Phosphodiesterase 5 Inhibitor Relaxes Isolated Rat Trachea in Endothelin-1-induced Contraction

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***Abstract*—*Phosphodiesterase* 5 inhibitor or sildenafil relaxes airway smooth muscle in endothelin-1 (ET-1) induces rat tracheal ring contraction, but the mysterious relationship between ET-1 and sildenafil unclear; therefore, this study shows interactions of sildenafil with ET-1 and roles of ET-1 receptor antagonists on rat tracheal ring in ET-1-induced tracheal contraction. Rat tracheal rings (intact and denuded) isolated and transferred into organ chamber. The rings preincubated with selective ET-1A receptor (BQ-123) and ET-1B receptor (BQ-788) antagonists and then ET-1 dose–response curve performed for the rat tracheal rings. Preincubation of BQ-123 markedly reduced the ET-1 potency, whereas BQ-788 did not reduce ET-1 pD2; also in denuded isolated rat trachea rings, preincubated with BQ-123 changed Emax values. L-NAME preincubation increased sildenafil pD2, whereas indomethacin preincubation is slightly decreased sildenafilpD2, whereas clotrimazole preincubation significantly increased sildenafilpD2 and highly elevated of sildenafil maximum efficacy (Emax). In conclusion, ET-1 induces tracheal smooth muscles contraction due to ET-1A receptor activity rather than ET-1B receptor activity, and tracheal epithelial cells highly contribute to tracheal contraction. Sildenafil relaxes rat tracheal rings of ET-1 precontraction mainly through NO-dependent mechanism, inhibition of COX, and cytochrome p450 epoxygenase pathways.**

***Index Terms*—Endothelin-1 receptors, Rats, Sildenafil, Trachea.**

I. Introduction

Endothelin-1 (ET) is a small peptide consist of 21 amino acids. ET-1 constricts vascular blood vessels and respiratory airway trachea (Nagase, *et al.*, 1997). Furthermore, ET-1 acts as a potent bronchoconstrictor [1,2] and then increases the airway muscle contraction through nitric oxide - granulate cyclase pathway [3], and ET-1 acts on airway epithelial cells [4]. ET-1 is localized mainly to vascular endothelium and airway smooth muscles in the respiratory system, but its localization is reduced in the epithelium [5,6].

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ET-1 has two main type receptors: ET-1A and ET-1B receptors, the roles of ET-1 receptors in modulation airway smooth muscle tone not entirely understood, but [7] showed that, both ET-1 receptors (A and B) have roles to contract airway smooth muscle [8]. Concluded that airway smooth muscle contraction in response to elevation of ET-1 could modulate by ET-1A receptor antagonist (BQ-123), whereas ET-1B antagonist (BQ-788) had no significant effects on modulation air smooth muscle. Further, Janosi, *et al.* [9] suggested that ET-1 causes the airway contraction; also, both ET-1A and ETB receptor antagonists exert modulation effects in different ways. Radioligand binding and immunohistochemistry indicated the expression of both ET_A and ET_B receptors confirms in the epithelium cells [10]. However, in guinea pig airway smooth muscles, the ET- 1B receptor has roles in contraction and ET-1A receptors mediate airway muscle contraction through release of COX metabolites (Bruno, *et al.*, 1994). ET-1-induced vascular smooth muscle cell proliferation is mediated by cytochrome p-450 arachidonic acid metabolites [11].

Sildenafil prevents hydrolyze cGMP and regulates airway smooth muscle relaxation (Calzetta, *et al.*, 2013). For instances, Toward, *et al.* (2004) reported that the relaxation of sildenafil is not dependent on the cellular endogenous NO levels, but [12] concluded that the vasorelaxant activity of sildenafil mostly due to NO-dependent mechanism. In addition, sildenafil reduces the intensity of ET-1 and induces airway smooth muscle contraction, but according to our knowledge, the exact mechanism of sildenafil bronchodilators on rat tracheal rings of ET-1 precontraction not fully understood. Therefore, the present study investigated the mechanism of sildenafil airway smooth muscles relaxation as precontracted by ET-1 and pointed on the roles of ET-1 receptor antagonists (BQ 123 and BQ 788) on modulation of induced ET-1 rat tracheal ring contraction.

