Blackwell Publishing

Asian J Androl 2007; 9 (3): 377–381
DOI: 10.1111/j.1745-7262.2007.00241.x

AJA
www.asiaandro.com

·*Original Article*·

Assessment of heme oxygenase-1 (HO-1) activity in the cavernous tissues of sildenafil citrate-treated rats

M. Talaat Abdel Aziz[1], M. Farid Al-Asmar[2], Taymour Mostafa[3], Hazem Atta[1], Laila Rashed[1], Dina Sabry[1], Shedeed Ashour[3], Ahmed T. Abdel Aziz[1]

[1]*Molecular Biology Unit, Medical Biochemistry Department, Faculty of Medicine, Cairo University, Cairo 11553, Egypt*
[2]*Medical Biochemistry Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt*
[3]*Andrology Department, Faculty of Medicine, Cairo University, Cairo 11553, Egypt*

Abstract

Aim: To assess heme oxygenase-1 (HO-1) activity in the cavernous tissue of sildenafil citrate-treated rats. **Methods:** One hundred and ninety-two Sprague-Dawley male rats, divided into four equal groups, were investigated. Group 1, the control group, received regular animal chow; group 2 received sildenafil citrate by intragastric tube; group 3 received sildenafil and HO inhibitor (zinc protoporphyrin, ZnPP); and group 4 received sildenafil and nitric oxide synthase (NOS) inhibitor L-nitroarginine methyl ester (L-NAME). Twelve rats from each group were killed after 0.5 h, 1 h, 2 h and 3 h of drug administration. Then HO-1 activity, cGMP levels and NOS enzymatic activity in the cavernous tissues were estimated. **Results:** In cavernous tissue, HO-1 activity, NOS enzymatic activity and cGMP concentration increased significantly in sildenafil-treated rats compared to other groups throughout the experiment. Rats receiving either HO or NOS inhibitors showed a significant decrease in these parameters. HO-1 cavernous tissue activity and NOS enzymatic activity demonstrated a positive significant correlation with cGMP levels ($r = 0.646$, $r = 0.612$ respectively; $P < 0.001$). **Conclusion:** The actions of PDE_5 inhibitor sildenafil citrate in the cavernous tissue are partly mediated through the interdependent relationship between both HO-1 and NOS activities. *(Asian J Androl 2007 May; 9: 377–381)*

Keywords: erectile dysfunction; heme oxygenase; sildenafil citrate; nitric oxide synthase; carbon monoxide

1 Introduction

Penile erection is reached through increased blood flow to the cavernous tissues, mediated by opening of penile resistance vessels (helicine arteries), relaxation of cavernous tissue cells and occlusion of the venous outflow. This erectile response is known to depend on nitric oxide (NO) released from nerves and vascular endothelium, activating soluble guanylate cyclase (sGC) that increases the concentration of cGMP. cGMP functions as a signaling mediator in the vasodilatation and the regulation of vascular tone. It activates cGMP-dependent protein kinase, which activates the Ca^{2+}/ATPase pump that extrudes Ca^{2+} into the endoplasmic reticulum.

Correspondence to: Prof. Taymour Mostafa, Andrology Department, Faculty of Medicine, Cairo University, Cairo 11553, Egypt.
Tel: +20-1051-50297 Fax: +20-2363-2297
E-mail: taymour1155@link.net
Received 2006-05-06 Accepted 2006-09-22

It also activates the Ca^{2+}/K efflux pump that extrudes Ca^{2+} across the plasma membrane. Consequently smooth muscle cell relaxation occurs as a result of decreased intracellular Ca^{2+} [1].

Thus, erectile function is determined by tight regulation of relaxation or contraction of corpus cavernosal smooth muscle, which is the result of a long and complex chain of molecular events. Control of erectile function resides in the signaling pathways of the central and peripheral nervous systems and in intracellular events in the penile smooth muscle. This knowledge has led to the development and current availability of effective oral treatments for erectile dysfunction, including the selective phosphodiesterase type 5 (PDE_5) inhibitors [2].

Sildenafil citrate was the first introduced PDE_5 inhibitors. Since its launch in 1998, it has proven effective in the treatment of male erectile dysfunction. Besides its proved erectogenic action it has demonstrated different beneficial, non-erectogenic actions. Sildenafil had been useful in the management of pulmonary hypertension, female sexual dysfunction, enhanced female genital blood flow and endometrial thickening. Sildenafil also exerts several effects that are of clinical relevance in gastrointestinal disorders. In patients with heart failure, the PDE_5 inhibitor improved the capacity for exercise in endothelial dysfunction. Moreover, in the treatment of Raynaud's phenomenon, a disease that as yet has no highly effective medical treatment, sildenafil has some has shown some promise [3].

