

Nicotine in High Concentration Causes Contraction of Isolated Strips of Rabbit Corpus Cavernosum

Hoai Bac Nguyen¹, Shin Young Lee², Soo Hyun Park³, Jun Hyun Han⁴, Moo Yeol Lee^{3,*}, and Soon Chul Myung^{1,*}

¹Advanced Urogenital Disease Research Center; Research Institute for Translational System Biomics; Department of Urology, Chung-Ang University Hospital, Seoul 156-755, ²Department of Urology, Seoul Medical Center, Seoul 131-795, ³Department of Physiology, College of Medicine, Chung-Ang University, Seoul 156-756, ⁴Department of Urology, Hallym University Dongtan Sacred Heart Hospital, Hwaseong 445-170, Korea

It is well known that cigarette smoke can cause erectile dysfunction by affecting the penile vascular system. However, the exact effects of nicotine on the corpus cavernosum remains poorly understood. Nicotine has been reported to cause relaxation of the corpus cavernosum; it has also been reported to cause both contraction and relaxation. Therefore, high concentrations of nicotine were studied in strips from the rabbit corpus cavernosum to better understand its effects. The proximal penile corpus cavernosal strips from male rabbits weighing approximately 4 kg were used in organ bath studies. Nicotine in high concentrations ($10^{-5} \sim 10^{-4}$ M) produced dose-dependent contractions of the corpus cavernosal strips. The incubation with 10^{-5} M hexamethonium (nicotinic receptor antagonist) significantly inhibited the magnitude of the nicotine associated contractions. The nicotine-induced contractions were not only significantly inhibited by pretreatment with 10^{-5} M indomethacin (nonspecific cyclooxygenase inhibitor) and with 10^{-6} M NS-398 (selective cyclooxygenase inhibitor), but also with 10^{-6} M Y-27632 (Rho kinase inhibitor). Ozagrel (thromboxane A₂ synthase inhibitor) and SQ-29548 (highly selective TP receptor antagonist) pretreatments significantly reduced the nicotine-induced contractile amplitude of the strips. High concentrations of nicotine caused contraction of isolated rabbit corpus cavernosal strips. This contraction appeared to be mediated by activation of nicotinic receptors. Rho-kinase and cyclooxygenase pathways, especially cyclooxygenase-2 and thromboxane A₂, might play a pivotal role in the mechanism associated with nicotine-induced contraction of the rabbit corpus cavernosum.

Key Words: Contraction, Cyclooxygenase, Nicotine, Rabbit corpus cavernosum, Rho-kinase

INTRODUCTION

Clinical and basic science research studies provide strong indirect evidence that smoking may affect penile erections by impairing endothelium dependent smooth muscle relaxation [1,2]. In addition, cigarette smoking appears to amplify the association between erectile dysfunction and car-

diovascular risk factors such as coronary artery disease [1].


Nicotine, an alkaloid derived from the plant *Nicotiana glauca*, acts as an agonist of nicotinic receptors [3,4]. Currently, the exposure to nicotine is increasing worldwide not only due to the global use of tobacco but also the wide use of medications such as nicotine replacement therapy to assist smoking cessation [3,5].

Many studies have reported the effects of nicotine on the cardiovascular system. In chronic nicotine-administered rat, the chronic nicotine administration impaired aortic reactivity, probably via redox imbalance and vascular remodeling mechanism [6]. In humans, cigarette smoking also increases blood pressure by 5~10 mmHg for 15~30 min [7]. However, hypertension is not more common among cigarette smokers compared to non-smokers [8]. This discrepancy may be caused by a transient blood pressure increase for a short duration,

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Corresponding to: Soon Chul Myung, Department of Urology, College of Medicine, Chung-Ang University, 221 Heukseok-dong, Dongjak-gu, Seoul 156-756, Korea. (Tel) 82-2-6299-1785, (Fax) 82-2-6294-1406, (E-mail) uromyung@cau.ac.kr, Moo Yeol Lee, Department of Physiology, College of Medicine, Chung-Ang University, 84 Heukseok-ro, Dongjak-gu, Seoul 156-756, Korea. (Tel) 82-2-820-5687, (Fax) 82-2-817-7115, (E-mail) heeyun@cau.ac.kr

*These authors contributed equally to this work (co-corresponding author).

