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Research Article

DETERMINATION OF THE EFFECTIVENESS OF SILDENAFIL ON HUMAN AORTIC SMOOTH MUSCLE CELLS

Harun Alp[1], Hidayet Tutun[2], Ergin Şingirik[3],
Halil Mahir Kaplan[4], Muhammet Mükerrem Kaya[2], Levent Altıntaş[5]

[1]*Karamanoglu Mehmetbey University, Medicine Faculty, Medical Pharmacology Department, Karaman, Turkey*
[2]*Burdur Mehmet Akif Ersoy University, Veterinary Faculty, Pharmacology and Toxicology Department, Burdur, Turkey*
[3]*Cukurova University, Medicine Faculty, Medical Pharmacology Department, Adana, Turkey*
[4]*Cukurova University, Medicine Faculty, Pharmacology Department, Adana, Turkey*
[5]*Ankara University, Veterinary Faculty, Pharmacology and Toxicology Department, Ankara, Turkey*

**Corresponding Author:*
E-mail: laltintas@ankara.edu.tr

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ABSTRACT. Erectile dysfunction is defined as the inability to gain and/or maintain a penile erection sufficient for satisfactory sexual performance. Sildenafil is a phosphodiesterase type 5 (PDE_5) inhibitor used in the treatment of erectile dysfunction. The aim of this study is to investigate the effect of sildenafil (0.8% in cell media) on the mechanism of human aortic smooth muscle contraction. Human smooth muscle cells treated with sildenafil for 1 and 4 days. These cells were then homogenized, and enzymes and proteins responsible for the mechanism of intracellular contraction were analysed. Rho-kinase (ROCK) activity, RhoA, ROCK II, CPI-17α, PDE_{5a}, and PLC levels were evaluated by using ELISA. In this study, it was observed that sildenafil increased RhoA, PDE_{5a} and CPI-17α levels, and decreased ROCK enzyme activity.

Keywords: *Contraction mechanism, human aortic smooth muscle cell, sildenafil.*

INTRODUCTION

Cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) are essential secondary messengers, which regulate a wide variety of processes, including processes such as signal transduction and regulation of physiological responses, blood pressure control, neurotransmission, platelet aggregation and dissociation, in many different tissues of the body [1,2]. Phosphodiesterase (PDE) isoenzymes, a heterogeneous group of hydrolytic enzymes, cause degrade cellular cAMP and cGMP. Currently, there are known to be 11 different PDE gene families containing different isoforms and splice variants [1]. Sildenafil citrate (Viagra), a PDE_5 inhibitor, fostering cGMP accumulation by inhibiting the activity of PDE_5 enzyme and initiates a cGMP-driven cascade of reactions, thus enhancing vasodilation and treating erectile dysfunction (ED). It was the first prescription drug that's approved to treat ED [3, 4]. In addition, inhibition of this enzyme provides clinical benefit in the treatment of several urological disorders such as female sexual dysfunction, priapism, Peyronie's disease, overactive bladder, lower

urinary tract symptoms, premature ejaculation, benign prostate enlargement, and urinary tract stones [5].

Nitric oxide (NO) is generated by NO synthase (NOS) enzymes and activates guanylate-cyclase (GC) to produce intracellular cGMP. High levels of cGMP after PDE_5 inhibition or NO stimulation cause smooth muscle relaxation due to activation of protein kinase G (PKG) [6-8]. Increased vasodilatory activity results in improved erectile function [9]. Sildenafil, a potent and selective PDE_5 inhibitor, causes smooth muscle relaxation by increasing the level of cGMP in the cell. Mechanisms regulating erectile function include arterial dilatation and smooth muscle relaxation. After the sexual stimulation, the erectile response is triggered by the release of NO which is the main vasoactive nonadrenergic, noncholinergic neurotransmitter mediating penile erection and then the intracellular messenger cGMP is produced. It activates cGMP-dependent protein kinases and in turn phosphorylate certain proteins and ion channels, resulting in opening of the potassium channels and hyperpolarization. After the hyperpolarization, accumulation intracellular calcium by the endoplasmic reticulum, and inhibition of voltage-dependent calcium channels resulting in blocking calcium influx. As a result, resulting in the reduction of cytosolic free calcium leads to smooth muscle relaxation [10, 11].