Heme oxygenase (HO) enzyme catalyzes the rate limiting step in oxidative degradation of heme to biliverdin, releasing equimolar amounts of carbon monoxide (CO) and iron. Two isoforms of HO exist as products of distinct genes. HO-1 is an inducible isoform distributed in the mammalian tissues and HO-2 is constitutively expressed, predominantly found in the central nervous system [4]. The relationship between the CO/HO system and NO/NOS system is controversial. While some studies reported synergism between NO and CO [5], others reported that inhibition of NO synthesis promoted renal production of CO [6]. Abdel-Aziz *et al.* [7] indicated that the induction of NOS or HO is equally effective in enhancing erectile function via the upregulation of their gene expression and the local tissue levels of cGMP, the mediator of vasodilatation.

The aim of the present work is to assess HO-1 activity in the cavernous tissue of sildenafil citrate-treated rats.

2 Materials and methods

2.1 Animals

One hundred and ninety-two Sprague-Dawley male rats (weight range 130–150 g) were used in this work. They were bred in the animal house at the Faculty of Medicine, Cairo University Hospital according to NIH guidelines. They were divided into four equal groups. Group 1, the control group, received regular animal chow; group 2, received the same diet in addition to sildenafil citrate (4 mg/kg bodyweight) through a gastric tube (equivalent to 50 mg dose adjusted according to Paget's table of experimental studies) [8]; group 3 received sildenafil citrate and HO inhibitor (zinc protoporphyrin, ZnPP; 50 μg/kg); and group 4 received the same dose of sildenafil citrate and NOS inhibitor L-nitroarginine methylester (L-NAME; 250 mg/kg).

2.2 Samples

The rats were killed in groups of twelve by cervical dislocation after 0.5, 1, 2 and 3 h. Cavernous penile tissues were dissected and divided into two portions. The first was put in homogenization buffer to estimate HO enzyme activity. The buffer contained sucrose (0.25 mol/L), 0.1 mol/L KCl, 1 mmol/L Tris HCl and 0.4 mol/L phenyl methyl sulfonyl fluoride (PMSF), adjusted to pH 7.8. The second portion was kept in 0.1 mol/L HCl to inhibit phosphodiesterase enzyme, stored at –80°C and used for cGMP assay by ELISA.

2.3 cGMP assay

Cavernous tissue samples stored in 0.1 mol/L HCl were homogenized and centrifuged at 6 000 × *g*, at 4°C for 10 min. The supernatant was used for cGMP assay by ELISA kit (R&D Systems, Minneapolis, MN, USA).

2.4 HO-1 enzyme activity assay

Cavernous tissue homogenized samples were incubated in medium consisting of heme (50 μmol/L), rat liver cytosol (5 mg/mL), $MgCl_2$ (2 mmol/L), glucose-6-phosphate dehydrogenase (1 unit), glucose-6-phosphate (2 mmol/L) and NADPH (0.8 mmol/L) in 0.5 mL of 0.1 mol/L phosphate buffer saline (pH 7.4) for 60 min at 37°C. Reaction was stopped by cooling the tubes on crushed ice and then the bilirubin product was extracted with chloroform. The concentration of bilirubin was monitored at 464 nm and 520 nm by a spectrophotometer and was then calculated by using an extinction coef-

ficient of 40.0 mmol/L [9].

2.4 NOS enzymatic activity

Nitrite and nitrate were used as markers for assay of the activity of NOS. Nitrite was measured by using the Griess reaction, the griess reagent (1 g/L sulfanilamide , 25 g/L phosphoric acid and 0.1 g/L N-1-naphthylethylenediamine) was added to the homogenized cavernous tissue samples. After 10 min of color development at room temperature, the absorbance was measured at 543 nm. Nitrate was measured as nitrite after enzymatic conversion by nitrate reductase [10].

2.5 Statistical analysis

Numerical data were expressed as mean ± SD and range. Comparisons were performed by one-way ANOVA test. Correlations were tested by Spearman's test. Comparisons and correlations were considered statistically significant if $P < 0.05$.