II. Materials and Methods

A. Animals and Housing

Male rats (*Rattus norvegicus*) housed in the animal facility belonged to Biology Department, College of Science - Salahaddin University - Erbil. The animals kept in plastic cages bedded with wooden chips and the rat diet is standard pellets; the ambient animal facility conditions were (12/12 h) photoperiod and temperature

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(22 ± 4°C). Animal research ethics committee in Biology Department - Salahaddin University - Erbil had approved ethical animal care and experimental design.

B. Tissue Preparation

Preparation of rat tracheal rings

Animals anesthetized with the intraperitoneal injection of ketamine (40 mg/kg) and xylazine (10 mg/kg). Then, the chest cavity opened near to cranial side to obtain the optimal length of the trachea. After that, the rat trachea cleaned of surrounding connective tissues and cut into small rings about 3–4 segments, and they transferred into a Petri dish filled with cold Krebs-Henseleit solution aerated with about 95% oxygen in 37°C. Krebs solution is a physiological buffered solution that contains (NaCl 0.118, $NaHCO_3$ 0.025, $MgSO_4$ 0.0012, KCl 0.0047, KH_2PO_4 0.0012, $CaCl_2$ 0.0025, $C_6H_{12}O_6$ 0.011, and EDTA 0.001) in mole/L with pH 7.4. Two stainless steel hooks passed through the lumen of the trachea and transferred into organ bath chamber with 10 mL Krebs solution aerated about 95% O_2 and 5% CO_2. The denuded trachea obtained by gently rubbing the lumen of the trachea to remove the epithelial cells. To ensure that the epithelium was denuded, histological preparations performed**.**

Isometric tension recording in isolated trachea

The tracheal rings held up by two stainless steel clamps (Tissue clamp, Model Le 0140, Panlab Harvard Apparatus, USA): The first clamp connected by the hook and the bottom of the organ bath jacket, and the second clamp connected to the thread and force transducer. Thread vibrations and rat tracheal ring isometric tension detected by the transducer and then signals recorded by amplifier (Quad bridge amplifier Powerlab 8/35), after that LabChart 7.1 software acquisition and analyzed tracheal ring contractions or relaxations. During data acquisition, the rat tracheal rings immersed in 10 mL Krebs solution inside organ chambers, and the Krebs solution was maintained at pH 7.4 with continuously aerated with carbogen, nearly 95% oxygen and 5% carbon dioxide at 37°C (LE 13206 Thermostat, Panlab Harvard Apparatus, USA). Rat tracheal rings set on 1 g tension and then 45 min inside organ bath incubated. When tension reached the steady state, 60 mM KCl used for rat tracheal rings viability. Rat tracheal rings several times washed by 10 mL Krebs to return of initial force tension; later, ET-1 (Bachem AG Company, Germany) dose–response curve (10^{-10}–10^{-7} M) obtained and EC50 for ET-1 calculated for time interval 8 min of organ bath for both intact and denuded rat tracheal rings.

Protocols and experimental design

Rat tracheal rings (intact and denuded) preincubated with 0.3 μM selective ETA receptor antagonist (BQ-123) and 1 μM selective ETB receptor antagonist (BQ-788) for 20 min. ET-1 cumulative contracting dose-response curve (DRC) (10^{-10}–10^{-7} M) and EC50 obtained for 8 min time interval of rat tracheal rings; further, sildenafil cumulative relaxing DRC (1×10^{-6} M-1×10^{-4} M) obtained for 3 min time interval of rat tracheal rings. In addition, the intact trachea rings preincubated for 20 min with 10 mM L-NAME, 10^{-5} M indomethacin, 30 mM clotrimazole, 3 mM MB, 1 mM$BaCl_2$, 1 mM TEA, and 10-5M nifedipine, then contracted with 10^{-8} M ET-1, after that sildenafil cumulative relaxing DRC (1×10^{-6} M-1×10^{-4} M) and pD2 obtained for 3 min time interval of rat tracheal rings.