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ABBREVIATIONS: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PSS, physiological salt solution; TP receptor, thromboxane receptor; S.E.M., standard error of means; ANOVA, analysis of variance; W/O, washout; NO, nitric oxide; Rho-kinase, Rho-associated protein kinase; COX, cyclooxygenase; PG, prostaglandin; TXA₂, thromboxane A₂; PGF_{2α}, prostaglandin F_{2α}; PGI₂, prostacyclin; PGE₂, prostaglandin E₂; FP receptors, F-prostanoid receptors.

during and after smoking. In contrast to the effects on the cardiovascular system, currently there is no evidence showing that nicotine has direct effects on erectile function.

While the nicotine effect on the penile vascular smooth muscles has been extensively reported, its direct effects in high concentrations on the cavernosal smooth musculature remain poorly understood [4]. The goal of this study was to determine the effects of nicotine on erectile function. Thus, an organ bath study was conducted to investigate the effects of nicotine in high concentrations on isolated rabbit corpus cavernosal strips and the associated mechanisms.

METHODS

Preparation of rabbit corpus cavernosal strips and tension recording

Experiments were carried out according to the guidelines of the Committee for the Protection of Persons and Animals at the Institute of Medical Science, Chung-Ang University, Seoul, Korea. A total of 34 New Zealand white rabbits (approximately 4 kg) were used. The rabbits were anaesthetized with an overdose of pentobarbital (60 mg/kg, intraperitoneal injection) and then sacrificed by incision of the carotid artery. The whole penis was detached from the animal and placed in a Petri dish containing cold (4°C) HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffered physiological salt solution (PSS) with 100% O₂ saturation.

A ventral corporotomy was made on each side of the penis and the penile erectile tissue was carefully dissected from the surrounding tunica albuginea. Two strips of the proximal corpus cavernosum were obtained from each animal. The strips of corpus cavernosum were trimmed to a standard size of 1×1×8 mm. Each strip was suspended in a 30 ml organ bath containing PSS with the following composition: 114 mM NaCl, 26 mM NaHCO₃, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM NaH₂PO₄, and 11 mM D-glucose. During the experiments, the baths were maintained at 37°C and continuously bubbled with gas containing 95% O₂ and 5% CO₂, maintaining a pH of 7.3~7.4. For the experiments, each corpus cavernosal strip was connected to a force transducer (52-9545, Harvard Apparatus, UK). Analog signals were converted to digital signals that were recorded on a MacLab 4e recording system (AD Instruments, Australia). The passive tension was adjusted to 1 g over an equilibration period of 120 minutes, with several changes of PSS.

Measuring strip responses to nicotine

To determine nicotine-induced contraction, the strips were contracted by adding cumulative concentrations of nicotine from 10⁻⁸ M to 10⁻⁴ M to the organ bath, and the responses were recorded.

Investigating the involvement of nicotinic receptors on nicotine-induced contraction

First, nicotinic receptor involvement with the nicotine-induced contraction was examined by incubating the tissue with 10⁻⁵ M hexamethonium (nicotinic receptor antagonist; N,N,N',N',N'-hexamethylhexane-1,6-diaminium) in the organ bath for 10 minutes in separate experiments; then the nicotine-induced contraction was recorded as described in the above protocol.

Determining involvement of the Rho-kinase pathway in the nicotine-induced contraction

To investigate whether the Rho-kinase pathway was involved in the mechanism associated with nicotine-induced contraction, the experimental strips were pretreated with a Rho-kinase inhibitor by adding 10⁻⁶ M Y-27632 ((1R, 4R)-4-((R)-1-aminoethyl)-N-(pyridin-4-yl)cyclohexanecarboxamide) to the organ bath for 10 minutes, then adding cumulative concentrations of nicotine from 10⁻⁸ M to 10⁻⁴ M to the organ bath. Changes in the nicotine contractile amplitude of the studied strips were measured.

