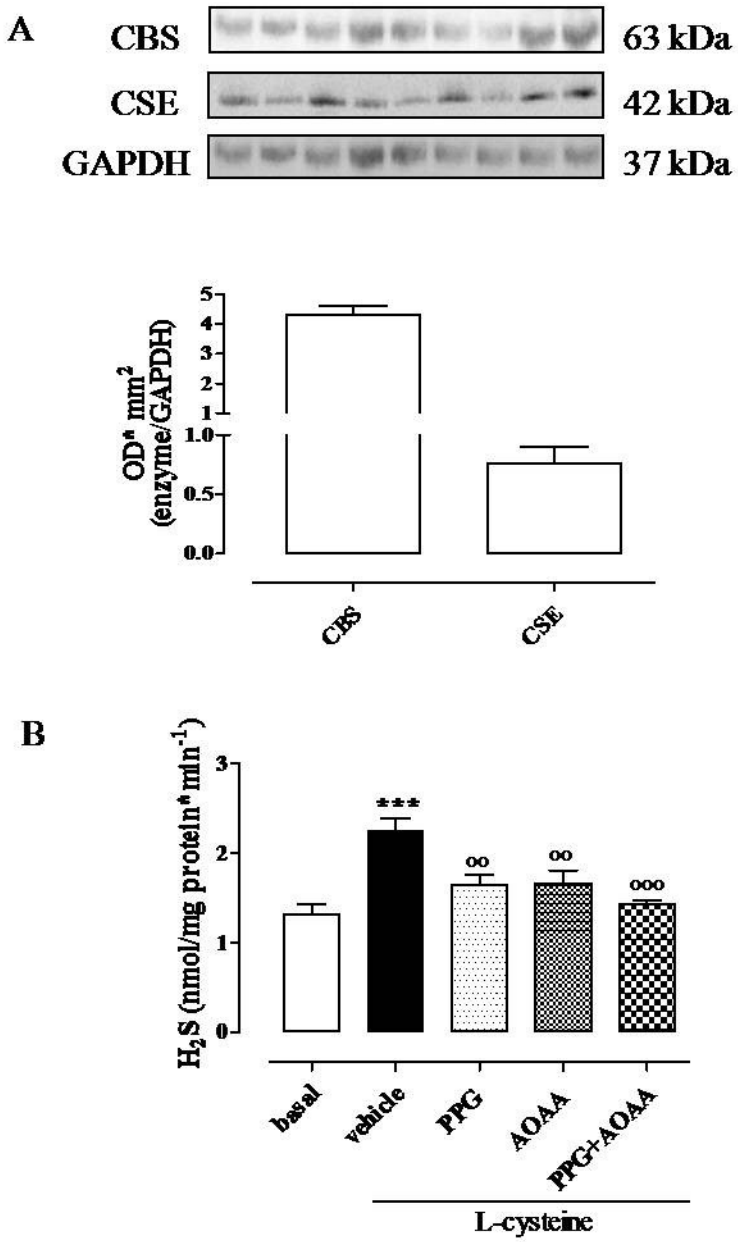

Figure 13*. Immunoblot for CBS and CSE and enzymatic activity in human bladder dome.** (A) Representative Western blot for CBS and CSE and relative optical densitometry (OD) in human bladder samples obtained from nine patients. (B) H_2S production in human tissue homogenates from five patients under basal condition and after incubation with L-cysteine 10 mM. L-cysteine caused a significant increase in H_2S production ($p < 0.001$ vs basal), significantly reverted by CSE inhibitor (PPG; 10 mM) and/or CBS inhibitor (AOAA; 1 mM) (°°$p < 0.01$ and °°°$p < 0.001$, vs L-cysteine, respectively). Data were calculated as nanomoles per milligram of protein per minute and expressed as mean ± SEM.

5.2 H_2S and sildenafil effect on detrusor reactivity

On a stable contraction induced by carbachol (1 μM), NaHS (0.1 μM to 1 mM) or L-cysteine (0.1 μM to 1 mM) elicited a similar concentration-dependent relaxation of human bladder strips (***Figure 14A***). Pre-treatment with PPG (10 mM) or AOAA (1 mM) significantly inhibited L-cysteine induced relaxation (***Figure 14B***; $p < 0.01$ and $p < 0.05$, respectively). Interestingly, sildenafil caused a concentration-dependent relaxation of human tissues (***Figure 14C***) significantly reduced by blockade of CSE and CBS enzymes by using a combination of PPG (10 mM) and AOAA (1 mM) inhibitors (***Figure 14C***; $p < 0.001$). These findings suggest an involvement of H_2S signalling in sildenafil induced relaxation of human detrusor muscle

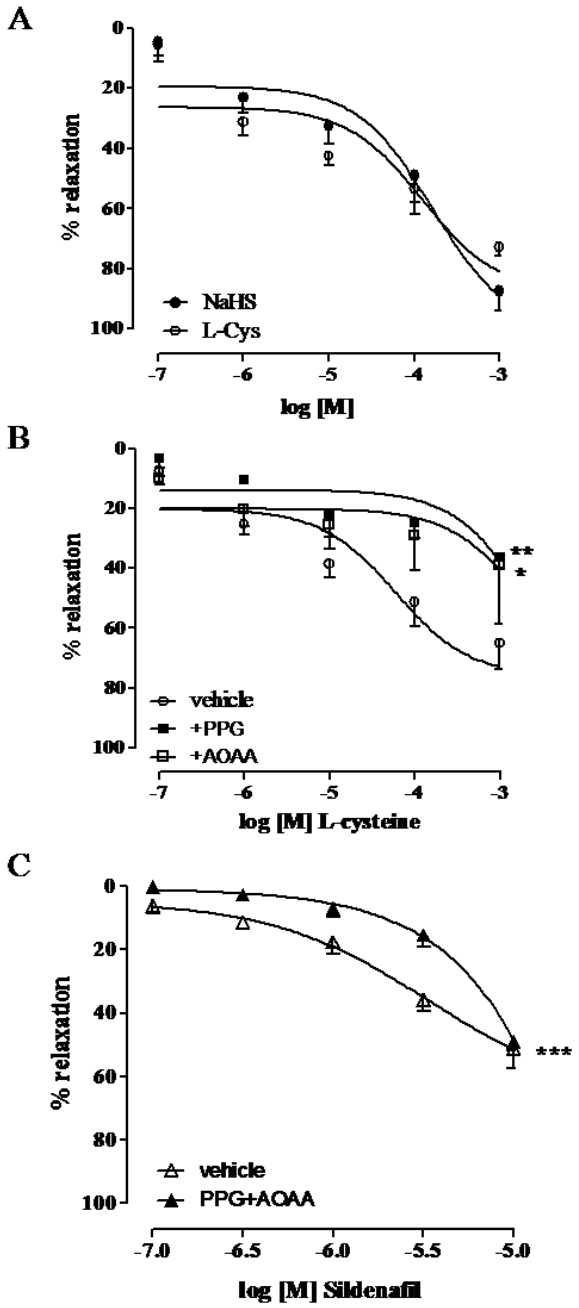

***Figure 14*. Sodium hydrogen sulfide (NaHS), L-cysteine, or sildenafil effect on carbachol induced contraction of human bladder dome strips.** (A) NaHS or L-cysteine caused a relaxation of human detrusor in a concentration-dependent way. (B) Relaxation induced by L-cysteine was significantly reduced by CSE inhibitor (PPG, 10 mM) or CBS inhibitor (AOAA, 1 mM) (**$p < 0.01$ and *$p < 0.05$ vs vehicle, respectively). (C) Sildenafil caused a concentration-dependent relaxation, significantly reduced by the combination of CBS and CSE inhibitors (PPG 10 mM and AOAA 1mM; ***$p < 0.001$ vs vehicle). Data were calculated as percentage of relaxation on carbachol induced contraction and expressed as mean ± SEM. Number of patients per study is equal to six.

5.3 H_2S production induced by sildenafil treatment in human bladder

Pre-incubation of human bladder samples with sildenafil (10 μM) induced an increase in H_2S production in a time-dependent manner (***Figure 15A***; $p < 0.001$ *versus* vehicle; $p < 0.01$ *versus* 30 min or 45 min). Pre-treatment with sildenafil for 30 min resulted the best incubation time. Furthermore, different concentration of sildenafil (1, 3, 10, 30 μM) caused a significant concentration-dependent increase in H_2S production when compared to vehicle (***Figure 15B***, $p < 0.05$ and $p < 0.01$ *versus* vehicle). Interestingly, the inhibition of CSE and/or CBS exerted by PPG (10 mM) and AOAA (1 mM) markedly reduced the increase of H_2S production induced by sildenafil (***Figure 15C***; $p < 0.05$ and $p < 0.01$ *versus* sildenafil, 10 μM; $p < 0.01$ *versus* vehicle).

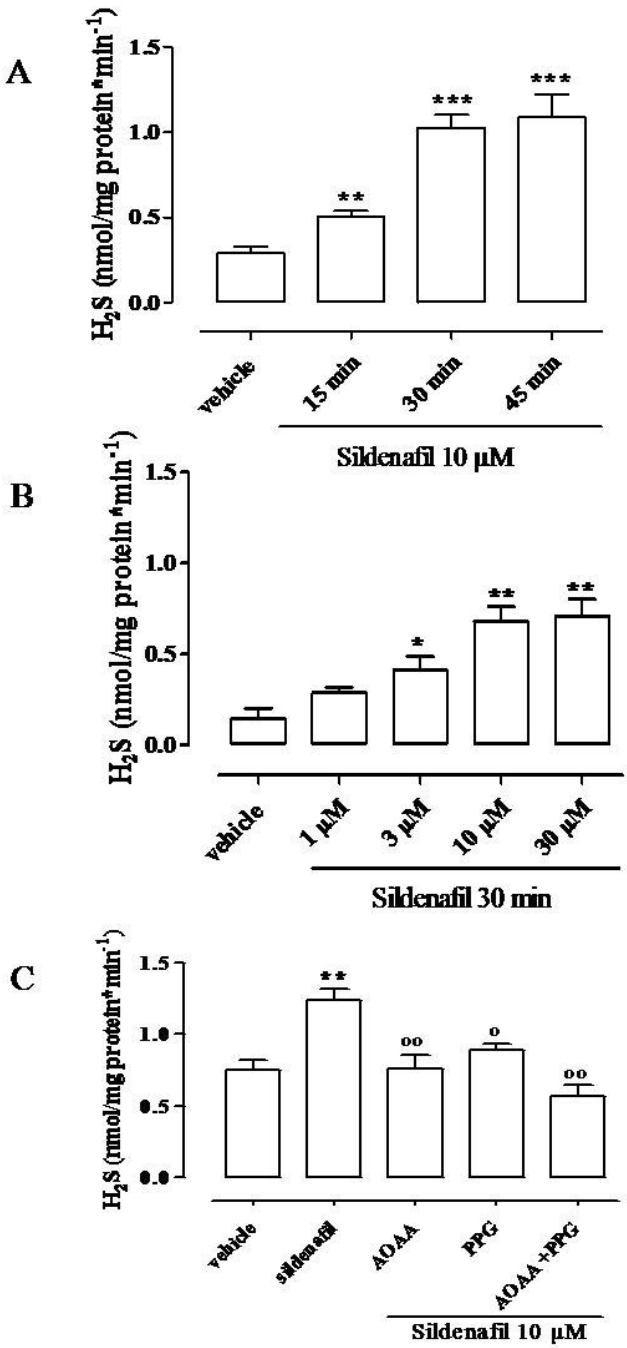

Figure 15*. Induction of H_2S production in human bladder dome by sildenafil treatment**. (A) Sildenafil treatment (10 µM) caused a time-dependent increase in H_2S production ($p < 0.001$ vs vehicle; **$p < 0.01$ vs 30 min and 45 min). (B) Sildenafil incubation for 30 min caused a concentration-dependent increase in H_2S production compared to vehicle (*$p < 0.05$ and **$p < 0.001$). (C) CBS and/or CSE inhibitors (AOAA, 1mM and PPG, 10 mM) significantly inhibited the increase of H_2S production induced by sildenafil (°$p < 0.05$ and °°$p < 0.01$ vs sildenafil 10 µM; **$p < 0.01$ vs vehicle). Data were calculated as nanomoles per milligram of protein per minute and expressed as mean ± SEM. Number of patients per study is equal to five.

5.4 H_2S production induced by stable analogues of cGMP and cAMP in human bladder

Pre-treatment with 8-Br-cGMP (100 μM) and d-cAMP (100 μM), two stable analogues of cGMP and cAMP, resulted in a significant increase of H_2S production similar to that induced by sildenafil (***Figure 16***; $p < 0.05$ *versus* vehicle).

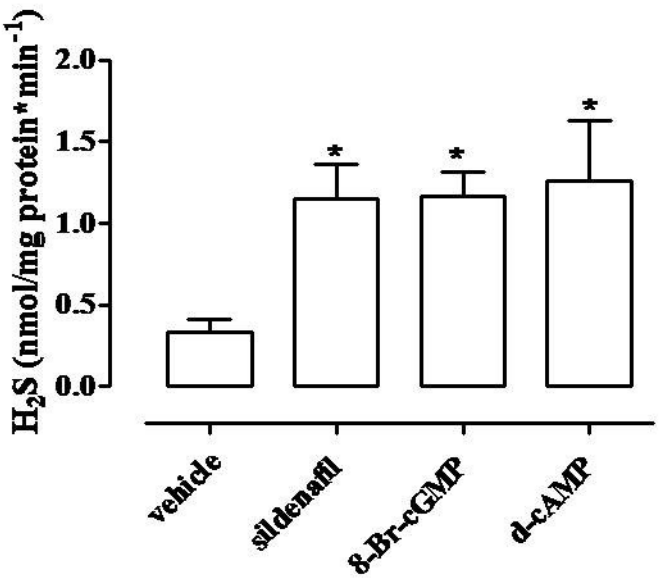

***Figure 16*. cGMP and cAMP induced H_2S production in human bladder.** Incubation of 8-bromo-cGMP (100 μM) or dibutyryl-cAMP (100 μM) for 30 min elicited a significant increase in H_2S production, in a similar way of sildenafil (10 μM) (*$p < 0.05$ vs vehicle). Data were calculated as nanomoles per milligram of protein per minute and expressed as mean ± SEM. Number of patients per study is equal to five.