C. Statistical Analysis

ET-1-induced contractions expressed as a percentage, and for each dose–response curves, the Emax and the concentration of ET-1, which produced half of Emax (EC50) were calculated. Emax and EC50 expressed as mean ± S.E.M. Statistical comparisons of EC50 (pD2) values of the dose–response curves for ET-1 obtained with the different treatments in the trachea achieved by unpaired *t*-test. The concentration of the sildenafil producing IC_{50} or pD2 determined after logarithmic transformation of the normalized concentration–response curves, using GraphPad Prism version 7. Results expressed as means ± SEM and the values compared by ANOVA and a Bonferroni's test to estimate significance among groups. Values considered to be statistically significant different when $P < 0.05$.

III. Results

Table I and Fig. 1a and b show that the pD2 and Emax of ET-1 DRC with its receptor A antagonist (BQ-123) and receptor B antagonist (BQ-788) in intact isolated tracheal rings. Here, it is obviously shown that preincubation of BQ-123 for 20 min markedly reduced pD2 from 7.538 ± 0.2232 in control to 6.623 ± 0.1828 in BQ-123 group. As well as the ET-1, DRC shifted to the right significantly begins from dose 10^{-8} M to 3×10^{-7} M; in contrast, BQ-788 did not change ET-1 DRC regarding pD2 values (Table I and Fig. 1a and b). In addition, in denuded isolated rat trachea rings, BQ-123 preincubation changed Emax values, but neither the potency of the ET-1 (EC50 or pD2) nor Emax values did not change statistically in BQ-788 preincubation (Fig. 1c and d).

Statistical analysis revealed that preincubation of L-NAME for 20 min increased pD2 (pIC50) from 3.806±0.2267 of sildenafil (control group) to 2.964±0.3308 of L-NAME group. On the other hand, L-NAME preincubation significantly shifted the DRC of sildenafil to the right and changed the Emax (%) from 64.71±15.05 to 69.30±0.2267 (Fig. 2a). Furthermore, as seen in Fig. 2b, the isolated tracheal rings preincubated with MB significantly decreased Emax from 90.81 ± 2.813 of control group to 62.93 ± 8.249 of MB

TABLE I Maximum Efficacy (Emax) and pD2 Values of Dose–response Curves for the Effects of ET-1 Receptor Antagonists on Response to ET-1 in Intact and Denuded Rats Isolated Tracheal Rings

Groups	Intact trachea		Denuded trachea	
	pD2	Emax	pD2	Emax
Control	7.538±0.2232	150.5±24.37	7.817±0.1259	104.2±7.581
BQ-123	6.623±0.1828**	314.4±97.65*	7.479±0.6105	120.7±54.68
BQ-788	7.417±0.2048	136.9±22.80	7.830±0.3003	98.32±17.99

Values are means ± S.E. Emax: Maximum efficacy, $pD_2 = -\log EC_{50}$. *Means $P < 0.05$; ** means $P < 0.01$