Penile erection is a complex neurovascular condition that begins with the stimulation of the central and peripheral systems [12]. Male sexual dysfunction, observed at high rates in developed countries, is considered to be an important health problem and may occur due to reasons such as chronic heart failure, high cholesterol, diabetes, smoking, alcoholism or drug addiction, stress, eating habits [13]. In the flaccid state of penis, the sympathetic effect is dominant and neurotransmitter substances such as noradrenaline are released, causing cavernosal smooth muscle contraction. The parasympathetic effect increases after sexual arousal, and therefore, the release of NO synthesized by neuronal NOS from nerve endings in the penis increases. NO triggers corporal smooth muscle relaxation by activating the cGMP / PKG signal from the muscle. Reduced peripheral resistance of cavernosal arterioles and the driving force of systemic blood pressure, leading to increase blood flow into the penis. It causes friction (shear) stress that activates endothelial NOS (eNOS) to produce NO with increased blood flow. As a result, the sinusoidal spaces fill with blood, which creates a pressure to squeeze the venules against the tunica albuginea, thus limiting the venous outflow. This veno-occlusion mechanism greatly increases intracavernosal pressure and causes penile erection [14, 15].

Vascular smooth muscle contraction is controlled by the Ca^{2+} dependent and Ca^{2+} sensitive mechanisms initiated by activation of receptors or depolarization of the plasma membrane [16, 17]. In the classical Ca^{2+} dependent pathway, agonist ligand-induced G-protein-coupled receptor (GPCR) activation increases phospholipase C (PLC) activity, increasing the formation of inositol trisphosphate (IP_3) and di acyl glycerol (DAG). IP_3 then causes Ca^{2+} release from the sarcoplasmic reticulum, which raises intracellular Ca^{2+} levels. Ca^{2+} binding to calmodulin leads to a conformational change in its conformation, enables Ca^{2+}/calmodulin to activate myosin light chain (MLC) kinase (MLCK). Phosphorylation of regulatory MLC by MLCK leads to force generation. DAG promotes contraction by activating protein kinase C (PKC) along with Ca^{2+} and by phosphorylation of L-type Ca^{2+} channels, or other proteins that regulate the cross-bridge cycle. As a consequence, voltage-operated Ca^{2+} channels in the membrane open in response to membrane depolarization caused by vascular smooth muscle tension [15, 18]. This process involves the Ca^{2+} dependent regulation and the monomeric GTP binding protein

RhoA [19]. The Rho family belongs to the Ras superfamily of GTPases, including five subfamilies; Rho-like (Rho A, B and C), Rac-like (Rac1, 2 and 3 and RhoG), Cdc42-like, RhoBTB-like and Rnd-like (Rnd 1, 2 and 3) [20]. RhoA plays important roles in the regulation of smooth muscle contraction, and stress fiber formation, cell proliferation, migration, and apoptosis. The best characterizing effector of RhoA is Rho-kinase (ROCK), which directly participates in smooth muscle contraction.

It has been reported that ROCK increases Ca^{2+} sensitivity by phosphorylating CPI-17 (a phosphatase inhibitor enhanced with 17 kDa PKC). CPI-17 is a myosin phosphatase phosphorylation dependent inhibitor and has two isoforms, CPI-17α and CPI-17β [21]. CPI-17 is a downstream target of PKC, which plays an important role in smooth muscle contraction [22]. IP_3 binds into a particular receptor after being formed by PLC activity and serves as a second intracellular messenger for smooth muscle contraction. The IP_3 receptor is in the sarcoplasmic reticulum channel protein that activated by IP_3 and it allows Ca^{2+} to flow into the cytoplasm, causing the smooth muscle to contract [23]. The IP_3 receptor is one of the best-known substrates of PKG. The phosphorylation of the IP_3 receptor leads to decreased channel activity in response to IP_3, a decrease in Ca^{2+}, and smooth muscle relaxation [23, 24]. In summary, an increase in PKG activity likely inhibits IP_3 formation. It is unclear whether this is a direct result of PLC inhibition or a result of G-protein inhibition. The reduction in Ca^{2+} released from intracellular stores results in smooth muscle relaxation in both cases [24]. There have been limited studies on the impact of chronic sildenafil usage on human smooth muscle cell contraction. In this study, it was aimed to investigate the effects of sildenafil on the contraction of human aortic smooth muscle cells (HASMCs) contraction *in vitro*.

MATERIALS AND METHODS

Cell culture and procedures

Primary HASMCs were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 1% penicillin-streptomycin (Hyclone), 10% Fetal Bovine Serum (Hyclone), 1% L-Glutamine (Hyclone). The cells were incubated in 24-well cell culture plates ($1x10^5$ cells per well) at 37 ºC temperature, 5% CO_2 and 95% relative humidity. The control group (99.2% culture medium and 0.8% PBS) and the experimental group treated with sildenafil (99.2% culture medium and 0.8% sildenafil) were formed. The groups were treated the drug for both 24 and 96 hours. After treatment, the cells were homogenized for protein analysis.