6. AIM II

We previously showed that H_2S is involved in human bladder homeostasis. Indeed, CBS and CSE are expressed in bladder and both NaHS, H_2S donor, or L-cysteine, endogenous substrate of H_2S-producing enzymes, relax full thickness bladder strips. In addition, the PDE5-I sildenafil and analogues of either cGMP (8-Br-cGMP) and cAMP (d-cAMP) trigger H_2S production in bladder biopsy. This finding raises the feasibility that drugs such as PDE5-Is or β_3-agonists may affect the H_2S signalling pathway through the modulation of intracellular cyclic nucleotides and subsequently induce post-translational modification of proteins[205, 206]. Furthermore, it is widely accepted that urothelium contributes to bladder homeostasis and influences the detrusor smooth muscle tone by releasing a number of signalling molecule[207-209]. Thus, it was very interesting to define the molecular mechanisms by which cGMP and cAMP enhance the H_2S production in human bladder and investigate deeply the H_2S signalling in urothelium tissue. In particular, it has been demonstrated that CBS activity is regulated by post-translational modification through small ubiquitin-like modifier protein which is correlated with the enzyme localization in nucleus and its leading to a diminished activity[210, 211]. Despite post-translational activation of CBS in response to oxidative stress has been demonstrated[212], the molecular mechanism(s) and its pertinence to H_2S production are still not fully understood. Therefore, our next interest was to investigate whether cGMP and/or cAMP, through the involvement of PKG and/or PKA, could trigger CBS phosphorylation and consequently enzyme activation.

7. MATERIAL AND METHODS II

7.1 Human tissue

Full thickness bladder dome samples were obtained from patients as previously described in Material and Methods I. Tissue collecting and experimental procedures were approved by the Local Ethical Committee (School of Medicine and Surgery, University of Naples Federico II, via Pansini, 5; 80131, Naples, Italy). All patients gave their informed consent. The samples were cleared of blood, adherent tissue and serosal layer as well as adventitia. Urothelium and detrusor muscle were carefully dissected, separated and immediately frozen.

7.2 RNA extraction and RT-qPCR

Total RNA from human urothelium was extracted by using the TRIzol® reagent (Sigma-Aldrich, Milan, Italy) according to the manufacturer's instructions and quantified by spectrophotometric analysis. RNA extract was retained DNA- and protein-free when the ratio 260/280 nm was ≥1.7. mRNA was reverse-transcribed in cDNA by using iScript kit (Bio-Rad, Milan, Italy). The RT-qPCR was carried out in CFX384 real-time PCR detection system (Bio-Rad, Milan, Italy) with specific primers from SYBR® Green (Bio-Rad, Milan, Italy). Samples were amplified simultaneously in triplicate in one-assay run with a non-template control blank for each primer pair to control for contamination or dimer formation. Ribosomal protein S16 was used as housekeeping gene to normalize the Ct values. Gene expression was analyzed using the $2^{-\Delta Ct}$ formula.

7.3 T24 cell culture and transfection

Human T24 cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM) with glutamax (Invitrogen, Carlsbad, CA, USA) supplemented with fetal bovine

serum 10% (FBS), L-glutamine 2 mM and penicillin-streptomycin 50 U/ml. A stable T24 cell line silenced for CBS, CBSΔT24, was cultured in DMEM supplemented with FBS 10%, non-essential amino acids 0.1 mM (Euroclone), L-glutamine 2 mM, penicillin-streptomycin 50 U/ml and puromycin 0.5 μg/ml (Sigma-Aldrich, Milan, Italy). Plasmids were transfected into T24 and CBSΔT24 cells (2.5 x 10^6 cells, 60 mm-well plate) by using Lipofectamine 2000 (Invitrogen, Life Technology, Monza, Italy) according to the manufacturer's instructions [213, 214].

7.4 CBS silencing

T24 cells were transfected with CBS short hairpin RNA (shRNA, 2 μg) expressing vector (Santa Cruz Biotechnology, CA, USA). 1 μg/ml of puromycin (Sigma-Aldrich) for 7 days was used to select transfected cells with shRNA and CBS depletion was evaluated by Western blot using anti-CBS antibody (Santa Cruz Biotechnology, DBA, Milan, Italy). The cell clone with lowest CBS expression was selected and used for further experiments. A short hairpin non-silencing construct was used as control.

7.5 CBS site-directed mutagenesis

The wild type (WT)-CBS cDNA was cloned into a version of the eukaryotic expression vector pcDNA4/HisMax C (Invitrogen, Life Technology, Monza, Italy) containing the hemagglutinin (HA) epitope. The constructs containing the substitution of single Ser with Ala, i.e. TCC/GCC mutation, were obtained by PCR site-directed mutagenesis using the QuickChange® Lightning Site-Directed Mutagenesis Kit (Agilent Technology, Agilent Technology, Milano, Italy) and the WT-CBS cDNA as template as previously described [215]. All constructs were verified by DNA sequencing.

7.6 Immunoprecipitation and Western blot

For immunoprecipitation assay, T24 cells were starved for 16 hours and incubated with 100 μM of 8-Br-cGMP or d-cAMP (Tocris, UK) for 5, 15 or 30 min. Samples were analyzed as previously described [216, 217]. The eluted proteins were used in cell free kinase assay, phosphorylation assay and Western blot.

For Western blot aliquots of immunocomplexes and protein samples (30 μg) were resolved by using 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed as previously described[218, 219]. The membranes then were probed with anti-CBS, anti-HA (Santa Cruz Biotechnology), anti-PKA (Cell Signaling Technology), anti-PKG (Cell Signaling Technology), anti-CSE (Novus Biologicals), anti-GAPDH (Sigma Aldrich) and anti-phospho-(Ser/Thr) (anti-pS/T; Cell Signaling Technology). Proteins were visualized with enhanced chemiluminescence detection reagent according to the manufacturer's instructions (Pierce, Rockford, IL, USA).

7.7 H_2S determination: methylene blue assay

H_2S production was evaluated as described in Material and Methods I. Human urothelium samples were incubated with 8-Br-cGMP (100 μM; Tocris, UK) or d-cAMP (100 μM; Tocris, UK) for 30 min. T24 cells were incubated with 8-Br-cGMP (100 μM) or d-cAMP (100 μM) for 5, 15 and 30 min. Treatment at 15 min was selected as the best time condition and used in all successive experiments in both T24 or CBSΔT24 cells. The selective inhibitors of PKG (KT5823, 10 μM, Tocris, UK) or PKA (KT5720, 10 μM, Tocris, UK) were incubated 20 min prior to challenge with vehicle, d-cAMP or 8-Br-cGMP.

7.8 Cell free phosphorylation assay

CBS-WT and CBS mutants immuno-complexes (HA-CBS WT, HA-CBS S32A, HA-CBS S227A, HA-CBS S525A) were incubated at 30° C in a kinase buffer pH 7.4,

containing 10 mM of $MgCl_2$, 20 mM of Tris-HCl, and 0.05 μM of PKG II (Sigma-Aldrich, Milan, Italy). Phosphorylation reactions were started by the addition of 50 μM ATP containing 0.5 μCi [γ-^{32}P] ATP, carried out for 30 min and stopped by the addition of 10 μL of Laemmli SDS stop solution. Proteins were then resolved by 12% SDS-PAGE. Incorporation of ^{32}P was visualized by autoradiography.

7.9 Phosphorylation assay in T24 cells

T24 cells were starved and then incubated with 10 μM of PKG inhibitor (KT5823) or PKA inhibitor (KT5720) for 20 min and treated with or without 100 μM of 8-Br-cGMP or d-cAMP for 5, 15 or 30 min respectively. CBS was immunoprecipitated from protein extracts. The presence of phosphorylation in CBS protein was evaluated by Western blot analysis using anti-phospho-(Ser/Thr) (anti-pS/T,Cell Signaling Technology, DBA, Milan, Italy).

Starved T24 cells were transiently transfected with constructs encoding for HA-CBS WT or mutants and treated with or without 100 μM of 8-Br-cGMP for 15 min. Samples were collected and WT or mutant proteins were specifically precipitated by using antibodies against the epitope HA. Immunoprecipitated proteins were separated by 12% SDS–PAGE and the presence of phosphorylated CBS was analyzed by Western blot.

7.10 Generation of specific anti-pCBS^{Ser227}

Peptide containing amino acids 225-234 (NAS^{227}NPLAHYD) of CBS protein with a single phosphorylated site, Ser227, has been selected and used to obtain polyclonal antibodies, anti-pCBS^{Ser227}, in rabbits (PRIMM s.r.l., Milano, Italy). This antibody was used for Western blot at 1:400 dilutions.

7.11 Statistical analysis

Data were expressed as mean ± SEM. Statistical analysis was determined by using t Student' test or ANOVA followed by Bonferroni as a post hoc test. Differences were considered statistically significant when p value was < 0.05.

8. RESULTS II

8.1 Human tissue: CBS and CSE are expressed in urothelium and produce H_2S

Both CBS and CSE were expressed in human urothelium (***Figure 17A***) as demonstrated by Western blot analysis and mRNA levels of these enzymes were similar as showed by RT-qPCR (***Figure 17B***). Human urothelium homogenates produce detectable amount of H_2S in basal condition (i.e. vehicle), that is significantly increased following the incubation with L-cysteine (***Figure 17C***, $p < 0.01$).

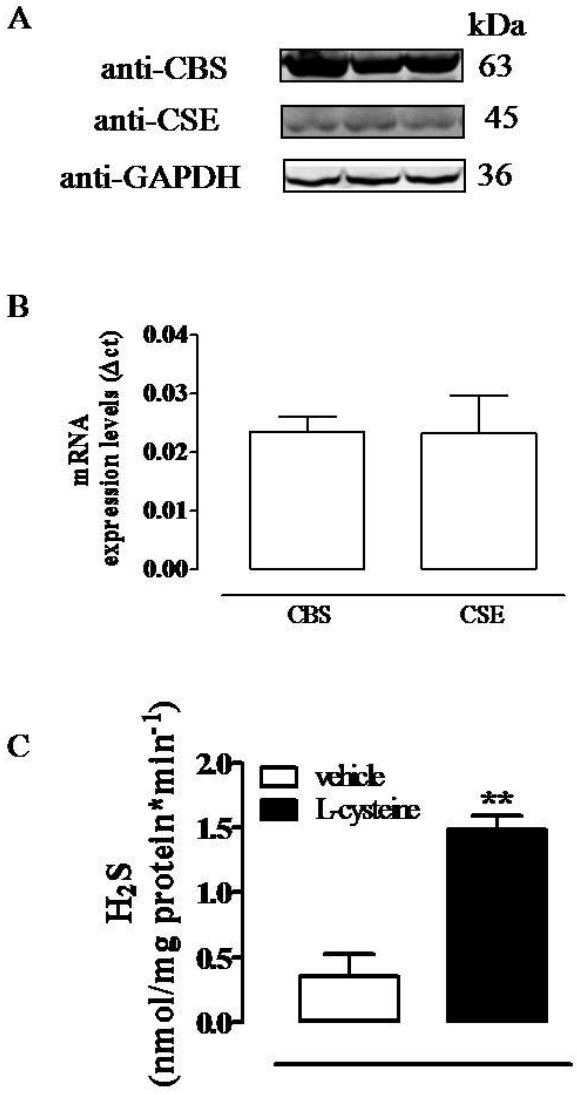

Figure 17. **Immunoblot and RT-qPCR for CBS and CSE and enzymatic activity in human urothelium.** (A) Both CBS and CSE were expressed in human urothelium as demonstrated by the Western blot. (B) CBS and CSE mRNA levels were similar in human urothelium as determined by RT-qPCR. Data were calculated as ΔCt and expressed as mean ± SEM of four separate specimens. (C) H_2S production in human urothelium under basal condition and after incubation with L-cysteine 10 mM. Urothelium homogenate incubation with vehicle generated detectable amount of H_2S, that was significantly increased following L-cysteine addition (**$p < 0.01$). H_2S levels were calculated as nanomoles per milligram of protein/minute and expressed as mean ± SEM of five different specimens.

8.2 8-Br-cGMP but not d-cAMP increases H_2S levels in human urothelium

Incubation of human urothelium with 8-Br-cGMP, a stable analogue of cGMP, significantly increased H_2S production as compared to vehicle (***Figure 18***, $p < 0.05$). This H_2S induction was reverted by the pre-treatment with KT5823, a selective inhibitor of PKG (***Figure 18***, $p < 0.05$). Conversely, H_2S levels were not modified by the treatment with d-cAMP, a stable analogue of cAMP. PKA blockade induced by the selective PKA inhibitor, KT5720, did not modify H_2S levels (***Figure 18***).

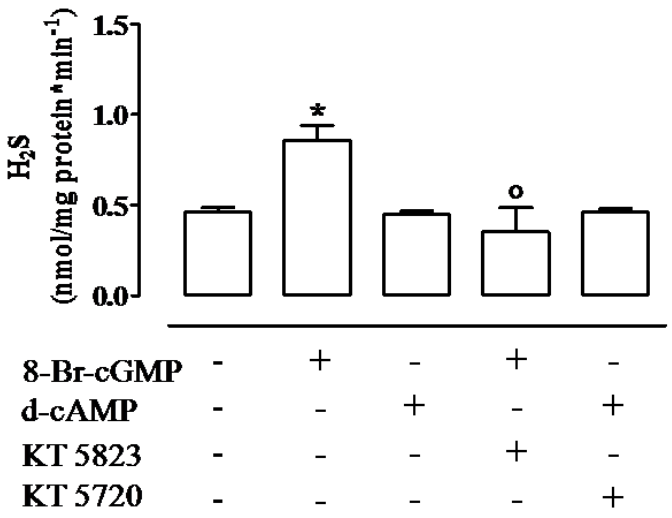

***Figure 18*. H_2S production following incubation with 8-Br-cGMP or d-cAMP in presence and absence of PKG or PKA inhibitor in human urothelium.** The incubation of human urothelium with 8-Br-cGMP significantly increased H_2S production compared to vehicle (*$p < 0.05$). This effect was abrogated by the pretreatment with KT5823, a selective PKG inhibitor (°$p < 0.05$). The stimulation with d-cAMP did not affect H_2S production either in presence or in absence of KT5720, a selective PKA inhibitor. H_2S levels were calculated as nanomoles per milligram of protein/minute and expressed as mean ± SEM of five different specimens.

8.3 T24 cell line: CBS and CSE are expressed in human T24 cells and produce H_2S

In order to further investigate the role of cyclic nucleotides in the regulation of H_2S production in human urothelium, we used the human urothelial T24 cell line. Interestingly, T24 cells expressed CBS and CSE (***Figure 19A***) and generated detectable amount of H_2S in basal condition (***Figure 19B).*** Furthermore, the H_2S production was significantly increased in presence of L-cysteine when compared to vehicle (***Figure 19B***, $p < 0.05$).