3 Results

Cavernous tissues HO-1 activity (pmol bilirubin/mg protein/min) was significantly higher in the sildenafil-treated group compared to other groups, where it reached a peak 1 h after administration, and then dropped gradually for 3 h but remained higher than the control levels. In rats that received sildenafil added to either HO (group 3) or NOS inhibitors (group 4) exhibited equally significant decreases in HO activity compared to groups 1 and 2 (Figure 1). NOS enzymatic activity (μmol/mg protein) in the cavernous tissues was also significantly higher in sildenafil citrate-treated groups compared to all other groups. Rats that received sildenafil added to the NOS inhibitor demonstrated significant decreases compared to those that received either sildenafil or sildenafil added to the HO inhibitor (Figure 2). cGMP levels (pmol/mg) in cavernous tissue were also significantly higher in sildenafil citrate-treated groups compared to all other groups. Rats that received sildenafil added to the NOS inhibitor demonstrated a significant decrease in cGMP levels compared to those that either received sildenafil or sildenafil added to the HO inhibitor (Figure 3). Cavernous tissue HO-1 activity demonstrated a positive significant correlation with cGMP levels and NOS enzymatic activity in all groups ($r = 0.646$, $r = 0.612$; $P < 0.001$).

4 Discussion

Clinical studies have demonstrated that sildenafil citrate successfully treats erectile dysfunction of varied etiologies. The impact of sildenafil has stimulated academic, clinical and industrial researchers to conduct experiments aimed at understanding the mechanism(s) underlying erectile function, hoping to develop better treatment modalities. PDE_5 is the major cGMP hydrolyzing enzyme in the penile cavernous tissues and is an important regulator of NO-mediated smooth-muscle re-

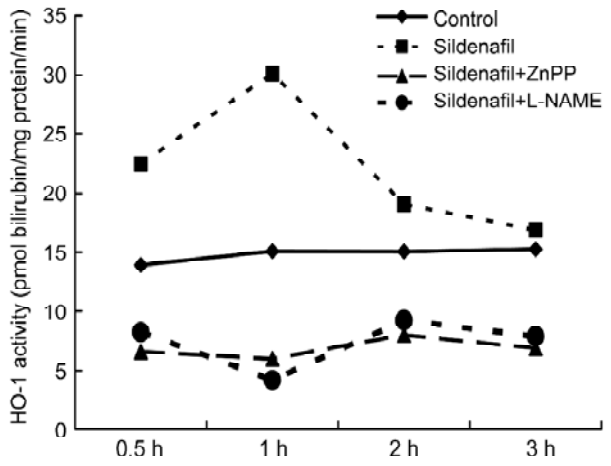

Figure 1. Time course study of cavernous tissue HO-1 activity in studied groups. ZnPP, zinc protoporphyrin; L-NAME, L-nitroarginine methylester.

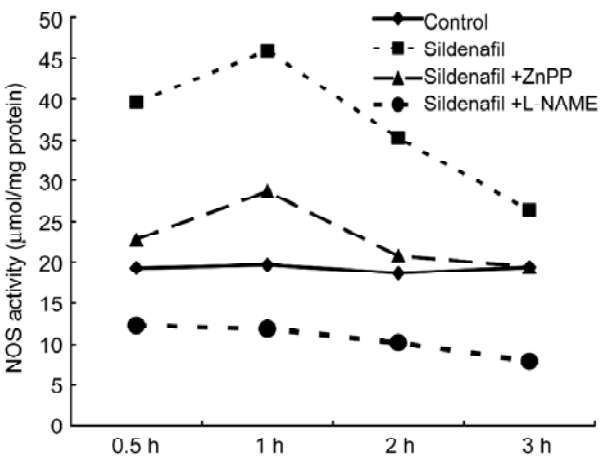

Figure 2. Comparison of NOS activity in cavernous tissues of studied groups. ZnPP, zinc protoporphyrin; L-NAME, L-nitroarginine methylester.

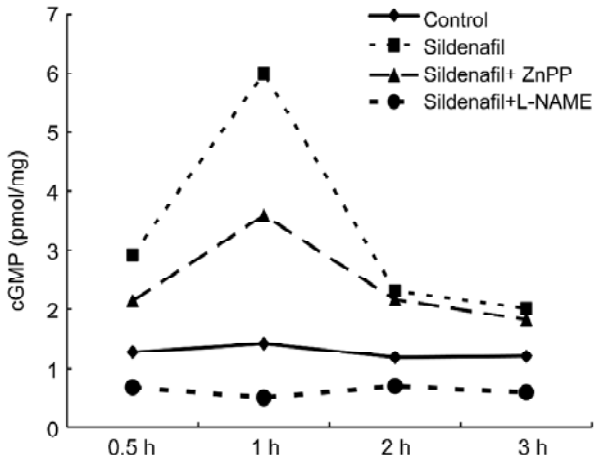

Figure 3. Comparison of cGMP concentration in cavernous tissue of studied groups. ZnPP, zinc protoporphyrin; L-NAME, L-nitroarginine methylester.

laxation [11]. NO is formed from oxidation of L-arginine by NOS isoforms: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS). NO plays a crucial role in initiating and maintaining an increase in intracavernous pressure, penile vasodilatation and penile erection-dependent cGMP [12].