Determining involvement of the cyclooxygenase pathway on nicotine-induced contractile effects

To identify cyclooxygenase pathway involvement with the nicotine-induced contraction, the experimental strips were exposed to nonselective cyclooxygenase inhibitor and selective cyclooxygenase-2 inhibitor by separately incubating with 10⁻⁵ M indomethacin (2-{1-[(4-chlorophenyl)carbonyl]-5-methoxy-2-methyl-1H-indol-3-yl}acetic acid) and 10⁻⁶ M NS-398 (N-[2-(Cyclohexyloxy)-4-nitrophenyl]methanesulfonamide) in the organ bath for 10 minutes in each experiment. Consequently, nicotine was added in a concentration dependent manner to the bath. The nicotine induced contractile amplitude was recorded.

Determining involvement of the thromboxane A₂ in nicotine-induced contractile effects

To investigate whether the thromboxane A₂ was involved in nicotine-induced contraction, the experimental strips were pretreated with thromboxane A₂ synthase inhibitor and highly selective TP (thromboxane) receptor antagonist; ozagrel 10⁻⁷ M ((2E)-3-[4-(1H-imidazol-1-ylmethyl)phenyl]acrylic acid) and SQ-29548 10⁻⁷ M ([1S-[1 alpha,2 beta (5Z),3 beta,4 alpha]]-7-[3-[[2-[(phenylamino) carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1] hept-2-yl]-5-heptenoic acid) were separately added to the organ bath in each experiment for 10 minutes, then two different concentrations of nicotine, 10⁻⁵ M and 10⁻⁴ M, were added. Changes in the nicotine associated contractile amplitude of the experimental strips were measured.

Drugs

Nicotine, hexamethonium and Y-27632 were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Indomethacin, NS-398, ozagrel, and SQ-29548 were ordered from Cayman Chemical Company (Ann Arbor, MI, USA). We used nicotine, indomethacin soluble in ethanol and Y-27632 soluble in water.

Data analysis

The experiments were repeated more than five times. Results are presented as the means±standard error of means (S.E.M.). Each contraction measurement of a corpus cavernosal strip is expressed as microN per mg of wet strip weight. The maximum tension value was calculated and obtained from regression plots, and each regression plot was constructed with four to five points using the logistic sigmoidal fitting model (MicroCal Origin, version 7.5). For all

experiments, "n" refers to the number of strips. For the statistical analysis, the Student *t*-test and repeated measures analysis of variance (repeated measures ANOVA) were used to evaluate differences and a $p < 0.05$ was considered significant.

RESULTS

Nicotine-induced contractile effects

The strip responses to nicotine were inconsistent. A majority of the experimented strips contracted in a dose dependent manner in response to nicotine; however, the remaining strips showed contraction followed by relaxation, or only relaxation or no response. The contractile responses of the corpus cavernosal strips to cumulative exposures to nicotine (10^{-8} M ~ 10^{-4} M) were dose-dependent (Fig. 1). The maximum tension value was 6.82 ± 0.11 microN per mg of wet strip weight. The initial contraction was observed at the concentration of 10^{-5} M. However, the higher concentration of nicotine, the more unstable strips became, due to the nicotine toxicity.

Involvement of nicotinic receptors in nicotine-induced contractions

Hexamethonium pretreatment itself evoked no response at resting strips. However, nicotine-induced contraction was inhibited almost completely in the strips pretreated with hexamethonium with the maximum tension values of 3.89 ± 0.06 microN per mg of wet strip weight (Fig. 2). The responses of the hexamethonium-pretreated strips were significantly different from the control group ($p < 0.05$, $n=12$).

The Rho-kinase pathway in association with nicotine-induced contractions

10^{-6} M Y-27632 pretreatment itself evoked no response at resting strips. However, compared with the control, the pretreatment with 10^{-6} M Y-27632 significantly inhibited the nicotine-induced contraction with the maximum tension values of 3.83 ± 0.25 microN per mg of wet strip weight ($p < 0.05$, $n=12$) (Fig. 3).