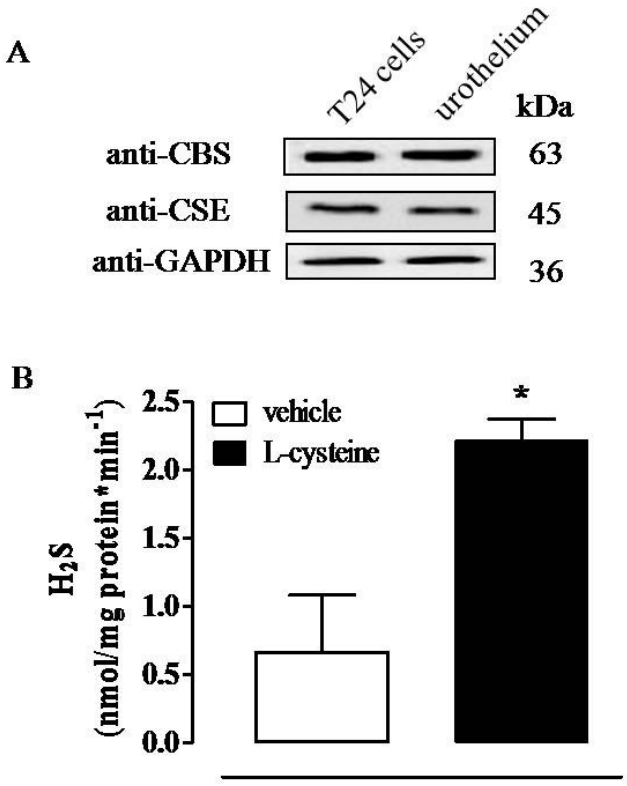

***Figure 19*. Immunoblot for CBS and CSE and enzymatic activity in human T24 cell line.** (A) CBS and CSE protein expression in T24 cells and human urothelium tissue. (B) T24 cells generated detectable amount of H_2S in basal (vehicle) or in stimulated (L-cysteine) conditions (*$p < 0.05$). Data were calculated as nanomoles per milligram of protein/minute and expressed as mean ± SEM of six different experiments.

8.4 8-Br-cGMP but not d-cAMP increases H_2S levels in T24 cells

As already observed in human urothelium, the incubation of T24 cells with 8-Br-cGMP caused a significant increase in H_2S production as compared to vehicle, reaching the highest H_2S values at 15 min of treatment (***Figure 20***, $p < 0.01$ and $p < 0.05$ at 15 and 30 min, respectively). As expected, pre-treatment with PKG inhibitor, KT5823, abolished the 8-Br-cGMP-induced H_2S production (***Figure 20***, $p < 0.001$). Interestingly, the incubation of T24 cells with either d-cAMP or KT5720, the PKA inhibitor, did not affect H_2S production (***Figure 20***).

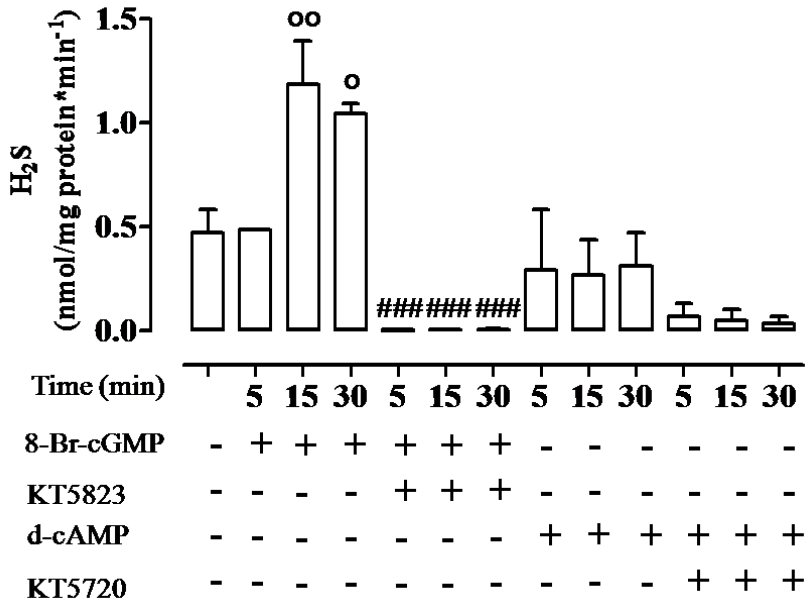

***Figure 20*. H_2S production following incubation with 8-Br-cGMP or d-cAMP in presence and absence of PKG or PKA inhibitor in human T24 cells.** 8-Br-cGMP incubation caused a significant increase in H_2S production at 15 or 30 min of treatment ($^{\circ\circ}p < 0.001$ and $^{\circ}p < 0.05$ at 15 and 30 min, respectively). Incubation with PKG inhibitor, KT5823, prior to 8-Br-cGMP challenge, abrogated the H_2S production ($^{\#\#\#}p < 0.001$). d-cAMP incubation for 5, 15 and 30 min in presence or in absence of PKA inhibitor, KT5720, did not affect H_2S production. Data were calculated as nanomoles per milligram of protein/minute and expressed as mean ± SEM of six different experiments.

8.5 H_2S levels in CBS silenced T24 cells (CBSΔT24)

CBS depletion from T24 cells was carried out by transfecting shRNA specific for CBS. Total cell lysates from puromycin-resistant clones (clones A-F) were analyzed by Western blot using anti-CBS antibody. The clone F, characterized by the lowest CBS expression, specifically CBSΔT24, was selected for the next experiments (***Figure 21A***). Interestingly, the H_2S production observed in CBSΔT24 cells was almost abolished when compared to T24 cells (***Figure 21B***, $p < 0.05$).

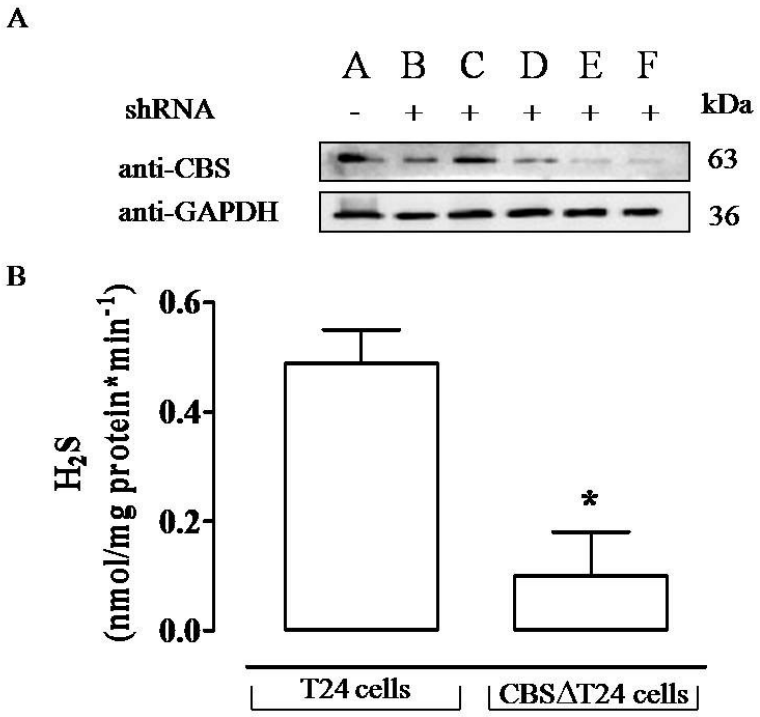

***Figure 21*. Effect of CBS silencing on H_2S production in human T24 cells.** (A) Generation of CBSΔT24 cells. Five puromycin resistant clones were analyzed for CBS expression by Western blot. Clone F was selected for the further experiments. (B) The H_2S production in CBSΔT24 was markedly lower when compared to control T24 cells (*$p < 0.05$). Data were calculated as nanomoles per milligram of protein/minute and expressed as mean ± SEM of four different experiments.

8.6 CBS phosphorylation

To investigate whether CBS could be a substrate of phosphorylation for PKG and/or PKA, the enzyme was specifically immunoprecipitated from T24 cells incubated with 8-Br-cGMP or d-cAMP in presence or absence of KT5823 or KT5720, selective PKG or PKA inhibitors, respectively. Incubation with 8-Br-cGMP caused an increase of phosphorylated form of CBS (pCBS) at 15 and 30 min of treatment (***Figure 22A***, $p < 0.001$ and $p < 0.05$, respectively). The maximum effect in CBS phosphorylation was observed at 15 min of treatment with 8-Br-cGMP, significantly reverted by the inhibition of PKG with KT5823 (***Figure 22A***, $p < 0.001$ and $p < 0.01$ at 15 and 30 min, respectively). Conversely, neither d-cAMP or KT5720, a PKA inhibitor, modified pCBS levels (***Figure 22B***).

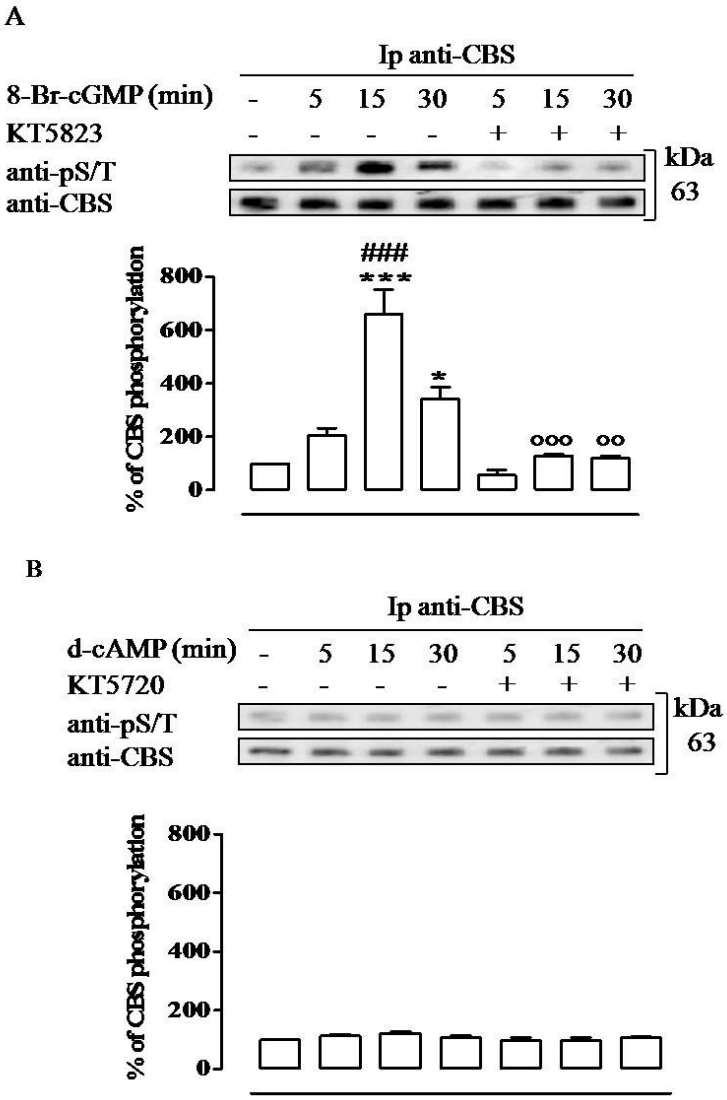

***Figure 22*. CBS phosphorylation mediated by PKG in T24 cells.** (A) CBS was immunoprecipitated from starved T24 cells treated with or without 8-Br-cGMP in presence or absence of PKG inhibitor. CBS phosphorylation was detected by Western blot using anti-pS/T. 8-Br-cGMP treatment caused a time-dependent increase of CBS phosphorylation (*$p < 0.05$, ***$p < 0.001$ vs vehicle; $^{###}$ $p < 0.001$ vs 5 and 30 min.). PKG inhibitor (KT5823) abolished CBS phosphorylation induced by the 8-Br-cGMP (°°$p < 0.01$ and °°°$p < 0.001$). (B) CBS was isolated by immunoprecipitation from starved T24 cells treated with or without d-cAMP in presence or absence of PKA inhibitor. CBS phosphorylation was detected by Western blot using anti-pS/T. The treatment with d-cAMP in presence or in absence of PKA inhibitor, KT5720, did not cause any changes in CBS phosphorylation levels. Data were calculated as % of CBS phosphorylation and expressed as mean ± SEM of three separate experiments.

8.7 PKG and PKA expression in human T24 cells, urothelium, detrusor and full thickness bladder

Previously, in Results I, we demonstrated that incubation of full thickness bladder samples with d-cAMP increased H_2S production; the finding that c-AMP did not modify H_2S levels either in human urothelium or in T24 cell line and did not induce CBS phosphorylation suggested us to investigate on PKA and PKG expression in human T24 cells, urothelium, detrusor and full thickness bladder. Western blot analysis clearly showed that PKG is widely expressed in all samples i.e. human full thickness bladder, urothelium and detrusor, as well as in T24 cells (***Figure 23***), while PKA is abundant in human detrusor and full thickness bladder and very weakly expressed in human urothelium or T24 cells (***Figure 23***). This finding clearly indicated that the lack of effect on H_2S production and pCBS levels were due to the weak expression of PKA in urothelium as well as T24 cells.

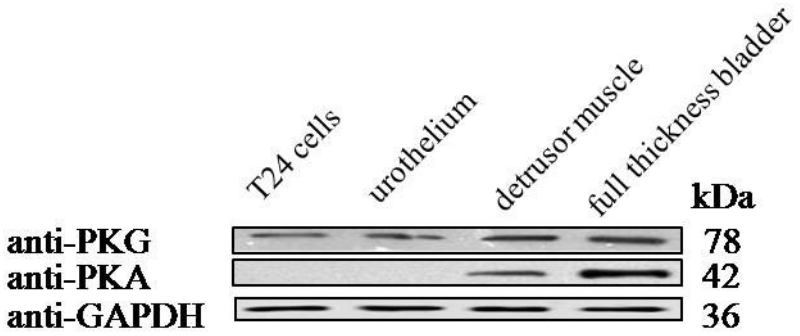

***Figure 23*. Representative Western blot of PKG and PKA in human T24 cells, urothelium, detrusor muscle and full thickness bladder**. PKG was expressed in all samples. PKA was widely expressed in detrusor and full thickness bladder and weakly expressed in T24 cell line and urothelium. The Western blot is representative of three separate experiments.

8.8 PKG mediates CBS phosphorylation at Ser227 and Ser525 in a cell free assay

To further characterize CBS phosphorylation, we used the GPS 2.1 software (Group-based Prediction System, version 2.1) to identity the putative PKG phosphorylation sites of CBS [220, 221]. Among the sites predicted by computational analysis, we chose those with the highest score e.g. Ser32, Ser227 and Ser525 (***Figure 24A***). To identify which of these predicted sites was subjected to phosphorylation by PKG, three constructs encoding for mutated forms of CBS, in which Ser32, Ser227 or Ser525 were mutated in Ala namely HA-CBS S32A, HA-CBS S227A and HA-CBS S525A, were generated by site-directed mutagenesis and then transfected in T24 cells. After transfection, HA-CBS WT and mutants were specifically immunoprecipitated and subjected to a cell free kinase assay by using catalytically active PKG. A significant reduction of labelling with ^{32}P of HA-CBS S227A and HA-CBS S525A but not HA-CBS S32A proteins was observed when compared to HA-CBS WT (***Figure 24B***, $p < 0.001$).