In the present work, HO-1 and NOS enzymatic cavernous tissue activities were significantly higher in the sildenafil-treated group, compared to the controls after one half hour and reached a peak after 1 h. A gradual drop was observed after that and this continued for 3 h. However, it was always higher than that of the controls. These activities in rats treated with the same dose of sildenafil citrate added to either HO or NOS inhibitors exhibited a dramatic decline of these activities, and reached levels even below that of the controls. Meanwhile, cavernous tissue cGMP level coincided with HO-1 and NOS activity changes; significantly increased in the sildenafil-treated group compared to the controls after one half an hour with a peak after 1 h, followed by a gradual decline for 3 h. This finding was supported by demonstrating a positive correlation between HO-1, NOS activities and cGMP levels in the investigated cavernous tissues. NOS inhibitor showed a stronger influence in declining c-GMP than HO inhibitor when added to sildenafil-treated rats. Based on these data, the increase of HO-1 activity by sildenafil citrate is mainly an indirect effect of NO, not sildenafil citrate. This new and important observation could point to the signaling mode of action(s) taking place in this process. These results were in agreement with Vidavalur *et al*. [13] who reported that human coronary arteriolar endothelial cells exposed to sildenafil demonstrated significant induction of HO-1 enzyme activity. In addition, Bivalacqua *et al*. [14] reported sildenafil significantly increased expression levels of eNOS and cGMP in streptozitocine (STZ) treated rats and that the erectile response was greater in STZ-diabetic rats receiving eNOS gene therapy plus sildenafil than STZ-diabetic rats receiving either sildenafil or eNOS gene therapy alone.

sGC is a heme-dependent enzyme that catalyzes the formation of guanosine 3',5'-cGMP after the binding of NO to the iron of the heme group. The purified heme-containing form of sGC has also been reported to be activated by CO. Endogenously produced CO has been shown to possess intriguing signaling properties affecting numerous critical cellular functions including but not limited to inflammation, cellular proliferation, and apoptotic cell death. The discovery that endogenously produced gaseous molecules such as NO and CO can exert potent physiological and biological effecter functions truly represented a paradigm shift and unraveled new avenues of intense investigations [15]. CO exhibits physiological properties similar to NO and it is believed that these actions are mediated in part by the ability of CO to act as an activator of sGC. Like NO, CO binds to the heme moiety of sGC leading to its activation and an increase in cGMP levels. After the induction of HO-1 in the rat aorta, the tissue cGMP content was greatly enhanced and was believed to be a part of the mechanism that underlies CO vasodilator activity [16].

Abdel-Aziz *et al*. [7] indicated that induction of either NOS (using L-arginine) or HO (using hemin) was equally effective in enhancing erectile function via the upregulation of gene expression of the two signaling molecules, NOS and HO-1, and through concomitant upregulation of the local tissue levels of cGMP. Hedlund *et al*. [17] found that the cholinergic nerve terminals in the human corpora cavernosa and corpus spongiosum contain NOS, HO-1 and HO-2 genes and enzymes, suggesting that these terminals comprise a distinct population of parasympathetic cholinergic nerves and that HO/CO systems have a complimentary role in erection [18]. The role of CO as an NO-like signaling molecule is also supported in studies of HO and NOS knockouts. Furthermore, both HO-1- and HO-2-derived CO have a positive and negative effect on sGC and cGMP levels in vascular endothelial cells [19]. Nevertheless, the CO-

releasing molecule CORM-3 has been shown to have a potent vasodilator effect in normal and diabetic rats [20]. Also, HO-1 overexpression increased vascular relaxation and NO bioavailability [21]. Ryter *et al*. [5] stated that HO protects NO through the scavenging of reactive oxygen species (ROS). Thus, HO prevents NO from reacting with ROS, preventing the formation of peroxynitrite and its subsequent degradation, confirming that CO tissue levels parallel NO levels.