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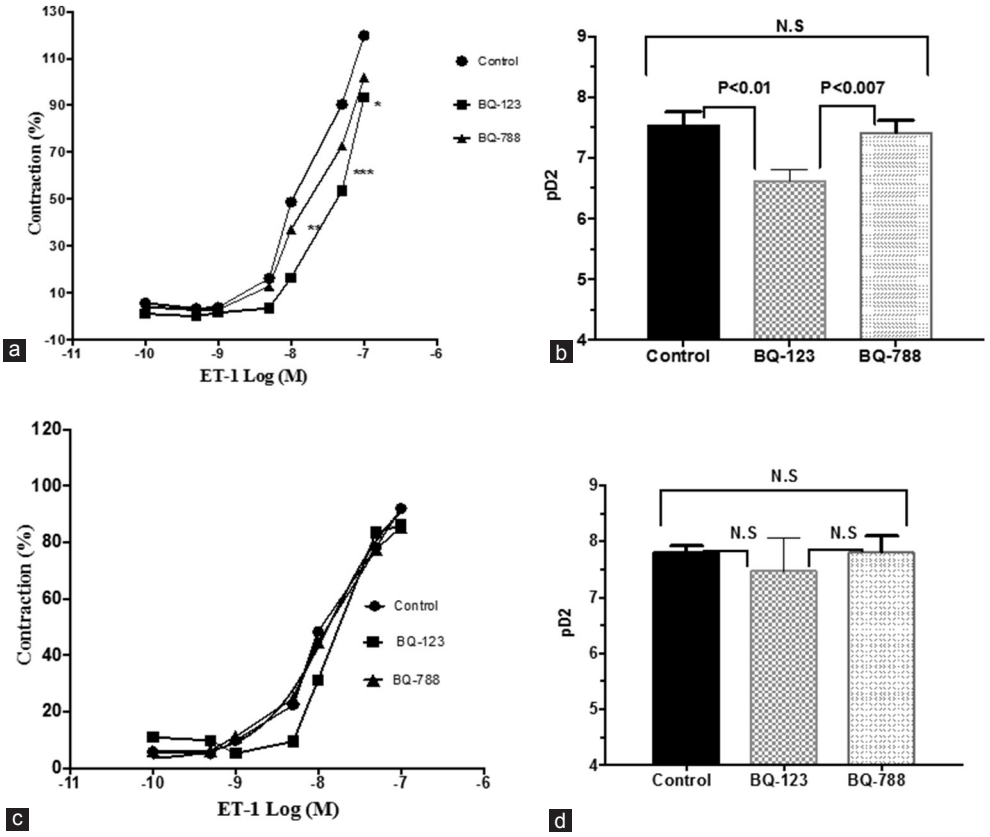

Fig. 1. Endothelin-1 (ET-1) cumulative contractile dose–response curve intact (a) and denuded (b) isolated tracheal rings with the absence (control) and presence of ETA receptor antagonist (BQ 123, 0.3 μM and BQ-788, 1 μM) in rats. The pD_2 value for ET-1 in intact tracheal (b) and denuded rings (d). Values represented as mean ± S.E.

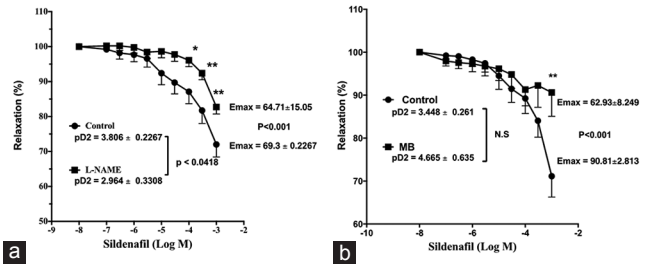

Fig. 2. Sildenafil cumulative relaxation response curves in isolated trachea precontracted with 10^{-8} M ET-1 in the (a) absence and presence of L-NAME and (b) MB. Values represented as mean ± S.E.

group. Meanwhile, the DRC of sildenafil markedly shifted to the left by BM preincubation. However, pIC50 (pD2) was slightly increased but not significantly in MB group.

When rat isolated trachea preincubated with indomethacin for 20 min slightly reduced pD2 of the sildenafil, whereas it markedly shifted the DRC of sildenafil to the left side (Fig. 3a). Moreover, the efficacy of the sildenafil (Emax) was statistically ($P < 0.05$) elevated from 61.41 ± 8.249 to 81.09 ± 30.25. As shown in Fig. 3b, statistical analysis revealed that clotrimazole preincubation significantly increased pD2 values from 3.413 ± 0.252 in control group (without clotrimazole preincubation) to 7.366 ± 1.120 in clotrimazole group. Furthermore, the curve of the sildenafil DRC in high significant sifted to the left with highly elevation in Emax from 61.41 ± 8.249 of control group to 105.2 ± 1.001 of clotrimazole group.

Fig. 4a-c shows the preincubation of Kir channel blocker ($BaCl_2$) and Kca++ channel blocker (TEA). Neither sildenafil potency (pD2) nor its efficacy (Emax) was changed when these blockers preincubated.