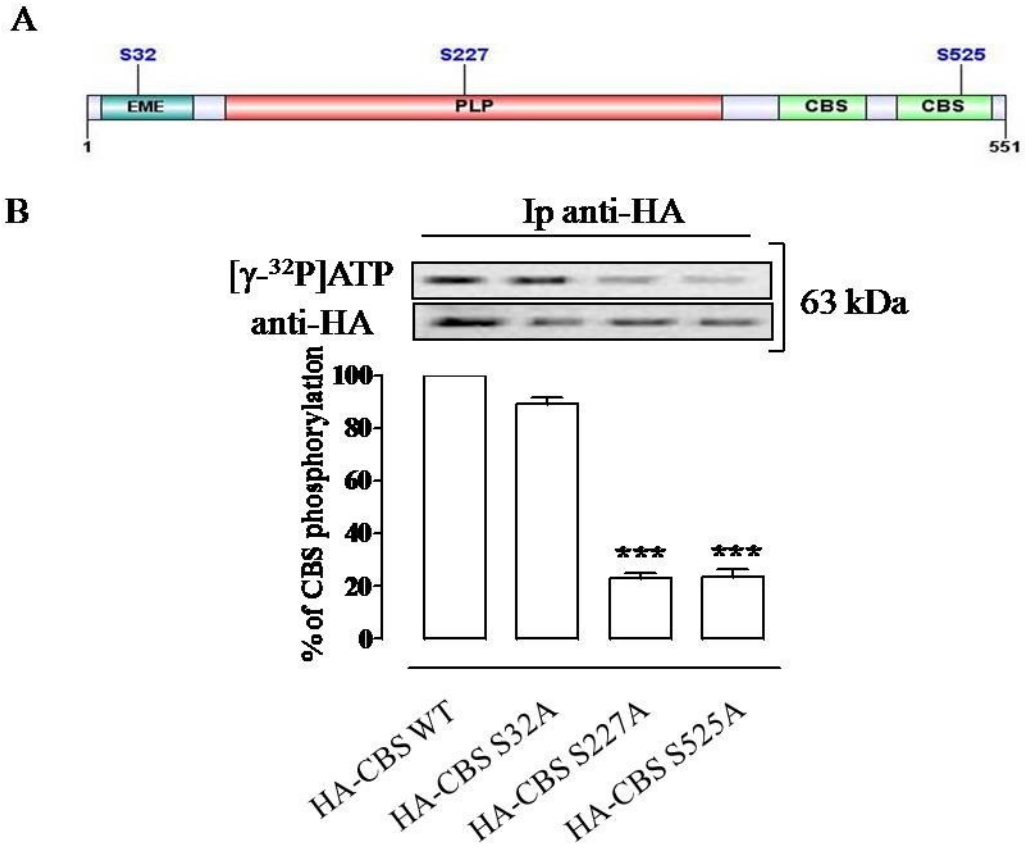

Figure 24*. PKG mediated CBS phosphorylation at Ser227 and Ser525.** (A) Schematic representation of CBS protein domains and predicted PKG phosphorylation sites by GPS 2.1 software. (B) HA-CBS WT or HA-CBS mutants proteins (HA-CBS S32A, HA-CBS S227A, HA-CBS S525A) were subjected to a cell free kinase assay, then separated by SDS–PAGE and analyzed for incorporation of ^{32}P by autoradiography. A significant reduction of labeling with ^{32}P in HA-CBS S227A and HA-CBS S525A proteins was observed when compared to HA-CBS WT ($p < 0.001$). Data were calculated as % of CBS phosphorylation and expressed as mean ± SEM of three separate experiments.

8.9 PKG mediates CBS phosphorylation at Ser227 and Ser525 in T24 cells

To better define the role of Ser227 and Ser525 in PKG-mediated CBS phosphorylation, a kinase assay was performed in T24 cells. T24 cells were transiently transfected with HA-CBS WT and HA-CBS mutants and treated with or without 8-Br-cGMP. WT and mutants proteins were specifically immunoprecipitated and analyzed by Western blot using anti-pS/T. The activation of PKG induced by 8-Br-cGMP treatment significantly increased CBS phosphorylation of ≈ 6 fold in T24 cells transfected with HA-CBS WT construct as compared with the matched control (***Figure 25***, $p < 0.001$). Similarly, phosphorylation of HA-CBS S32A mutant was increased of 5 fold compared with the control (***Figure 25***, $p < 0.001$). Interestingly, the PKG-induced phosphorylation of HA-CBS S227A and HA-CBS S525A mutants was significantly reduced when compared to HA-CBS WT (***Figure 25***, $p < 0.01$).The phosphorylation levels of HA-CBS S227A and HA-CBS S525A mutants were still increased when compared to their matched control (***Figure 25***, $p < 0.001$). These overall results indicate that Ser227 and Ser525 are mainly involved in PKG-mediated CBS phosphorylation.

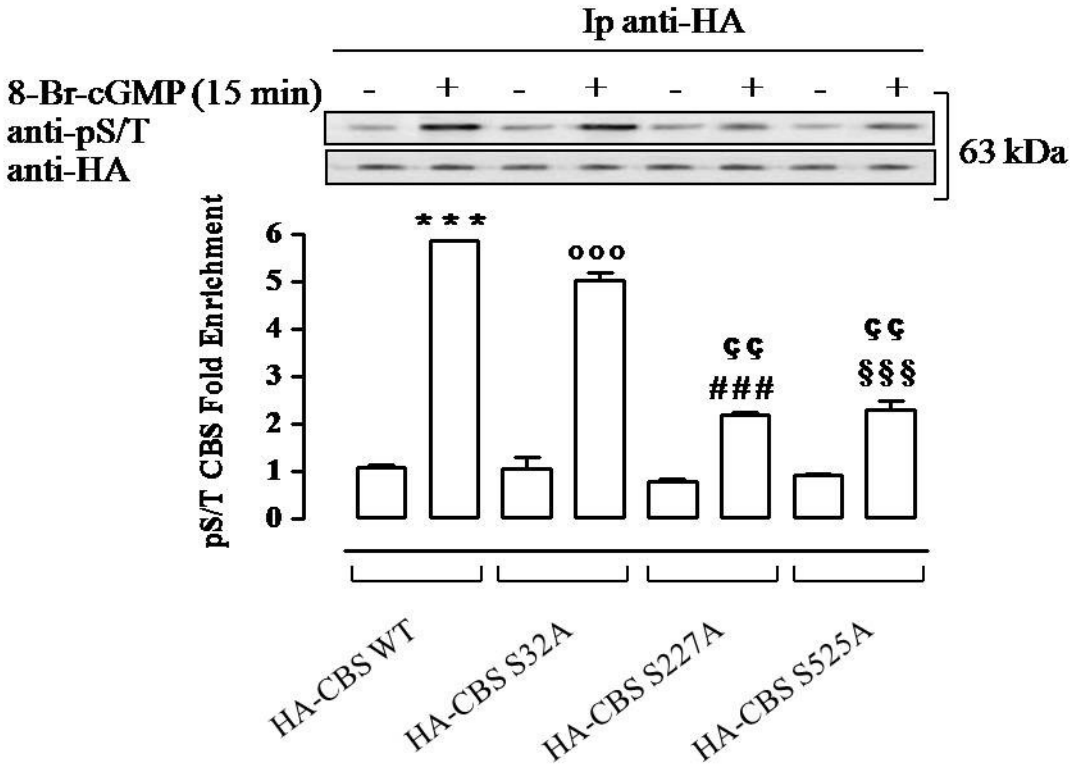

Figure 25*. PKG mediated phosphorylation at Ser227 and Ser525 in T24 cell line.** Proteins from transfected T24 cells with HA-CBS WT or HA-CBS mutants (HA-CBS S32A, HA-CBS S227A HA-CBS S525A), treated with or without 8-Br-cGMP were specifically immunoprecipitated, then separated by SDS–PAGE and immunoblotted with anti-pS/T and anti-HA. The activation of PKG by 8-Br-cGMP led to a significant increase of pCBS in cells transfected with HA-CBS WT and HA-CBS S32A mutant ($p < 0.001$ and °°°$p < 0.001$). Levels of pCBS in cells tranfected with HA-CBS S227A and HA CBS-S525A were increased as compared to control ($^{###}p < 0.001$ and $^{§§§}p < 0.001$). Levels of pCBS in HA-CBS S227A and HA-CBS S525A were significantly reduced when compared with HA-CBS WT ($^{ςς}p < 0.01$). Data were expressed as mean ± SEM of three different experiments.

8.10 The mutation of Ser227 impairs PKG-mediated increase of H_2S production

CBSΔT24 cells were transiently transfected with constructs HA-CBS WT, HA-CBS S227A or HA-CBS S525A mutants and treated with 8-Br-cGMP or vehicle. Ectopic expression of HA-CBS WT or HA-CBS mutants was evaluated by Western blot using anti-HA (***Figure 26A***). The same samples were analyzed for H_2S levels. HA-CBS WT generated detectable amount of H_2S. As expected, treatment with 8-Br-cGMP caused a significant increase in H_2S production (***Figure 26B***, $p < 0.05$). Interestingly, mutation of Ser227 led a significant reduction of basal H_2S ($p < 0.05$) and 8-Br-cGMP-mediated increase of H_2S production ($p < 0.001$) as compared to WT (***Figure 26B***). In contrast, mutation of Ser525 did not affect H_2S production either in basal condition or following 8-Br-cGMP treatment.

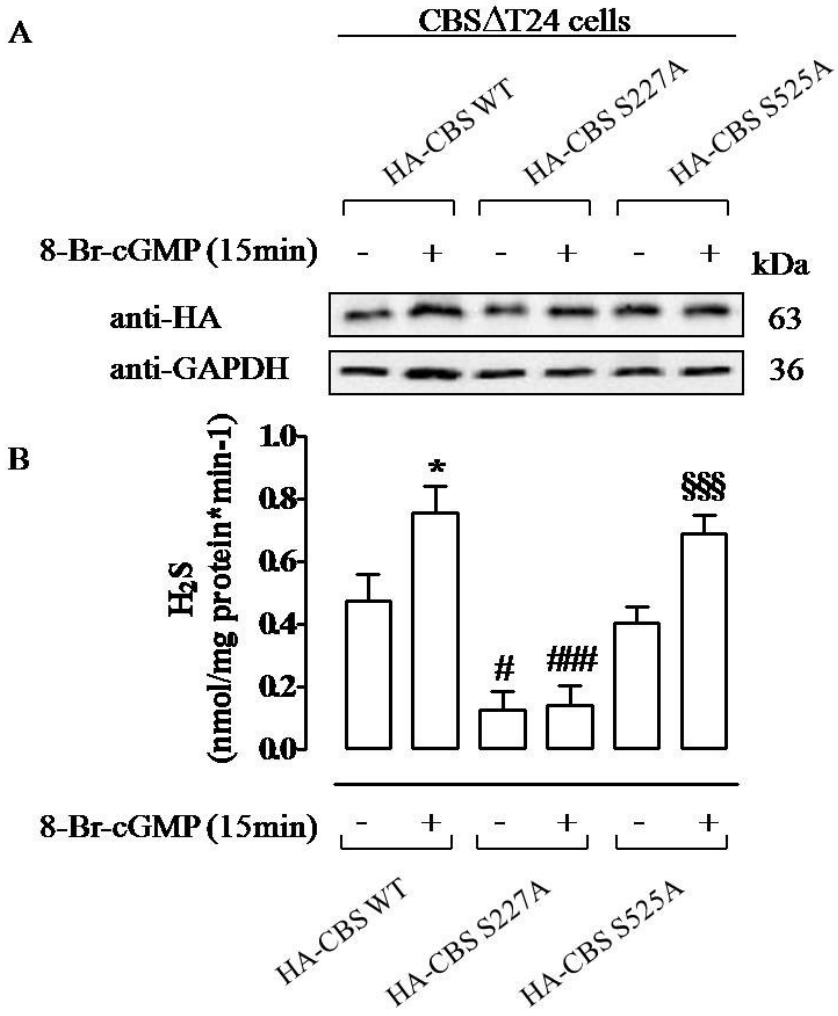

***Figure 26*. Ser 227 mutation impairs PKG-induced H_2S production in T24 cells.** (A) Western blot analysis of proteins from CBSΔT24 cells transiently transfected HA-CBS WT and HA-CBS mutants (HA-CBS S227A, HA-CBS S525A), treated with or without 8-Br-cGMP for 15 min. (B) Production of H_2S from the same samples. Mutation of Ser 227 significantly reduced H_2S production in basal condition and following incubation with 8-Br-cGMP compared with the paired WT ($^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$). H_2S levels were not affected by the mutation of Ser 525. Indeed, H_2S levels were similar to those obtained from HA-CBS WT either in basal condition or upon cyclic nucleotide stimulation. 8-Br-cGMP significantly increased H_2S production in HA-CBS WT and in HA-CBS S525A compared to vehicle ($^{*}p < 0.05$, $^{\S\S\S}p < 0.001$). Data were calculated as nanomoles per milligram of protein/minute and expressed as mean ± SEM of three different experiments.

8.11 Generation and validation of a novel antibody anti-pCBS^{Ser227}

A peptide of 10 amino acids corresponding to residues 225 through 234 of CBS protein including phosphorylated Ser227 was synthesized and used to obtain polyclonal antibodies in rabbit. Specificity of the antibody was tested by Western blot on lysates from human urothelium and T24 cells treated with or without 8-Br-cGMP. The phosphopeptide-specific CBS antibody (anti-pCBS^{Ser227}) efficiently recognized pCBS^{Ser227} protein in human urothelium and in T24 cells treated with 8-Br-cGMP (***Figures 27A-B***). No difference in CBS expression was observed in the same samples incubated with the anti-CBS (***Figures 27A-B***) in presence or absence of cGMP challenge. No other proteins were detected.

Specificity of the antibody anti-pCBS^{Ser227} was further evaluated in T24 cells transiently transfected with constructs HA-CBS WT or HA-CBS S227A. As showed in ***Figure 27C***, the Ser227Ala mutant CBS protein was not recognized by the anti-pCBS^{Ser227} demonstrating that this novel antibody is characterized by specificity at phosphorylated site Ser227.