Cell homogenization

Treated cells were lysed in RIPA buffer (TritonX-100, 20 mmol/L EGTA, 150 mmol/L NaCl, 0.5%, 1 mmol/L ditiotreitol, 25 mmol/L NaF, 1 mmol/L Na_3VO_4, 50 mmol/L Tris-HCI [pH 7.4]) for 15 minutes on ice and the lysate was centrifuged at 15000 rpm for 20 minutes, the supernatants were used for analysis**.**

Analysis of protein

3 ml RIPA lysis buffer (Abcam, ab156034) was added per well onto the cells. The lysate was sonicated on ice for 5 seconds, centrifuged at 10000 rpm for 10 minutes at 4

°C and then the supernatant was used for protein analysis. Total protein quantification from homogenized cells was done [25]. Protein quantification was performed for standardization of ELISA experiments.

ELISA Test

Levels of PDE5, RhoA, ROCK II, MYPT, PLC, CPI-17α (phospho-t38) enzymes were analyzed by ELISA test. Experimental protocols of ELISA kits vary for each enzyme. PDE_{5a}, RhoA, ROCK II, MYPT, PLC and CPI-17α protein levels were also measured with ELISA test kits (Shanghai Sunred Biological Technology Co., Ltd).

Statistics

One Way Anova "Bonferroni's Multiple Comparison Test" was used for comparisons between groups. $P<0.05$ was accepted as the statistical significance value.

RESULTS AND DISCUSSION

Corporal smooth muscle relaxation is critical importance for normal erectile function, and NO of neuronal and endothelial origin is considered to be the principal mediator of corporal smooth muscle relaxation [26, 27]. Relaxation is mediated by the dephosphorylation of the MLC by smooth muscle myosin phosphatase [28]. ROCK, a serine/threonine kinase, is a major regulator of MLCP. RhoA, a GTP-binding protein, has been shown to mediate agonist-induced ROCK activation 29 [17]. ROCK inhibits MLPC and directly increases cellular CA concentrations by phosphorylating MLC, resulting in smooth muscle contraction [30-32]. It has been reported that ROCK activity is important in the continuity of corporal vasoconstriction and penile detumescence [33].

ROCK is activated by RhoA which is a small GTP-binding protein. Activated ROCK (available in α and iso isoforms) phosphorylates the regulatory myosin phosphatase target subunit 1 (MYPT1) of MLCP at Thr-696, inhibiting its activity, promoting smooth muscle contraction [28]. In a study on rats, sildenafil given for a long time prolonged the duration of erection in elderly rats and increased the expression of eNOS phosphorylated at serine-1177 and Akt phosphorylated at serine-473 in the penis. Also, it has been shown that sildenafil increased the protein expression of PDE5 and phosphomyosine phosphatase target subunit 1 (phospho-MYPT1, p-MYPT1) which is a marker of ROCK function, in the penis of young rat. The authors reported that the lack of increased erectile ability with prolonged PDE_5 inhibition in young rats may be related to the limited NO signalling by PDE_5 upregulation, lack of Akt and eNOS phosphorylation, and increased ROCK signalling in the penis. Sildenafil had no effect on the protein expression of R-ks α and β [34]. In the current study, the effects of sildenafil on PDE_5, RhoA, ROCK activity (p-MYPT), ROCK II (enzyme expression), PLC and CPI-17α (phospho-t38) enzymes in the HASMCs are shown in Figure 1.