It is concluded that the actions of the PDE_5, sildenafil citrate, in the cavernous tissue are partly mediated through the interdependent relationship between HO-1 and NOS activities and the upregulation of the tissue levels of cGMP, the mediator of vasodilatation.

References

1 Hellstrom WJ. The molecular basis of erectile physiology: From bench to bedside. J Androl 2002; 23: S3–4.

2 Seftel AD. Phosphodiesterase type 5 inhibitors: molecular pharmacology and interactions with other phosphodiesterases. Curr Pharm Des 2005; 11: 4047–58.

3 Cremers B, Bohm M. Non erectile dysfunction application of sildenafil. Herz 2003; 28: 325 –33.

4 Wang J, Lu S, Moenne P, Montellano OR. Interaction of nitric oxide with human heme oxygenase-1. J Biol Chem 2003; 278: 2341–7.

5 Ryter SW, Morse D, Choi AM. Carbon monoxide: to boldly go where nitric oxide has gone before. Sci STKE 2004; 2004 (230): RE6.

6 Rodriguez F, Lamson BD, Gong W, Kemp R, Nasjletti A. Nitric oxide synthesis inhibition promotes renal production of carbon monoxide. Hypertension 2004; 43: 347–51.

7 Abdel-Aziz MT, El-Asmar MF, Mostafa T, Atta H, Abdel aziz M, Fouad H, *et al*. Effects of nitric oxide synthase and heme oxygenase inducers and inhibitors on molecular signaling of erectile function. Clin Biochem Nut (Japan) 2005; 37: 103–11.

8 Paget GE, Barnes GM. Evaluation of Drug Activities. Vol. 1, Academic Press: London. 1964.

9 Abraham NG, Lutton JD, Levere RD. Heme metabolism and erythropoiesis in abnormal iron states. Role of aminolevulinic acid synthetase and heme oxygenase. Exp Haemato 1985; 13: 833–43.

10 Moshag H, Kok B, Huizenga JR, Jansen LM. Nitrite and nitrate determination in plasma: A critical evaluation. Clin Chem 1995; 41: 892–6

11 Jackson G, Gillies H, Osterloh I. Past, present and future: a 7-year update of Viagra (sildenafil citrate). Int J Clin Pract 2005; 59: 680–91.

12 Toda N, Ayajiki K, Okamura T. Nitric oxide and penile function. Pharmacol Ther 2005; 106: 233–66.

13 Vidavalur R, Penumathsa SV, Zhan L, Thirunavukkarasu M, Maulik N. Sildenafil induces angiogenic response in human coronary arteriolar endothelial cells through the expression of thioredoxin, hemeoxygenase and vascular endothelial growth factor. Vascul Pharmacol 2006; 19; 45: 91–5.

14 Bivalacqua TJ, Usta MF, Champion HC, Leungwattanakij S, Dabisch PA, McNamara DB, *et al*. Effect of combination endothelial nitric oxide synthase gene therapy and sildenafil on erectile function in diabetic rats. Int J Impot Res 2004; 16: 21–9.

15 Ryter SW, Alam J, Choi AM. Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. Physiol Rev 2006; 86: 583–650.

16 Wang R, Wang Z, Wu L. Carbon monoxide-induced vasorelaxation and the underlying mechanisms. Br J Pharmacol 1987; 121: 927–34.

17 Hedlund P, Ny L, Anderson KE. Cholinergic nerves in human corpus cavernosum and spongiosum contain nitric oxide synthase and heme oxygenase. J Urol 2000; 164: 868–75.

18 Wang J, Lu S, Moenne P, Montellano OR. Interaction of nitric oxide with human heme oxygenase-1. J Biol Chem 2003; 278: 2341–7.

19 Abraham NG, Quan S, Mieyal PA, Yang L, Burke-Wolin T, Mingone CJ, *et al*. Modulation of cGMP by human HO-1 retrovirus gene transfer in pulmonary microvessel endothelial cells. Am J Physiol Lung Cell Mol Physiol 2002; 282: L1117–24.

20 Di Pascoli M, Rodella L, Sacerdoti D, Bolognesi M, Turkseven S, Abraham NG. Chronic CO level has a beneficial effect on vascular relaxation in diabetes. Biochem Biophys Res Commun 2005; 340: 935–43.

21 Ahmad M, Turkseven S, Mingone CJ, Gupte SA, Wolin MS, Abraham NG. Heme oxygenase-1 gene expression increases vascular relaxation and decreases inducible nitric oxide synthase in diabetic rats. Cell Mol Biol 2005; 51: 371–6.

Edited by Dr Steven M. Schrader