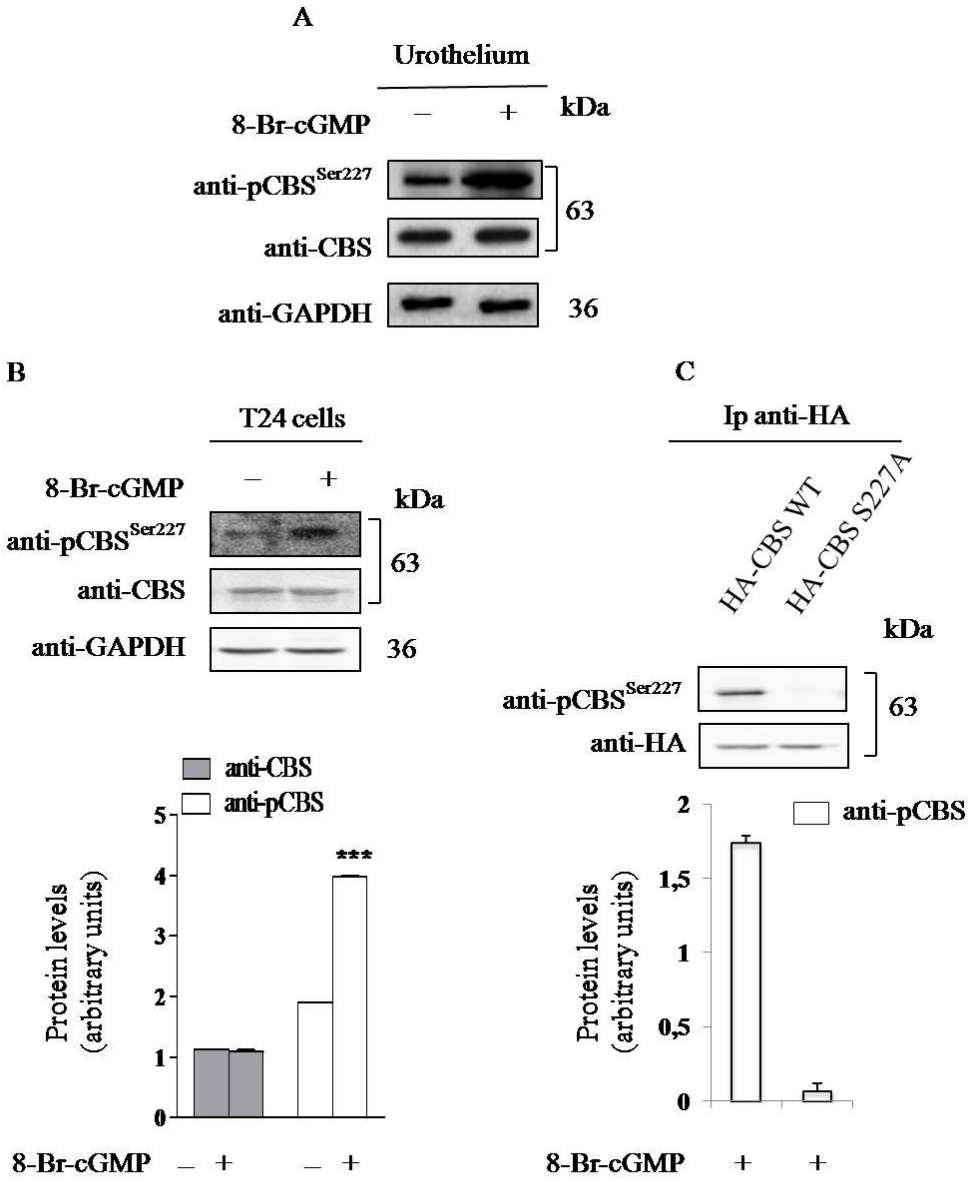

***Figure 27*. The pCBS^{Ser227} antibody selectively recognizes the phosphorylated CBS in human urothelium and T24 cells.** (A) Expression of CBS phosphorylated form in human urothelium tissue by using pCBS^{Ser227} antibody. (B) T24 cells treated with or without 8-Br-cGMP for 15 min. Protein extracts were analyzed by immunoblotting using anti-pCBS^{Ser227} and anti-CBS antibodies. The blot is representative of three separate experiments. (C) Proteins from T24 cells transiently transfected with plasmids expressing HA-CBS WT or HA-CBS S227A and treated with 8-Br-cGMP were specifically immunoprecipitated with anti-HA epitope. Immunoprecipitates were separated by SDS–PAGE and immunoblotted for anti-pCBS^{Ser227}. Note the absence of signal in cells transfected with HA-CBS S227A mutant construct. Data were expressed as mean ± SEM of three separate experiments.

9. DISCUSSION

Recently, PDE5 inhibitors, widely used as first line treatment for erectile dysfunction, have been indicated also in the treatment of LUTS. Although their mechanism/s of action is poorly understood, their beneficial effects may be due to smooth muscle relaxation of prostatic gland, urethra, and bladder as well as modulation of sensory nerves and pelvic vasodilatation[222]. Recently, a link between H_2S and PDEs has been reported in the current literature. Indeed, it has been shown that tadalafil, a PDE5-I, is cardioprotective in a model of myocardial ischemia reperfusion[223] through the H_2S signalling and that H_2S acts itself as an endogenous inhibitor of PDE activity[175]. In this study, we have demonstrated that H_2S pathway plays a role in bladder homeostasis. It was already indicated in literature that H_2S has either a contracting effect[224] or a relaxing effect[203] on the bladders of different animal species. The novelties of this project are the findings that i) the L-cysteine/H_2S pathway is present in the human bladder dome and contribute to bladder relaxation through endogenous production of H_2S and ii) sildenafil relaxes human bladder strips and, at similar doses, causes a concentration and time-dependent increase in H_2S production.

Oger et al.[107] demonstrated that sildenafil relaxes human bladder dome strips in a partially nitric oxide independent manner. Here, we have shown that sildenafil-induced relaxation involves also H_2S and its effect is significantly reduced by CBS and CSE inhibition. Furthermore, given that sildenafil determines the elevation of cGMP levels, we evaluated whether a stable analogue of cGMP could trigger similar effects observed with sildenafil. We reported that the increase in H_2S production was induced by both the stable analogue of cGMP and cAMP. These data are consistent with previous studies showing that inhibitors of cAMP-dependent PDEs relax human detrusor strips[79]. A valid explanation of this finding is that sildenafil inhibits also PDE4, even if with ≈ 375 times lower affinity than PDE5[225]. Thus, we can hypothesize that the stimulation of the L-cysteine/H_2S pathway is a common effect among all commercially available PDE5-Is. It is also well established that cGMP and cAMP activate PKG or PKA that in turn phosphorylates downstream proteins thereby

triggering signal transduction. On the basis of these results and available evidence, we have hypothesized that the increase in H_2S production triggered by 8-Br-cGMP or d-cAMP involves CBS phosphorylation leading to an increased catalytic activity. In order to address this issue, we focused our attention on urothelium rather than on the whole bladder (urotheloum *plus* detrusor) for two different reasons: i) the important role played by urothelium in bladder pathophysiology[208] and ii) the availability of a well characterized human urothelial T24 cell line. As first step, we investigated the expression of CBS and CSE in human urothelium and T24 cell line and their enzymatic activity. We have found that both enzymes are expressed and are catalytically active. Furthermore, the treatment with 8-Br-cGMP but not d-cAMP causes a significant increase in H_2S production in both human urothelium and T24 cells, and this effect is significantly reverted by KT5823, a selective inhibitor of PKG. These findings are not consistent with the previous one, given that we showed both cyclic nucleotides caused an increase in H_2S production in whole human bladder. In order to clarify this issue, we hypothesized a different distribution/expression of PKA and PKG in human bladder. Indeed, Western blot analysis reveals that PKG is strongly expressed in human urothelium, detrusor muscle and whole bladder as well as T24 cells, while PKA is widely expressed in detrusor muscle, whole bladder and weakly present in urothelium and T24 cells. Confirmed that human urothelium and T24 cell line display similar features, we stimulated T24 cells with 8-Br-cGMP and investigated CBS phosphorylation. Incubation of T24 cells with 8-Br-cGMP causes a time-dependent increase in CBS phosphorylation, reverted by using PKG selective inhibitor (KT5823) and by silencing T24 cells for CBS. Furthermore, as we expected, d-cAMP does not cause any increase in CBS phosphorylation in agreement with the weakly expression of PKA in T24 cells. Then, it was very interesting to identify the main CBS phosphorylation site of PKG. Through a computational approach, we identified Ser32, Ser227, and Ser525 as possible site of phosphorylation. Moreover through a phosphorylation assay on HA-CBS WT and HA-CBS mutants (HA-CBS S32A, HA-CBS S227A, and HA-CBS S525A), we have found that the mutation of Ser227 and Ser525 but not Ser32 causes

a drastic reduction of CBS phosphorylation in T24 cells. Furthermore, HA-CBS S227A and HA-CBS S525A mutants display a significant reduced level of phoshorylation when treated with 8-Br-cGMP. Considering all together these results, it is clear that among predicted PKG-phosphorylation sites, Ser227 and Ser525 are experimentally validated as sites of phosphorylation by PKG.

To assess the contribution of each phosphorylation sites on the enzymatic activity and on H_2S production, we transfected CBSΔT24 cells with HA-CBS S525A, HA-CBS S227A mutants or HA-CBS WT. Treatment of cells with 8-Br-cGMP does not induce any significant changes in H_2S production in S227A mutant, suggesting that this site is responsible of CBS activation and that it can be enhanced selectively in a cGMP/PKG-dependent manner. Then, to confirm and validate our data we have generated a specific antibody, anti-pCBSSer227. It recognizes the phosphorylated CBS in both urothelium and T24 cell line, treated with 8-Br-cGMP. The specificity of our antibody is also confirmed by the finding that Ser227Ala mutant CBS protein is not recognized by the anti-pCBS^{Ser227}. Looking at Western blot we observed an amount of phosphorylated CBS also in basal condition and this finding may suggest that the enzyme is basally activate and it could be enhanced by an increase of cGMP level. In conclusion, it is well known that urothelium influences the contractile state of detrusor smooth muscle and contributes to bladder function and homeostasis by releasing several agents that modulate muscle contractility. Since cGMP/PKG dependent CBS phosphorylation occurs in human urothelium regulating H_2S production, we suggest that CBS-derived H_2S contributes to bladder homeostasis by modulating detrusor muscle contractility. Since sildenafil effect involves the H_2S pathway, it is feasible that this mechanism could contribute to explain the efficacy of PDE-5 inhibitors in LUTS therapy. Interestingly, even though Ser525 is also phosphorylated in a PKG-dependent manner, mutation at this residue does not affect H_2S production. Ser525 lies within a non-consensus CBS domain that is described as a regulatory region target of the allosteric modulator SAM. Thus, it is reasonable to postulate that phosphorylation of Ser525 may be implicated in the modulation of additional features of CBS protein not as yet determined.

10. H_2S RELEASED BY ZOFENOPRIL PROMOTES CARDIOPROTECTION THROUGH NRF2 SIGNALLING

On March 2014, as a visiting Ph.D. student I joined for one year the research group of Dr. David J. Lefer, Director of the Cardiovascular Center of Excellence at Louisiana State University Health Sciences Center, New Orleans, USA. My project focused on investigating the mechanisms of how ACE inhibitor-mediated cardioprotection is regulated by hydrogen sulfide (H_2S) and nitric oxide (NO) signalling.

10.1 INTRODUCTION

Recently, H_2S has been recognized as a physiological signalling molecule with cytoprotective effects in cardiovascular diseases, including myocardial ischemia and heart failure. H_2S is produced in mammalian tissue by three enzymes: cystathionine γ-lyase (CSE), cystationine β-synthase (CBS) and 3-mercaptopopyruvate sulfurtransferase (3-MST)[162, 226]. Previous studies have been reported on the involvement of reactive oxygen species (ROS) as a main cause of cardiomyocyte injury and dealth during myocardial ischemia/reperfusion (MI/R)[227]. It has been demonstrated that acute injection of H_2S, either prior to ischemia or at reperfusion, markedly ameliorates *in vivo* MI/R injury. Similarly, cardiac overexpression of CSE protects against acute MI/R injury by attenuating oxidative stress, inhibiting apoptosis and reducing inflammation[228, 229]. Recently, it has been demonstrated that H_2S exerts antioxidant actions in myocardium *via* nuclear factor (erythroid-derived 2)-like 2 (Nrf2) signalling. Nrf2 is a member of NF-E2 family of nuclear basic leucine zipper transcription factors, and regulates the gene expression of a number of enzymes that detoxify pro-oxidative stressors[227]. In addition, it has been reported that exogenous H_2S improves survival after cardiac arrest and cardiopulmonary resuscitation in an endothelial nitric oxide synthase (eNOS)-dependent manner[180].

Another recent study revealed that H_2S mediates cardioprotection against MI/R injury by enhancing eNOS activity and increasing myocardial NO bioavailability[230].

Angiotensin converting enzyme inhibitors (ACEIs), initially approved only for the treatment of hypertension, are actually recommended also for the management of myocardial ischemia and left ventricular dysfunction; however, their mechanism(s) of cardioprotection remain poorly understood. ACEIs can be divided in three groups based on their chemical molecular structure: sulfhydrylate (i.e. zofenopril), dycarboxilate (i.e. ramipril) and phosphonate (i.e. fosinopril) compounds. Among all currently available data regarding the anti-ischemic effects of ACEIs, some have shown efficacy for all three group of compounds tested[231]. However, some have failed to observe any anti-ischemic actions[232] and others have found efficacy only for sulfhydryl containing agents[233]. Because of these opposing findings, several hypotheses concerning the protective mechanism(s) of action of ACEIs have been proposed. In isolated heart models the anti-ischemic actions of ACEIs have been appointed to inhibition of cardiac angiotensin II formation[234], increase of NO bioavailability induced by the inhibition of the cardiac bradykinin breakdown[235], oxygen radical scavenging[236] or altered prostaglandin production[237]. Even though ACEIs have been shown to reduce cardiovascular morbidity and mortality in patients affected with left ventricular hypertrophy, and post-myocardial infarction, their cardioprotection may not be entirely dependent on inhibition of angiotensin II production.