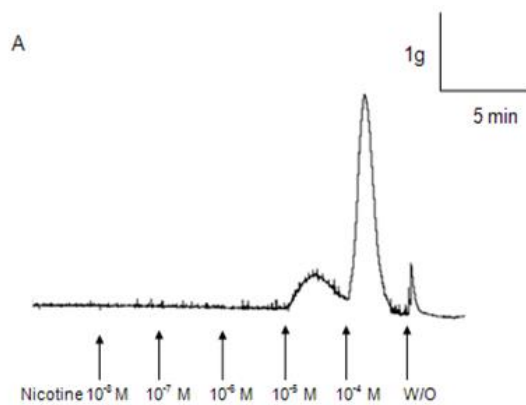


Fig. 1. Effect of nicotine on corpus cavernous strips. High-nicotine concentrations were associated with contraction of the rabbit corpus cavernosal strips ($n=12$). The typical recording of these results is representative of five independent experiments (A), and the contraction was expressed in grams (g). W/O=washout (B). Each point indicates the mean \pm standard error of means (S.E.M.).

Involvement of cyclooxygenase pathway in nicotine-induced contractions

NS-398 or indomethacin pretreatment itself evoked no response at resting strips. However, the nicotine-induced contraction was inhibited in the strips pretreated with NS-398 or indomethacin with maximum tension values of 3.93 ± 0.06 microN per mg of wet strip weight for the former and of 4.72 ± 0.36 microN per mg of wet strip weight for the latter. Compared with the control group, the changes in response of these strips were significant ($p < 0.05$, $n=12$) (Fig. 4).

The association of thromboxane A_2 in nicotine-induced contraction

Ozagrel and SQ-29548 pretreatment itself evoked no re-

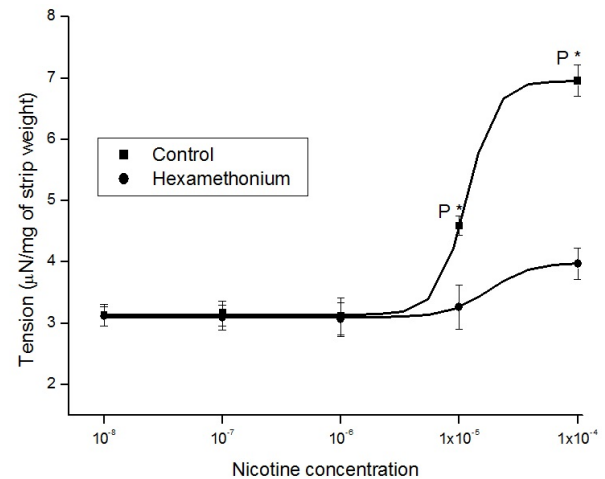
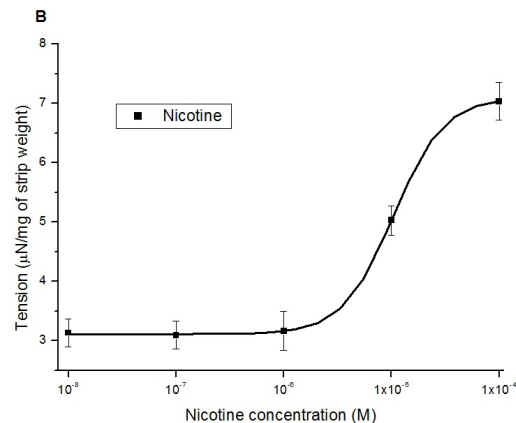


Fig. 2. Effect of hexamethonium (10^{-5} M) on nicotine-induced contractions. Hexamethonium almost completely inhibited the nicotine-induced contractions. Data are shown as the mean \pm standard error of means (S.E.M.) ($n=12$, $*p < 0.05$). For the statistical analysis, repeated measures analysis of variance (repeated measures ANOVA) was used to evaluate differences and a $*p < 0.05$ was considered significant.