V. Discussion

The present results revealed that when an intact isolated trachea incubated with ET-1A antagonist (BQ-123) significantly lowered pD2 (potency of the drug) and the efficacy of ET-1 (Emax) and it shifted the curve to the right side (Fig. 1a and b). In contrast to this, ET-1B antagonist (BQ-788) did not change the pD2 and Emax of ET-1 DRC (Table I and Fig. 1c and d). Previously, it has been shown that ET-1 can cause significant airway contraction in isolated tracheal rings (Frullini, *et al*., 2011). ET-1 induced–tracheal contraction via the mediation of NO-cGMP pathway [3]. ET-1 inhibits NO release and synthesis [13]. Furthermore, ET-1 induces the formation of IP3 as the second messenger (Li, *et al*., 2005) and as a consequence calcium released into the cytosol. All these pathways occur through ET-1A and B receptors [7,14]. However, the roles of ET-1A and B receptors in modulation airway smooth muscle tone are not fully explored. Uhlig and Featherstone [8] reported that

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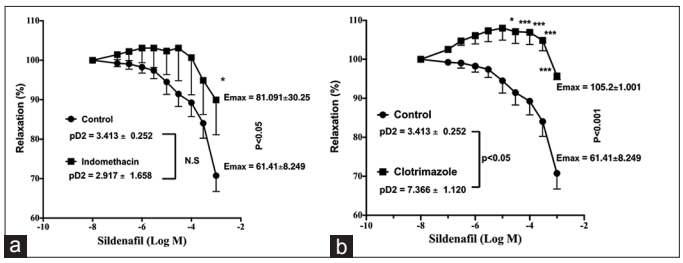

Fig. 3. Cumulative concentration–response curves for the phosphodiesterase 5 inhibitor (sildenafil, 1×10^{-6} M-1×10^{-4} M) in producing relaxation of isolated trachea precontracted with 10^{-8} M ET-1 in the absence and presence of indomethacin (a) and clotrimazole (b). Values represented as mean ± S.E.

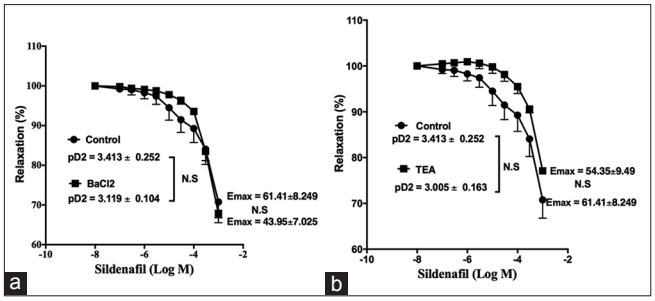

Fig. 4. Cumulative concentration–response curves for the phosphodiesterase 5 inhibitor (sildenafil, 1×10^{-6} M-1×10^{-4} M) in producing relaxation of isolated trachea precontracted with 10^{-8} M ET-1 in the absence and presence of BaCl2 (a) and TEA (b). Values represented as mean ± S.E.

tracheal smooth muscle response to ET-1 was significantly by ET-1A receptor antagonist (BQ-123), whereas ET-1B receptor antagonist (BQ-788) had no significant roles. On the other hand, Janosi, *et al.* [9] suggested that ET-1 receptors exert their modulation with differential effects. However, Goldie*, et al*. [15] resulted that the ET-1 binding sites on the bronchial smooth muscles are ET-1B receptors, and the induced contraction is not prevented by ET-1A antagonist, and conversely, it augmented by ET-1B receptor agonist. On the contrary, the present results indicated that denuded isolated trachea preincubated with BQ-123 and BQ-788 did not cause any change in ET-1 DRC regarding Emax and pD2 (Fig. 1c and d). Previously, it has been concluded that in the tracheal smooth muscle, ET-1 is mainly localized to the muscle and with low expression in the epithelium [16]. Our presented result is in contrast to Clancy, *et al.* [10] who showed that radioligand binding and immunohistochemistry indicated the expression of both ET- 1A and B receptors on the tracheal epithelial cells. Hence, one can conclude from the present results that ET- 1A receptor exerts its effects on both epithelial and smooth muscle cells in isolated tracheal rat rings. In other words, epithelial cells in the trachea mediated the important roles in airway smooth muscle tones.