Zofenopril is a sulphydryl-ACE inhibitor and a prodrug of the active compound, zofenoprilat. Both zofenopril and its metabolite are characterized by high lipophilicity, long-lasting tissue penetration, selective cardiac ACE inhibition and antioxidant activities resulting in robust cardioprotection[238, 239]; furthermore, they are effective even using only a single daily oral administration[240]. Several studies have investigated the effects of zofenopril in experimental models of MI/R injury. Zofenopril has been shown to improve cardiac contractile force, reduce lactic dehydrogenase (LDH) release during reperfusion and the infarct size in isolated rat hearts subjected to global MI/R injury[241]. It has been shown that zofenopril stimulates

active calcium uptake through sarcoplasmatic reticulum cycling, thus enhances calcium handling in the cardiomyocytes, which could account for improvements in myocardial contractility following MI/R[242]. Moreover, the cardioprotection afforded by zofenopril could also be a result of the preservation of protein thiols at the end of ischemia due to its antioxidant properties[243]. It has become increasingly clear that zofenopril exerts a number of beneficial effects beyond ACE inhibition but the precise mechanisms remain poorly understood. Recently, experimental evidence suggest that zofenopril improves peripheral vascular function by potentiating H_2S signalling and independently by ACE inhibition[244]. Furthermore, it also has been demonstrated that zofenoprilat, the active metabolite of zofenopril, is an H_2S donor [244]. Based on the biological effects of H_2S, it can be hypothesized that the antioxidant, anti-inflammatory, endothelial and cardioprotective effects of zofenopril may be mediated through H_2S and NO dependent signalling. In the present study, we investigated *in primis* the effect of a single administration of zofenopril on myocardial and circulating H_2S bioavailability and then on NO levels in the same territories; we also determined the effects of zofenopril treatment on the extent of cardiac injury in a murine model of MI/R. Furthermore, we also investigated the effects of zofenopril therapy on oxidative stress and associated Nrf2 signalling.

10.2 MATERIAL AND METHODS

10.2.1 Animals

Male C57BL/6J mice were purchased from the Jackson Laboratory and were at 10-14 weeks of age at the time of experiments. All the experimental protocols were approved by the Institute for Animal Care and Use Committee at LSUHSC and conformed to the Guide for the Care and use of Laboratory Animals. Mice were treated per oral gavage with vehicle (carboxymethylcellulose 0.02% m/v; Santa Cruz), or zofenopril calcium {[(1(S), 4(S)]-1(3-mercapto-2 methyl-1-oxopropyl) 4-phenyl-thio-L-proline-S-benzoylester, 10 mg/kg; Menarini Ricerche S.p.A, Firenze, Italy} for 1, 8 and 24 hours. Mice were subjected to the surgical protocol for *in vivo* MI/R or sacrificed to collect heart tissue and plasma to perform molecular studies.

10.2.2 Measurement of H_2S

Free H_2S levels were measured in heart tissue and plasma obtained from mice treated with vehicle or zofenopril by gas chromatography coupled with sulfur chemiluminescence according to previously described methods[229].

10.2.3 Measurement of NO metabolites

Nitrite levels in plasma and heart tissue obtained from mice treated with vehicle or zofenopril were quantified using HPLC methods as described previously[245].

10.2.4 RT-qPCR

Total RNA was extracted from myocardial tissue and reverse transcribed to cDNA using synthesis kits from Bio-Rad. Sequence Detection System (Life Technologies)

was used to monitor the increase of fluorescence following PCR. PCR amplification was carried out using TaqMan PCR Master Mix (Life Technologies). RT-qPCR was used to evaluate the gene expression of H_2S producing enzymes CBS, CSE and 3-MST.

10.2.5 Western blot

Total extracts were prepared using myocardial tissue obtained from mice treated with or without zofenopril. Proteins samples were separated on Tris-HCl gel (4-20%, Biorad) and transferred to nitrocellulose membranes [246]. The membranes were probed with the following primary antibodies at 4°C overnight: CBS (Santa Cruz), CSE (Abnova), 3-MST (Novus), eNOS (BD Biosciences), p-eNOS^{1177} (Abcam), p-eNOS^{495} (Cell Signaling), GPX-1 (Santa Cruz), Trx-1 (Santa Cruz), Trx-2 (Santa Cruz), Nrf2 (Santa Cruz), Keap1 (Santa Cruz), and GAPDH (Santa Cruz). Immunoblots were then incubated with the appropriate fluorescence conjugate secondary antibodies and Odissey Infrared Imaging System (PCSH898) was used for protein visualization.

10.2.6 Myocardial infarction protocol

The surgical protocol for *in vivo* MI/R was similar to methods described previously[227, 245]. Male C57BL/6J mice at 10-14 weeks of age were pre-treated with or without zofenopril (10 mg/kg, o.g.). At 8 hours of treatment mice were subjected to 45 minutes of MI by occluding the left coronary artery followed by 24 hours of reperfusion.

10.2.7 Measurement of circulating cardiac troponin-I levels

At 4 hours of reperfusion, plasma samples were collected and used to measure circulating troponin-I levels as an additional index of cardiac injury by using a mouse-specific ELISA kit (Life Diagnostics).

10.2.8 Infarct size assessment

After 24 hours of reperfusion, mice hearts were excised and sliced in five sections. All the sections were incubated in 2, 3, 5 triphenyltetrazolium chloride (TTC) buffer for 3 minutes at 37° C. Myocardial area at risk (AAR) per left ventricle (LV), and myocardial infarct size (INF) per area at risk were determined by using Image J software.

10.2.9 MDA and AOPP measurement

Biomarkers of oxidative stress in heart tissue and plasma from mice treated with or without zofenopril were determined by measuring malondialdehyde (MDA) and advanced oxidation protein products (AOPP) levels according to methods described previously[247 248].

10.2.10 Statistical analysis

Data were expressed as mean ± SEM. Statistical analysis was determined by using Student's unpaired, two-tailed t-test or ANOVA followed by Dunnett as a post hoc test. Differences were considered statistically significant when p value was < 0.05.

10.3 RESULTS

10.3.1 A single administration of zofenopril increases H_2S bioavailability in myocardial tissue and plasma

To determine whether zofenopril has the ability to enhance H_2S production, male C57BL/6J mice were treated per oral gavage with a single administration of vehicle or zofenopril for 1, 8 and 24 hours. Mice were then sacrificed and heart tissues and plasma were collected to evaluate H_2S levels. As can be seen in ***Figures 28A and 28B***, zofenopril treatment resulted in a significant increase in H_2S bioavailability in both heart tissue ($p < 0.01$) and plasma ($p < 0.05$) at 8 hours of treatment when compared to vehicle. Thus, we considered 8 hours of zofenopril treatment as optimal for all subsequent experiments.

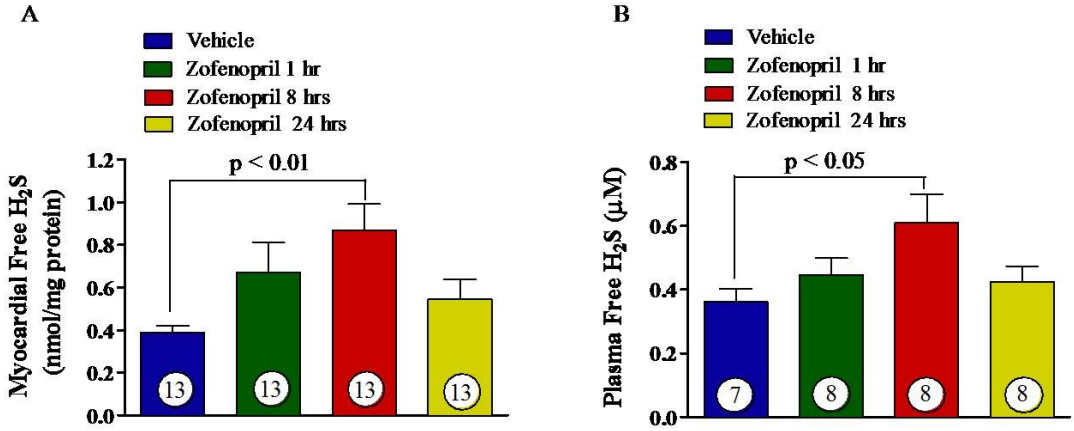

***Figure 28*. H_2S bioavailability induced by a single dose of zofenopril.** Effect of zofenopril treatment (10 mg/kg, o.g.) at 1, 8, 24 hours on H_2S levels in mice heart tissue (A) and plasma (B). 8 hours of zofenopril treatment induced a significant increase in H_2S levels in both myocardial tissue and plasma when compared to vehicle ($p < 0.01$ and $p < 0.05$, respectively). Number inside the bars denotes the number of animals used per each group.

10.3.2 A single dose of zofenopril enhances myocardial and circulating NO bioavailability

Given that zofenopril significantly increased tissue and circulating H_2S levels after 8 hours of treatment, it was of interest to determine the effect of drug treatment on myocardial and plasma NO availability. Therefore, heart tissues and plasma samples obtained from mice treated with or without zofenopril at 8 hours were analyzed for NO metabolites (***Figure 29***). A significant increase in nitrite levels was observed in heart tissues (***Figure 29A***, $p < 0.01$) and in plasma (***Figure 29B***, $p < 0.05$) obtained from mice treated with zofenopril when compared to vehicle.

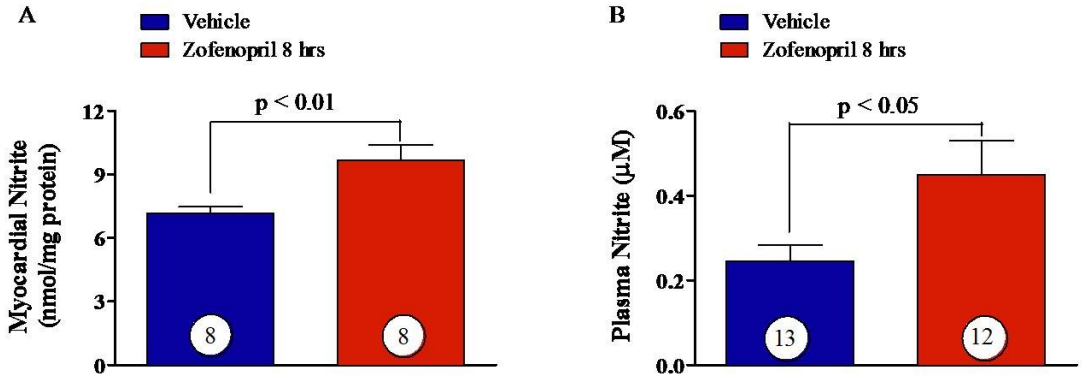

***Figure 29*. Nitrite bioavailability induced by a single zofenopril administration.**. Zofenopril acute therapy (10 mg/kg, o.g.; 8 hours) induced a significant raise of nitrite levels in both mice heart tissue and plasma when compared to vehicle (A, B; $p < 0.01$, $p < 0.05$ respectively). Number inside the bar denotes the number of animals used per group.

10.3.3 A single dose of zofenopril modifies H_2S producing enzymes 3-MST gene expression

Since tissue and circulating H_2S were increased after 8 hours of treatment with zofenopril, it was interesting to evaluate the gene expression of H_2S producing enzymes CBS, CSE and 3-MST (***Figures 30A-C***). Zofenopril therapy did not cause any change in mRNA levels of CBS (***Figure 30A***) and CSE (***Figure 30B***), while it

induced a significant increase in mRNA expression of cardiac H_2S producing enzyme 3-MST (***Figure 30C***) as compared to vehicle ($p < 0.05$).

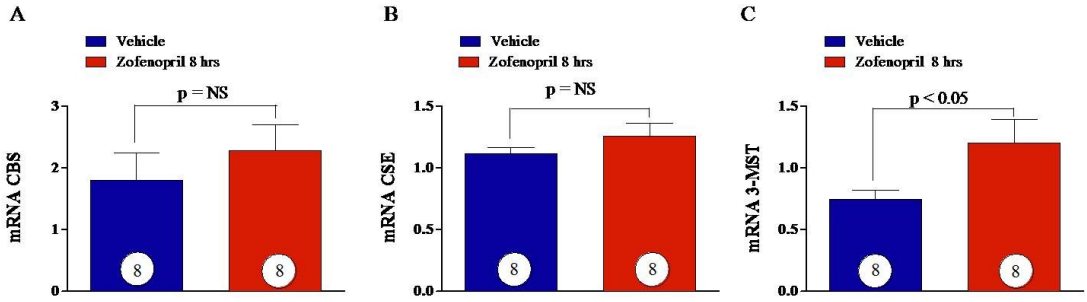

***Figure 30*. Effect of a single administration of zofenopril on myocardial H_2S producing enzymes gene expression.** Zofenopril treatment (10 mg/kg, o.g.; 8 hours) did not affect CBS (A), CSE (B) gene expression, while it induced a significant increase of 3-MST mRNA levels when compared to vehicle (C, $p < 0.05$). Number in the circle inside the bar denotes the number of animals used per each group.

10.3.4 A single administration of zofenopril does not alter H_2S producing enzymes CBS, CSE and 3-MST protein levels

Acute administration of zofenopril at 8 hours did not result in any change in protein expression of cardiac H_2S producing enzyme CBS, CSE, and 3-MST as compared to vehicle (***Figures 31A-D***). This finding, together with mRNA levels of CBS and CSE, suggests that the H_2S increase observed in myocardial tissue and plasma is not dependent on changes in the expression of endogenous H_2S producing enzymes but may be a result of direct H_2S generation from zofenopril[244].

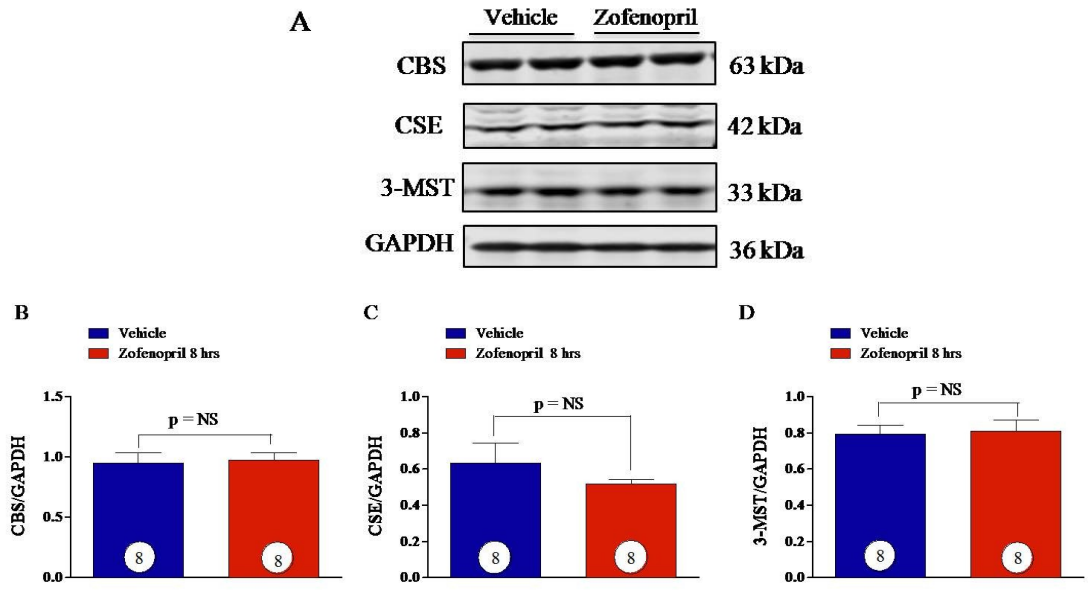

Figure 31. **Effect of a single administration of zofenopril on myocardial H_2S producing enzymes protein expression.** Immunoblot for CBS, CSE and 3-MST (A) with relative optical densitometry (B-C). Zofenopril treatment (10 mg/kg, o.g.; 8 hours) did not cause any change in CBS (A), CSE (B) and 3-MST (C) protein expression. Number in the circle inside the bar denotes the number of animals used per group.