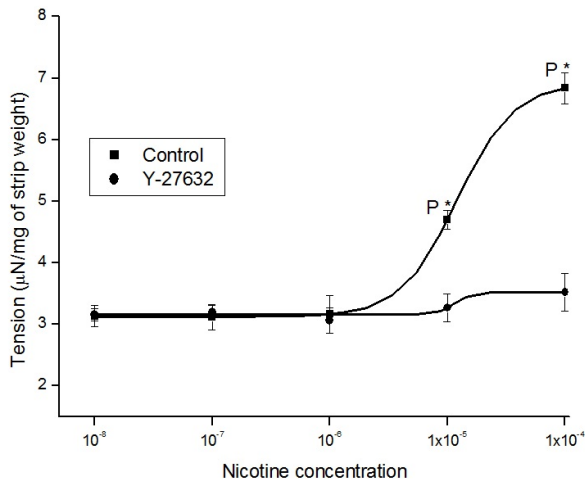


Fig. 3. Influence of Y-27632 on the corpus cavernosal strips response to nicotine. Y-27632 (10^{-6} M) pretreatment markedly reduced the nicotine effects on the study strips. Data are shown as the mean±standard error of means (S.E.M.) (n=12, *p<0.05). For the statistical analysis, repeated measures analysis of variance (repeated measures ANOVA) was used to evaluate differences and a *p<0.05 was considered significant.

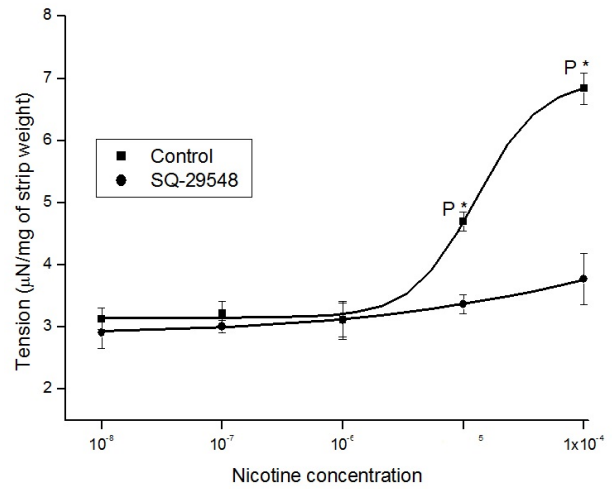


Fig. 5. Effects of SQ-29548 (10^{-7} M) on nicotine-induced contractions. SQ-29548 pretreatment almost completely reduced nicotine-induced contractions. Data are shown as the mean±standard error of means (S.E.M.) (n=12, *p<0.05). For the statistical analysis, repeated measures analysis of variance (repeated measures ANOVA) was used to evaluate differences and a *p<0.05 was considered significant.

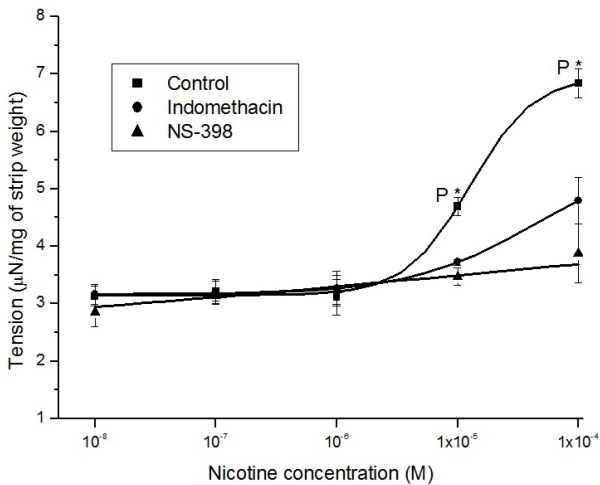


Fig. 4. Comparison of the effects of indomethacin (10^{-5} M) and NS-398 (10^{-5} M), nonselective COX and COX-2 inhibitors, on nicotine-induced contraction. Compared with the control, indomethacin (10^{-5} M) and NS-398 (10^{-6} M) significantly caused inhibition of the nicotine effects; however, NS-398 (10^{-6} M) effect might be more potent than indomethacin. Data are reported as the mean±standard error of means (S.E.M.) (n=12, *p<0.05). For the statistical analysis, repeated measures analysis of variance (repeated measures ANOVA) was used to evaluate differences and a *p<0.05 was considered significant.