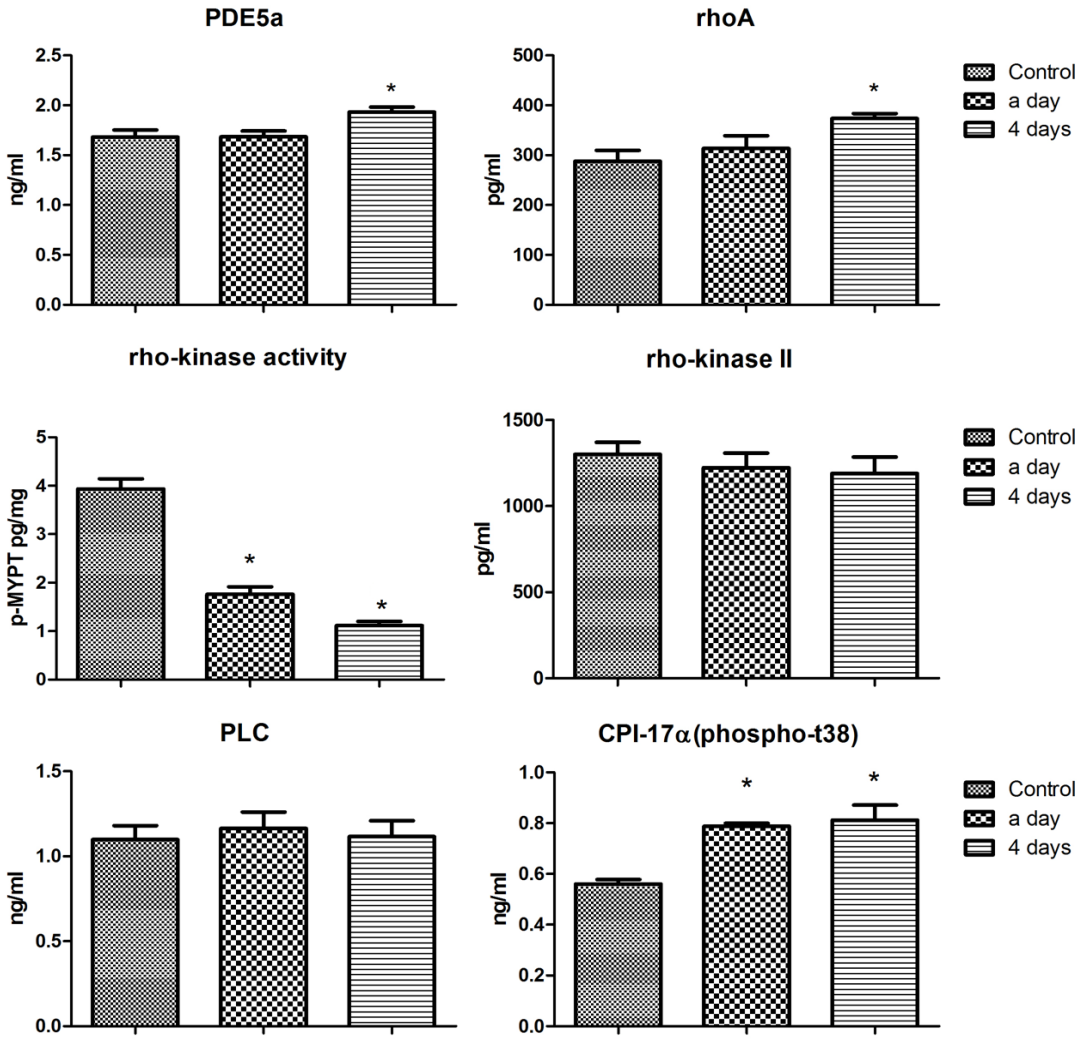

Fig. 1. *Effect of sildenafil on PDE5, RhoA, ROCK activity (p-MYPT), ROCK II (enzyme expression), PLC and CPI-17α (phospho-t38) enzymes in HASMCs (n=6).*
** : P<0.05.*

Although PDE_{5a} and RhoA enzyme levels in human smooth muscle cells did not increase after one day of sildenafil exposure, they increased significantly after four days of sildenafil exposure as compared to the control group. ROCK activity was significantly decreased after 1 and 4 days of sildenafil exposure compared to the control group. However, it was observed that sildenafil had no significant effect on ROCK II enzyme activity. Similar to other studies [34, 35], the current study indicates that the observed increase in PDE_{5a} protein expression may be due to chronic exposure to sildenafil and negative feedback in response to sustained sGMP elevation, thus preventing excessive sGMP accumulation and excessive erection.

After the agonist stimulation, CPI-17-mediated MLCP inhibition with concurrent MLCK activation is thought to be responsible for the significant rise in MLC phosphorylation and smooth muscle contraction [36, 37]. In the current study, it was observed that sildenafil significantly increased CPI-17α but did not have a significant effect on the PLC enzyme.

CONCLUSION

As a result, sildenafil has been found to relax smooth muscle by reducing ROCK enzyme activity, and thereby reflexively increase the levels of RhoA, PDE_{5a} and CPI-17α enzymes. In addition, it is predicted that this reflex response may indirectly lead to the development of a tolerance to the drug.

Conflict of Interest. "The authors declared that there is no conflict of interest."

Authorship Contributions. Concept: H.A., E.Ş., H.M.K., L.A. Design: H.A., H.T., E.Ş., H.M.K., M.M.K., L.A., Data Collection or Processing: H.A., H.T., E.Ş., H.M.K., M.M.K., L.A., Analysis or Interpretation: H.A., E.Ş., H.M.K., Literature Search: H.A., H.T., M.M.K., L.A., Writing: H.A., H.T., E.Ş., H.M.K., M.M.K., L.A., Reading and review: H.A., H.T., E.Ş., H.M.K., M.M.K., L.A.

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