10.3.5 Zofenopril administration induces eNOS activation through Ser1177 phosphorylation

Given that we observed an increase of NO derived metabolites in heart tissue and plasma after zofenopril treatment, we then evaluated the effect of this drug on myocardial eNOS, p-eNOS^{1177} and p-eNOS^{495} protein expression (***Figures 32A-D***). Acute administration of zofenopril at 8 hours induced an increase in myocardial eNOS phosphorylation at Ser^{1177} site (Ser^{1177} is an activation site of eNOS; ***Figure 32B***; p = 0.053) in absence of any difference in eNOS expression when compared to vehicle (***Figure 32D***). Furthermore zofenopril treatment did not cause any change in eNOS phosphorylation at the inhibitory site, Thr^{495} (***Figure 32C***).

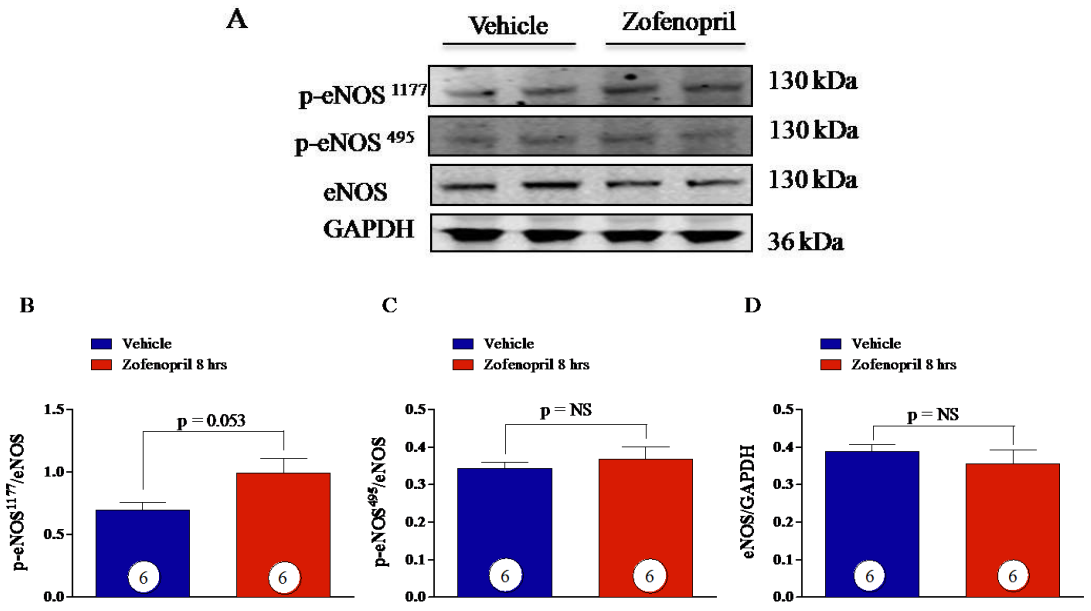

Figure 32. Effect of zofenopril treatment on myocardial p-eNOS^{1177}, p-eNOS^{495} and eNOS expression. (A) Immunoblot for p-eNOS^{1177}, p-eNOS^{495} and eNOS with relative optical densitometry (B-D). Zofenopril therapy (10 mg/kg, o.g.; 8 hours) induced a marked raise in myocardial eNOS phophorylation at Ser1177 (B, p = 0.053), while it did not have any effect on p-eNOS^{495} (C) and eNOS (D) expression. Number in the circle inside the bar denotes the number of animals used per group.

10.3.6 Zofenopril therapy protects against MI/R injury

An induction of H_2S bioavailability in heart tissue and plasma after 8 hours of zofenopril treatment let us to investigate on the effects of zofenopril on the extent of MI/R injury (***Figures 33A-C***). Mice were pretreated with or without zofenopril 8 hours before 45 min of ischemia and 24 hours of reperfusion. Pretreatment of 8 hours with zofenopril resulted in a significant ($p < 0.05$) reduction in infarct size per area at risk (INF/AAR) measured at 24 hours of reperfusion when compared to vehicle (***Figure 33B***). Furthermore, the area at risk per left ventricle (AAR/LV) was similar in both groups. Moreover, circulating troponin-I levels (***Figure 33C***) at 4 hours of reperfusion were markedly reduced by pretreatment with zofenopril, as compared to vehicle ($p < 0.01$). These findings clearly indicate that zofenopril exerts cardioprotective effect during MI/R injury.

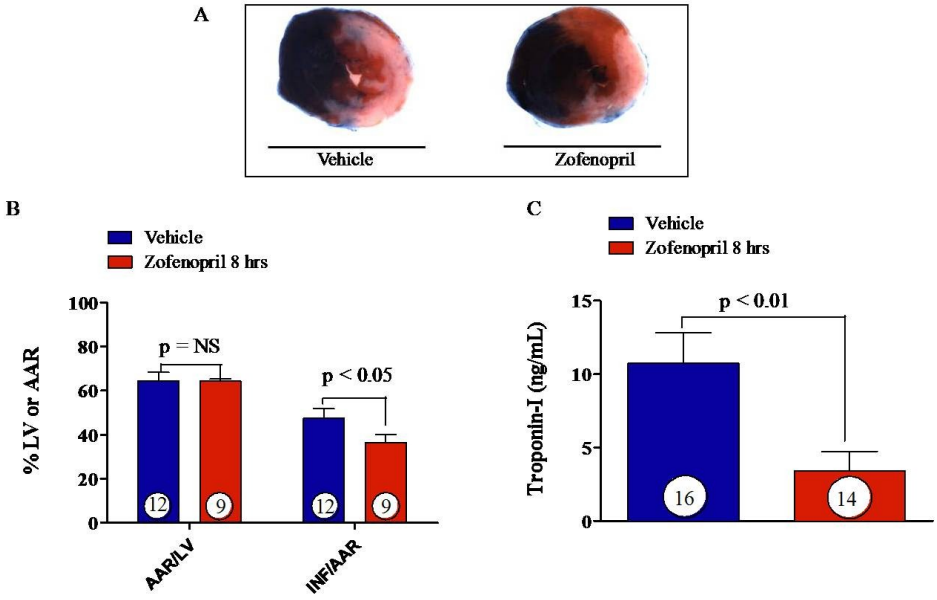

***Figure 33*. Cardioprotection induced by zofenopril therapy in a murine model of MI/R.** (A) Representative section of infarcted myocardium following vehicle and zofenopril pretreatment. (B) Zofenopril administration (10 mg/kg, o.g.) 8 hours before MI/R reduced significantly the infarct size per area at risk (INF/AAR) as compared to vehicle ($p < 0.05$). Furthermore, circulating troponin-I levels at 4 hrs of reperfusion (C) were significantly diminished by zofenopril treatment ($p < 0.01$). Number in the circle inside the bar denotes the number of animals used per group

10.3.7 Zofenopril induces up-regulation of total transcription factor Nrf2

It has been demonstrated that either H_2S exerts antioxidant properties through the activation of Nrf2 signaling and zofenopril acts itself as antioxidant. Thus, it was of our interest to investigate whether zofenopril treatment could modulate the expression of Nrf2 transcription factor and Keap1 levels, a cytosolic repressor of Nrf2 activity (***Figures 34A-C***). We found that zofenopril therapy induced a significant up-regulation of Nrf2 as compared to vehicle (***Figure 34B***, $p < 0.05$). Furthermore, zofenopril treatment did not affect Keap1 levels (***Figure 34C***).

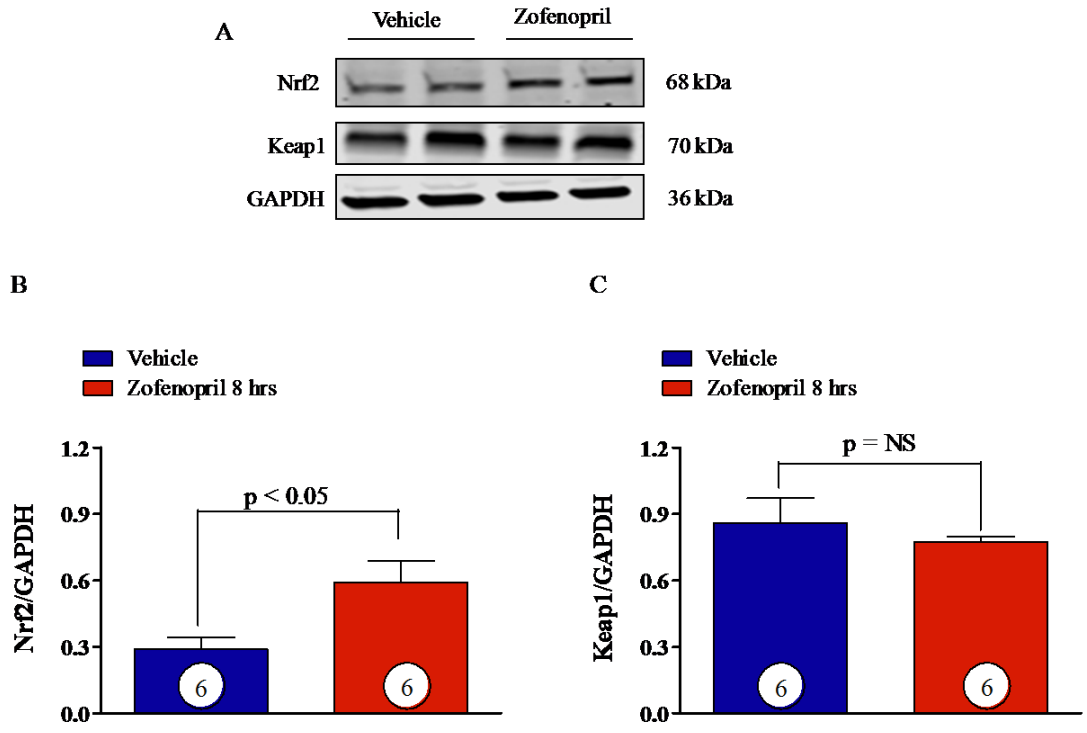

***Figure 34*. Induction of transcription factor Nrf2 expression induced by zofenopril treatment.** Immunoblot for Nrf2 and Keap1 in total protein extract (A) with relative optical densitometry (B, C). Zofenopril therapy (10 mg/kg, o.g.; 8 hours) induced a significant upregulation of myocardial transcription factor Nrf2 as compared to vehicle (B, $p < 0.05$). Zofenopril therapy did not have any effect on Keap1 protein expression (C). Number in the circle inside the bar denotes the number of animals used per group.

10.3.8 Zofenopril induces up-regulation of antioxidant proteins and decreases oxidative stress

It was next interest to evaluate the expression of Nrf2 related downstream antioxidant proteins (***Figures 35A-D***), such as glutathione peroxidase-1 (GPX-1), thioredoxin 1 (Trx-1) and thioredoxin 2 (Trx-2). Zofenopril therapy induced a significant up-regulation of GPX-1 and Trx-1 as compared to vehicle (***Figures 35B and 35C***, $p < 0.05$), while it did not alter Trx-2 expression (***Figure 35D***). Then, we also evaluated the effect of zofenopril on the biomarkers of oxidative stress in myocardial tissue and plasma (***Figures 35E and 35F***), such as malondialdehyde (MDA) and advanced

oxidative protein products (AOPP). We observed a significant reduction in MDA levels in heart tissue (***Figure 35E***; $p < 0.05$) and AOPP in plasma (***Figure 35F***; $p < 0.05$) induced by zofenopril treatment as compared with vehicle.

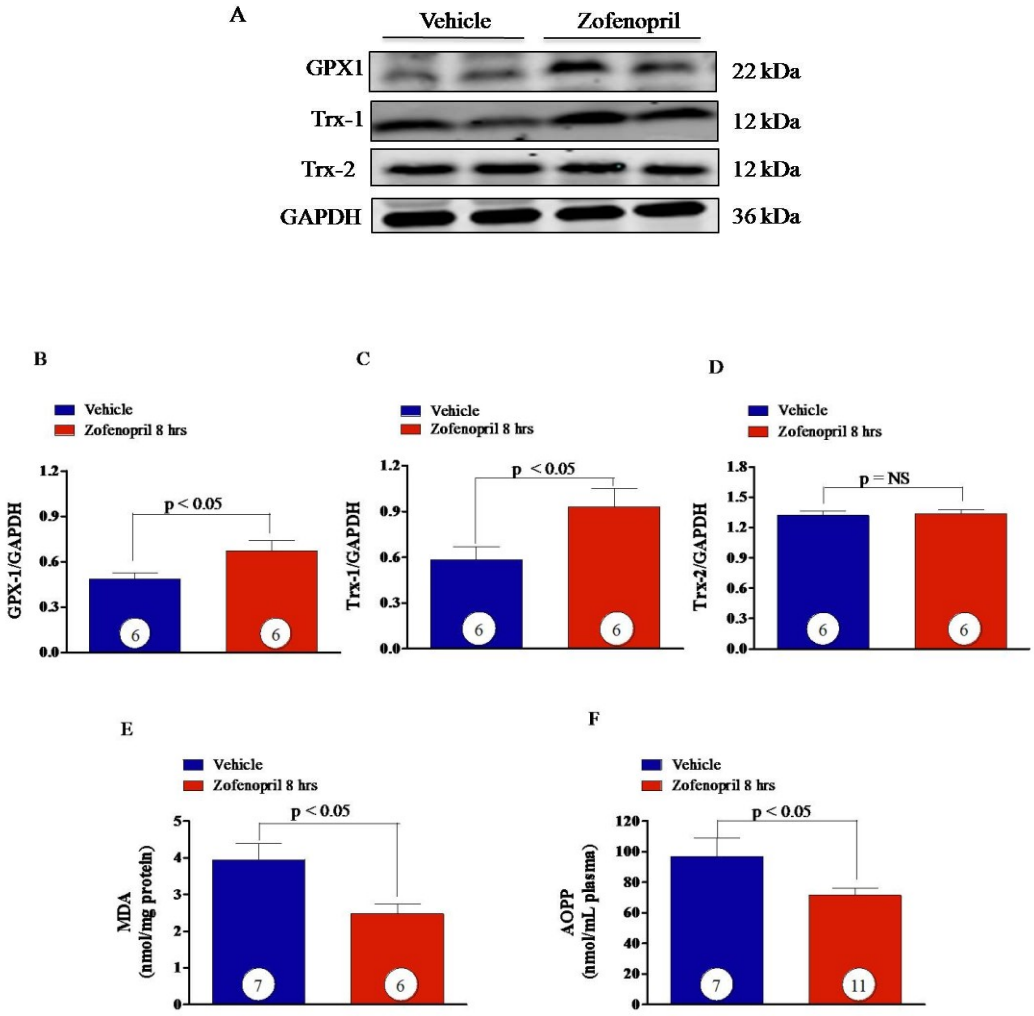

***Figure 35*. Upregulation of antioxidant proteins and reduction of biomarkers of oxidative stress induced by zofenopril treatment.** Immunoblot for GPX-1, Trx-1 and Trx-2 (A) with relative optical densitometry (B-D); tissue levels of malodialdehyde (MDA) and circulating advanced oxidation protein product (AOPP) (E, F, respectively). Zofenopril therapy (10 mg/kg, o.g.; 8 hours) induced a significant upregulation of myocardial antioxidant proteins, such as GPX-1 and Trx-1 (B, C; $p < 0.05$). Zofenopril therapy did not have any effect on Trx-2 protein expression (D). A single dose of zofenopril caused a significant reduction of MDA and AOPP levels as compared to vehicle (E, F, $p < 0.05$ respectively). Number in the circle inside the bar denotes the number of animals used per group

10.4 SUPPLEMENTAL RESULTS

10.4.1 Zofenopril effect on H_2S and NO bioavailability is dose-dependent

We investigated the effect of 8 hours of treatment with a single different dose of zofenopril (6 mg/kg, o.g.; 8 hours) on H_2S and NO bioavailability (***Figures 36A-D***). As can be seen in ***Figure 36A***, zofenopril at dose of 6 mg/kg did not induce any change in H_2S levels in myocardium tissue, but it increased significantly plasma H_2S as compared to vehicle (***Figure 36B***, $p < 0.05$). In addition, zofenopril administration augmented NO metabolites levels in heart tissue (***Figure 36C***, $p < 0.01$), but not in plasma (***Figure 36D***). These results clearly demonstrate that zofenopril effect on these signalling molecules depends on dosage.