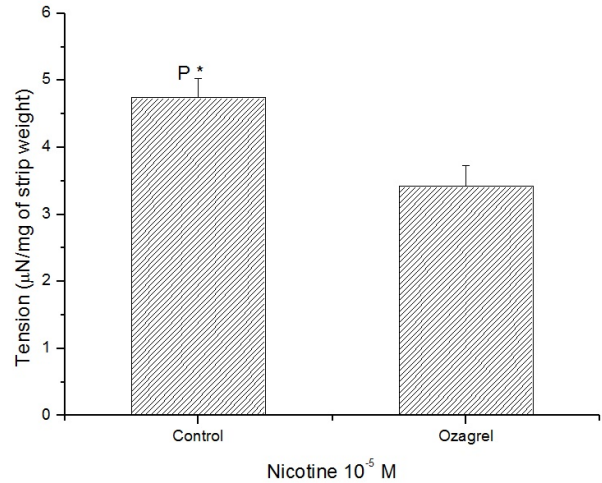


Fig. 6. The influence of ozagrel on the nicotine effects at a concentration of 10^{-5} M. Ozagrel pretreatment significantly reduced nicotine-induced contractions. Data are reported as the mean±standard error of means (S.E.M.) (n=12, *p<0.05). For the statistical analysis, the Student t-test was used to evaluate differences and a *p<0.05 was considered significant.

sponse at resting strips. However, ozagrel and SQ-29548 significantly reduced the nicotine-induced contractile amplitude. The responses in ozagrel and SQ-29548-pretreated strips were significantly different from the control group (p<0.05, n=12) (Figs. 5 and 6).

DISCUSSION

It is well known that anatomical and functional integrity of the cavernosal smooth musculature and the penile vascular smooth muscles plays a key role in the penile erectile process. Clinical and basic science studies on the penile vascular system provide strong indirect evidence that cigarette may affect penile erection by the impairment of endothelium dependent vascular smooth muscle relaxation [1]. Data on human subjects have suggested that cigarette smoking may not only cause acute vasospasm of the penile arteries

leading to reduction in arterial flow, but also increase restriction of the venous system [9]. Smoking decreased the penile brachial pressure index and the response to papaverine injections in smokers compared to nonsmokers. Furthermore, smoking cigarettes with a high nicotine concentration increased penile arterial vasoconstriction [1]. *In vivo*, nicotine administration is expected to produce penile arterial constriction by the predominant stimulation of adrenergic nerves, leading to impaired penile erection, even though cavernosal smooth muscle is relaxed [10].

There are various results about the effects of nicotine on corpus cavernosal strips. In the studies using isolated strips of bovine retractor penis muscle and rabbit corpus cavernosum, nicotine had been reported to induce relaxation [11,12]. The mechanisms underlying these effects were thought to occur as a result of nicotine activating inhibitory nerves located in the muscles of the penis or potentiating the release of nitric oxide (NO) from nitrergic nerve terminals in the corpus cavernosum [12,13]. In a study on canine corpus cavernosal strips, nicotine produced both contraction and relaxation responses [14]. Four out of 10 examined strips showed contraction followed by relaxation in response to nicotine. The six remaining strips responded by relaxation. The underlying mechanism associated with the nicotine-induced contraction is the release of norepinephrine in response to nicotine from adrenergic nerves in the penile corpus cavernosum that stimulates α 1-adrenoceptors and then elicits contraction [14].

Similarly, in the present study, rabbit corpus cavernosal strips responded inconsistently to nicotine. A majority of the examined strips contracted in a dose dependent manner. The remaining strips showed contraction followed by relaxation or only a relaxation response. The contraction response produced a sufficient magnitude for analysis without any precontraction by other agents and was initially observed at the high concentration of 10^{-5} M.

Nicotinic receptors are part of a superfamily of neurotransmitter-gated ion channels that mediate rapid intracellular communication. In the corpus cavernosum, nicotinic receptors have been shown to be located in the nerve endings (adrenergic and nitrergic endings) and the smooth muscle cells [15,16]. They are involved in many nicotine associated relaxation and contraction activities. Although many experiments reported the relaxation or both relaxation and contraction after phenylephrine precontraction [11,13], we carried out experiments without precontraction. As a result, nicotine in high concentration evoked contractions at resting state. Hayashida et al. reported that the contraction of isolated canine corpus cavernosal strips by nicotine was blocked by hexamethonium, suggesting the involvement of nicotinic receptors [14]. In the present study, nicotine-induced contraction was completely inhibited by 10^{-5} M hexamethonium. These findings imply that nicotine exerts its contractile effect on corpus cavernosal smooth muscle by activation of nicotinic receptors. Nicotine may act directly as a transmitter through activation of nicotinic receptors on smooth muscle cells.