Data analysis revealed that the DRC of phosphodiesterase 5 (PDE-5) inhibitor (sildenafil), the potency (pD2 or pIC50), and the efficacy of sildenafil (Emax). The trachea here contracted with ET-1 (EC50 =10–8M). Before that, the isolated tissues preincubated with L-NAME and MB and then they compared with control group (without preincubation). The sildenafil DRC of significantly shifted to the right side, pD2 and Emax values also markedly elevated when L-NAME preincubated (Fig. 2a). However, Toward, *et al.* [17] reported that the effectiveness of sildenafil is not dependent on the endogenous NO levels. The present result is in consistent with Yildirim, et al. [12] who concluded that the vasorelaxant activity of sildenafil is mainly due to NO-dependent mechanism. Accordingly, it can be found that sildenafil relaxed airway smooth muscle precontracted with ET-1 mostly through the NO dependent. Previously, it has been reported that sildenafil is mostly responsible for cGMP degradation and stimulating of soluble cGMP to generate intracellular cGMP [18,19]. Our results concerning the roles of sildenafil mediated with cGMP (inhibited by MB) are consistent with the previous results showing that PDE-5 inhibitor can prevent the hydrolyze cGMP which regulates the airway smooth muscle relaxation [20]).

Statistical analysis revealed that indomethacin pre-incubation for 20 min markedly shifted the DRC of sildenafil to the left and significantly increased the Emax values. Indomethacin is a non-selective COX antagonist it generally used to reduce the action of COX in vessels [21]. Prostaglandins synthesized from arachidonic acid which is converted to prostaglandin G2 and subsequently to prostaglandin H2 by COX. It is well known that the epithelial cells in tracheal tissues are abundant with their prostaglandin synthases and produce prostaglandins [22]. However, the role of the airway epithelium as the source of prostaglandins had not been recognized until evidence was gathered on epithelium-denuded tracheal tissues. The present result is consistent with the previous study showing that arachidonic acid-induced guinea pigs tracheal relaxation was first found to be inhibited by indomethacin [23].

Results from the present study indicated that clotrimazole preincubation statistically elevated the potency of sildenafil (pD2), and it also increased the sildenafil efficacy values (Emax). Clotrimazole is a non-specific IKca channel blocker and also a non-peptide inhibition of cytochrome P450 monoepoxygenase [24]. The roles of Kca channel in airway smooth muscle contraction is not fully understood yet. However, previous reports indicated that inactivation of Kca with inhibitors like clotrimazole increased smooth muscle tones [25]. Besides, clotrimazole affects airway smooth muscle contractions through inhibition epoxygenase pathway. Epoxyeicosatrienoic acids (EETs) have been suggested to account for epithelium-dependent hyperpolarizing factor-mediated relaxation in guinea pig airways [26]. Spector [27] reported that cytochrome p450 epoxygenase (CYP) which converts arachidonic to EET that produces smooth muscle relaxation by an activity of BKca. Taken together, one can be suggested from the present results that clotrimazole because of its inhibitory effects on CYP and its blocking of IKca could reduce the contraction of isolated airway smooth muscle tone.

As shown in Fig. 4a and b, preincubation of non-selective Kca channel blocker (TEA) and Kir channel blocker (BaCl2) slightly shifted the DRC of sildenafil to the left, but they did not change the pD2 values. However,

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it has been well established that potassium channels play a central role in the respiratory epithelium [28] and airway smooth muscle relaxation [29], but according to our knowledge, the exact mechanism of such roles has not been elucidated. Also, it is known that the opening of Kca or Kir channels in the airway smooth muscle cells induces hyperpolarization of the membrane potential, which have important bronchodilator actions [30]. However, in the current results, blocking of these channels slightly changed the DRC of sildenafil which mostly due to the preincubation of the isolated tracheal rings with the potent contractors, ET-1 which may close more these potassium channels.

In conclusion, the results suggested that ET-1-induced airway smooth muscles contraction mostly due to ET-1A receptor activity rather than ET-1B receptors and airway epithelial cells have the importance mediated tracheal contraction. On the other hand, PDE5 inhibitor relaxes airway trachea when it precontracted with ET-1 mainly through NO-dependent mechanism through inhibition of COX enzymes, CPY epoxygenase, and blocking IKca.

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