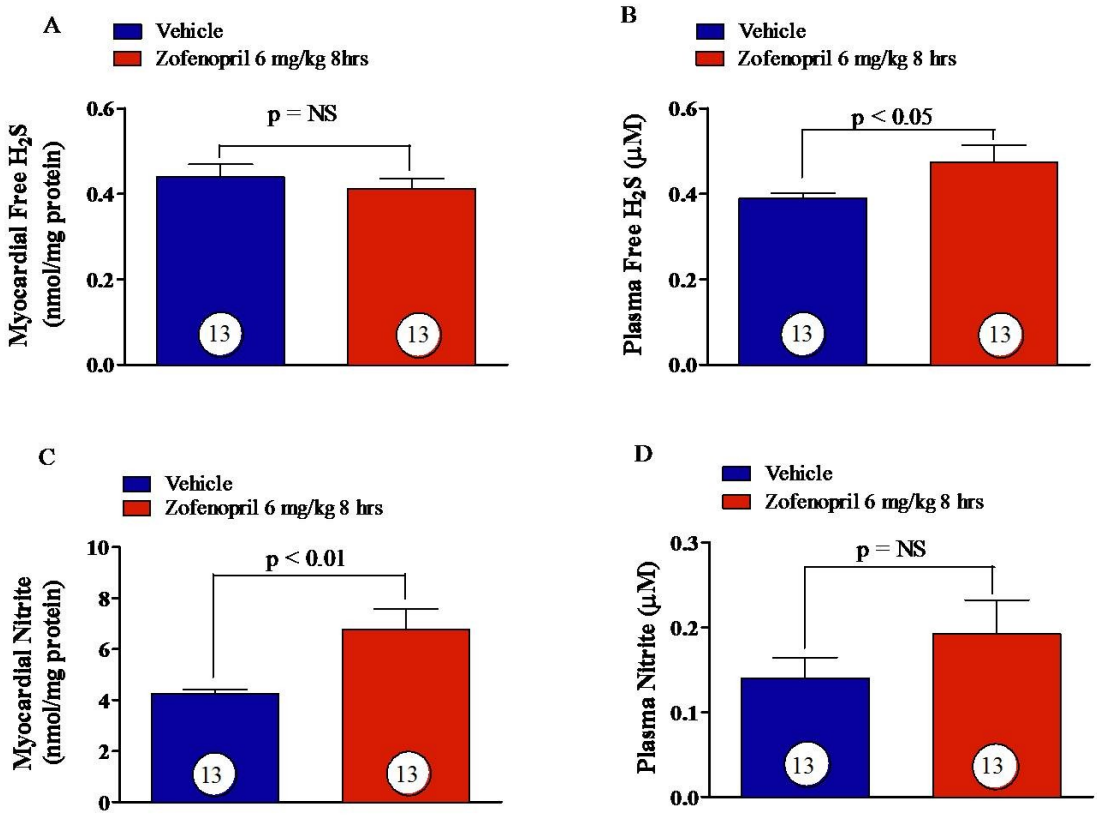

Figure 36. **Effect of a different dose of zofenopril on tissue and circulating H_2S and NO bioavailability.** A single administration of zofenopril (6 mg/kg, o.g.; 8 hours) did not modify H_2S levels in myocardium (A), while it induced a significant increase in plasma H_2S as compared to vehicle (B, $p < 0.05$). Zofenopril therapy showed an opposite effect on NO bioavailability in heart tissue and plasma. Acute zofenopril treatment induced a significant raise in myocardial nitrite as compared to vehicle (C, $p < 0.01$). This effect was not observed in plasma (D). Number in the circle inside the bar denotes the number of animals used per group.

10.4.2 A single administration of ramipril, a dycarboxilate ACE inhibitor, does not have any effect on H_2S and NO bioavailability

It was also our interest to evaluate the effects of ramipril, a non sulfhydryl ACE inhibitor, on H_2S levels (***Figures 37A-B***). Ramipril (3 mg/kg, o.g.) was administered 8 hours before mice sacrifice. We found that ramipril therapy did not induce any increase in H_2S bioavailability in heart and plasma compared to vehicle (***Figures 37A and 37B***). We also analyzed the effect of ramipril administration on NO levels.

Ramipril treatment did not cause any change in nitrite levels in both myocardial tissue and plasma (***Figures 37C and 37D***).

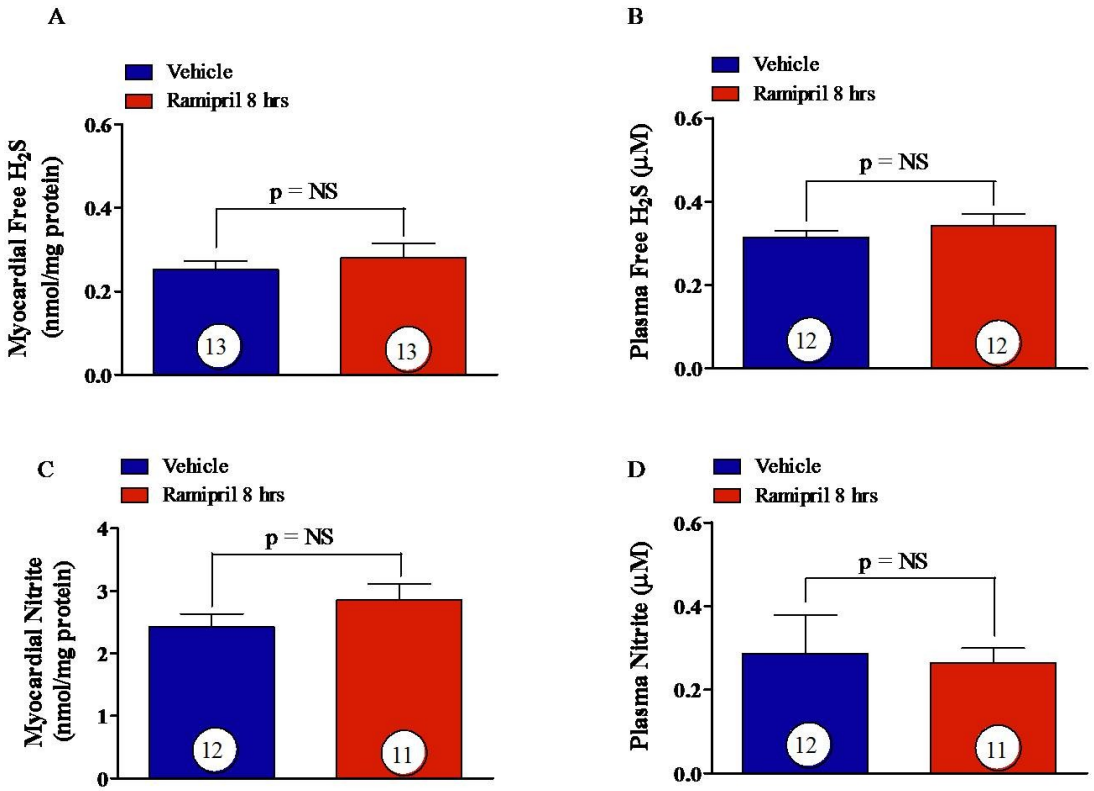

***Figure 37*. Ramipril effect on tissue and circulating H_2S and NO levels.** A single administration of ramipril (3mg/kg, o.g.; 8 hours) did not modify H_2S availability in heart tissue and plasma (A, B respectively) and NO metabolites levels in the same vascular territories (C, D respectively). Number in the circle inside the bar denotes the number of animals used per group.

10.5 DISCUSSION

Zofenopril is a highly lipophilic pro-drug, converted by esterase into the active metabolite zofenoprilat, which is characterized by a free thiol group. A number of clinical studies have demonstrated that the sulfhydryl ACE inhibitor zofenopril exerts additional effects beyond the ACE inhibition[249, 250]. Indeed, recently it has been reported that zofenopril potentiates the H_2S signalling through H_2S release and/or *via* H_2S producing enzymes induction[244]. In this study, we have investigated the bioavailability of myocardial and circulating H_2S and NO in mice after a single administration of zofenopril. Furthermore, we also evaluated the effect of zofenopril therapy in a murine model of MI/R injury. Our findings demonstrate that a single administration of zofenopril increases H_2S bioavailability and enhances NO levels in both heart tissue and plasma. The induction in H_2S availability in these cardiovascular territories occurs mainly in an non-enzymatic manner, while the augmented levels of NO appear to be due to enhanced phosphorylation of eNOS at the Ser^{1177} site. This activation of eNOS may be a result of increased H_2S following zofenopril treatment. Moreover, pretreatment with zofenopril before MI/R exerts cardioprotective effects through the reduction of the infarct size per area at risk and decrease of circulating troponin-I levels, an index of cardiac injury. The presence of the free radical scavenging sulfhydryl group in zofenoprilat molecular structure may be the explanation of the cardioprotective activity of zofenopril and its high potential in the prevention and therapy of cardiovascular diseases. During hypoxia and ischemia/reperfusion conditions, ROS are the main factor of cardiac tissue damage. Moreover, it is well known that H_2S exerts antioxidant properties and promotes Nrf2 activation and transcription of downstream antioxidant proteins related genes. Therefore, we evaluated whether zofenopril therapy could affect Nrf2 signalling and enhance tissue antioxidant defense preventing the ischemic injury. In this context, we have found that zofenopril treatment induces the up-regulation of the transcription factor Nrf2 and downstream antioxidant enzymes, such as Trx-1 and GPX1;

moreover, it reduces the oxidative biomarker levels of MDA in myocardial tissue and AOPP in plasma. These findings result very interesting when it is taken account of a recent evidence that demonstrate 3-MST activity requires the presence of thioredoxin[251]. However, further studies are necessary to investigate the role of 3-MST in cardioprotection and to better elucidate the mechanisms by which zofenopril promotes Nrf2 signalling activation. Thus, our findings support the concept that zofenopril released H_2S scavenges ROS directly and/or indirectly by activation of Nrf2 and subsequently increases antioxidant enzymes expression resulting in cardiac protection. In conclusion, it can be established that zofenopril may prevent cardiac injury during ischemic conditions primarily through the enhancement of cardiovascular antioxidant defense induced by H_2S activated Nrf2 signalling.

11. CONCLUSION

The findings reported in my PhD thesis strongly argue in favor of a role for H_2S in lower urinary tract and cardiovascular diseases.

Despite H_2S is well recognized as an important signalling molecule in the cardiovascular system, it still deserves a better characterization and elucidation by further studies in urinary system. To date, several evidence clearly indicate a deregulation of L-cysteine/H_2S pathway as the main cause of a number of cardiovascular disorders, i.e. myocardial ischemia, hypertension and heart failure together with the NO/cGMP pathway. On the other hand, intense research is actually under way to discover the association between lower H_2S levels and progression of urinary disorder, including chronic renal failure and renal ischemia reperfusion. A better understanding of the roles of H_2S in urinary tract can provide insights into potential therapeutic strategies of urinary diseases. More specifically, data available so far, strongly suggest that H_2S may become the next potent preventive and therapeutic agent for counteracting and ameliorating the symptoms of renal-associated diseases. Therefore, therapeutic interventions aimed at increasing H_2S levels might be beneficial for patients affected with cardiovascular or genito-urinary disorders. On the other hand, there are evidence that suggest a negative modulation of L-cysteine/H_2S pathway may be useful in some other pathologies such as cancer.

In this regard, the emerging data on the biological effects of H_2S support basically two approaches for the development of sulfide-based therapeutics: H_2S-releasing compounds or inhibitors of CSE and/or CBS, which suppress endogenous sulfide formation, in those diseases associated to H_2S overproduction. However, it is also conceivable to highlight whether modulation of endogenous H_2S pathway may contribute to the pharmacological actions of some of the already approved drugs and this may support the better selection of therapeutic prescription.

Regarding to this latter approach, we have demonstrated additional mechanisms of action for PDE5-Is and sulfhydryl ACE-Is, drugs already approved for treatment of ED and hypertension, respectively. Actually PDE5-Is are also suggested for treatment

of LUTS associated to BPH and/or ED, while ACE-Is for myocardial ischemia and heart failure.

It has been reported that beneficial effects showed by PDE5-Is are partially dependent on NO/cGMP signalling, while ACE inhibition is not the exclusive mechanism by which ACE-Is exert cardioprotection. In this context, we have taken in account sildenafil, a PDE5-I and zofenopril, a sulfhydryl-ACE-I, providing a further explanation of their effectiveness in LUTS and myocardial ischemia, respectively. In particular, we have revealed that both drugs potentiate H_2S signaling or *via* CBS activation (sildenafil) or acting as an H_2S donor *in vivo* (zofenopril).

In conclusion, these findings confirm that H_2S plays an important role in maintaining the homeostasis in physiopathological condition and by directing endogenous H_2S increases in production or applying exogenous H_2S, we may find novel solutions for preventing, interfering, and treating a wide spectrum of diseases.

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