Rho-associated protein kinase (Rho-kinase) is a target molecule of RhoA, a small G protein that plays an important role in the signal transduction pathway of the cell membrane. The Rho-kinase pathway has been suggested to play a pivotal role in the regulation of myosin light chain phosphatase activity and in the regulation of Ca^{2+} sensitivity in smooth muscle contraction [17]. To investigate the involvement of Rho-kinase associated with the nicotine-induced

contraction in the present study, the corpus cavernosal strips were incubated with Y-27632, an inhibitor of Rho-kinase. Y-27632 treatment significantly inhibited the nicotine-induced contraction. These findings suggest the possibility that Rho-kinase participates in the nicotine associated mechanisms. This result is in accordance with the findings reported by Rees that Y-27632 inhibited the contraction of human and rabbit corpus cavernosal strips elicited by noradrenergic nerve stimulation; that is, Rho-kinase is involved in the noradrenergic contractile pathway in the cavernosal smooth muscle of a penis [18]. In the rat, the Rho-kinase pathway was also shown to be associated with endothelin-1 and phenylephrine combined induction of contraction of corporal cavernosal tissues [19].

Cyclooxygenase (COX) is a membrane-bound functional enzyme that transforms arachidonic acid to the intermediate prostaglandin (PG) H_2 , which is then converted to prostanoids by specific synthases [20]. At least two distinct COX isoforms have been identified, namely COX-1 and COX-2. It is well known that COX-1 is found in most tissues and is thought to be involved in physiological processes in the stomach, kidneys, platelets, and smooth muscle; while COX-2 is preferentially expressed in cells and in the central nervous system [21]. Recently, the expression of COX-2 was also observed in smooth muscle and endothelium cells [22]. In the study reported by Ji X, COX-1 inhibitors (flurbiprofen and ketoprofen) did not cause any changes in the nicotine-induced endothelium-dependent contraction of the rat basilar arterial strips; however, COX-2 inhibitors (nimesulide, L-745,337 and celecoxib) attenuated, in a concentration-dependent manner, the contractions. Therefore, this study suggested that COX-2, but not COX-1, is involved in nicotine-induced endothelium-dependent contraction in the rat basilar artery [23]. In the present study, indomethacin 10^{-5} M and NS-398 10^{-6} M were used to examine whether cyclooxygenase was involved in the corpus cavernosal smooth muscle contraction caused by nicotine. Although both indomethacin and NS-398 significantly inhibited contraction, the inhibiting ability of NS-398 appear to be more potent than indomethacin suggesting the participation of COX-2 in nicotine-induced contraction. The involvement of COX-2 implies the importance of certain endogenous prostanoids.

The prostanoids are local mediators involved in many regulatory processes such as inflammation, platelet aggregation, and a control of vascular tone. In rabbit and human corpus cavernosal tissues, some prostanoids such as thromboxane A_2 (TXA₂), prostaglandin $F_{2\alpha}$ (PGF_{2 α}), prostacyclin (PGI₂), and prostaglandin E_2 (PGE₂) have been reported [24-26]. TXA₂ and PGF_{2 α} act through the activation of their specific receptors, thromboxane receptors (TP receptor) and F-prostanoid receptors (FP receptors), respectively, to regulate contractile processes in human corpus cavernosal tissues [27,28]. Angulo et al. reported that prostanoid-induced contraction was regulated mainly by thromboxane prostanoids (TP) in human corpus cavernosal tissues [27]. In the present study, ozagrel, a selective inhibitor of thromboxane A_2 synthase that specifically converts PGH₂ to TXA₂, significantly inhibited nicotine-induced contraction. This observation was also supported by the effect of the selective TP receptor antagonist (SQ-29548). These findings suggest the possibility that TXA₂, to some extent, is involved in the observed effects.

CONCLUSIONS

The results of this study showed that high nicotine concentrations caused contraction of isolated rabbit corpus cavernosal strips and that this contraction may be mediated by activation of nicotinic receptors. Rho-kinase and cyclooxygenase pathways, especially cyclooxygenase-2 and thromboxane A₂, might play a pivotal role in this process.

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