Korean J. of Pharmacology
Vol. 25, No. 1, pp.43-51, 1989

Responsiveness of Muscarinic and Alpha Adrenergic Activation on Endothelial Cell in Isolated Canine Renal Arteries

Soo Youn Chung, Ki Churl Chang and Jung Kyoo Lim*

Department of Pharmacology, College of Medicine, Gyeongsang National University, Chin Ju, Korea
*Department of Pharmacology, College of Medicine, Seoul National University, Seoul, Korea**

ABSTRACT

Responsiveness of muscarinic and alpha adrenoceptor activation on endothelial cells was studied in isolated canine renal artery rings. Ach (10-100 nM), dose dependently, relaxes endothelial intact rings precontracted with phenylephrine (IC_{50} of Ach was 34.5 nM). Selective mechanical destruction of the endothelium transformed the activity of this substance from vasodilatation to vasoconstriction. Acetylcholine induced relaxations could be selectively inhibited competitively by atropine, but could not be inhibited by cyclooxygenase inhibitor. Methylene blue, however, an inhibitor of soluble guanylate cyclase activity, inhibited Ach as well as sodium nitroprusside (SNP) induced relaxation. Relaxation produced by prostacyclin was not modified by methylene blue.

On the other hand, alpha adrenoceptor agonist did not relax but contract canine renal artery rings possessing an intact intima precontracted with U-46619. Clonidine, however, selective alpha-2 adrenergic agonist, is more susceptible than phenylepherine, selective alpha-1 adrenergic agonist, to the inhibitory effect of contraction.

These results suggest that in canine renal artery rings, 1) muscarinic receptor is responsible for releasing endothelium dependent relaxation factor (EDRF). 2) alpha-1 and alpha-2 adrenergic receptors are present in canine renal artery. 3) relaxation via EDRF is antagonized by methylene blue, providing further evidence that EDRF acts through a cGMP mechanism.

Key Words: EDRF, Renal artery, Acetylcholine, Clonidine, Methylene blue, Renal hypertension

Abbreviation: EDRF: Endothelium dependent relaxation factor, PE: Phenylephrine, Ach: Acetylcholine, MB: Methylene blue, SNP: Sodium nitroprusside, PGI_2: Prostacyclin, CRA; Canine renal artery, TX; Thromboxane

INTRODUCTION

The relaxing effect of acetylcholine (Ach) on rabbit aorta was mediated indirectly by a nonprostanoid substance released from the endothelial cells was first demonstrated by Furchgott *et al.* in 1980. Similar endothelium-dependent mechanisms have subsequently been shown in other arteries and for other vasodilator compounds (Cherry *et al.*, 1982, De May *et al.*, 1981, Singer *et al.*, 1983). However, only recently has the idea that vasoconstrictors, such as norepinephrine, serotonin and thrombin etc. (Cocks *et al.*, 1983, Cohen *et al.*, 1983, De May *et al.*, 1983) also release endothelium-dependent relaxation factor (EDRF) and vascular endothelial lining does modulate alpha adrenergic agoinist-induced vasoconstriction (Egleme *et al.*, 1983, Lues *et al.*, 1981) started to attract more interest to the role of this factor in both normal and diseased blood vessel. Even though many attempts have been made to elucidate the nature of EDRF (Chand *et al.*, 1981, Forstermann *et al.*, 1984, Griffith *et al.*, 1984, Singer *et al.*, 1984), the chemical nature of this factor, however, is still unknown. Furthermore, the fact that species-as well as site dependent differences of the factor exist (Angus *et al.*, 1986, Forstermann *et al.*, 1984) seems to make matters more complicated. On the other hand, considerable portion of hypertension secondary to renovascular disease is attributable to renal arterial stenosis due to ischemia and atherosclerosis of renal artery (Stephen, 1984). To test the possible etiology of the increased renal resistance observed in renal hypertension might be damage to arterial endothelium, we wish to know the following things in this study.

① Do the canine renal arteries respond to release EDRF? ② If so, for what kind of receptors is responsible? Will it be a muscarinic cholinergic and/or alpha adrenoceptor. Finally, ③ what is the mechanism of action of EDRF?

METHODS AND METERIALS

Preparation of renal arterial ring

Healthy mongrel dogs of either sex (25～35 kg) were anesthetized with phenobarbital sodium (20～30 mg/kg) and the renal artery and some of its side branches were dissected out and rinsed with Krebs-bicarbonate solution to remove remaining blood. The arteries were cleaned from connective tissue and special care was taken during the preparation not to damage the endothelium of the blood vessels. Endothelial cells were removed from some rings by gently rubbing the intimal surface, with a wooden stick. The cleaned canine renal artery (CRA) was then cut into 2.5 mm wide transverse rings, using a specially designed razor blade slicing device.

Isometric tension recording

Rings were mounted horizontally onto two L-shaped hooks in tissue baths containing 10 mL Krebs solution at 37℃ of the following composition (mM): NaCl 118.5, KCl 4.74, $NaHCO_3$ 1.18, $MgSO_4$ 1.18, $CaCl_2$ 2.5, Glucose 10, and EDTA 0.1; and bubbled with 95% O_2～5% CO_2. Rings were mounted under an optimal resting tension of 2 g and allowed to equilibrate for more than 90 min before the experiment. During the equilibration period, the tissue bathing solution was changed every 20 min. After the initial equilibration period, the artery preparations were exposed to maximally effective concentrations of one of the agonists to ensure stabilization of the muscles. After a stable plateau tension had developed, the agonist was removed by several washes with fresh buffer solution. A period of 30 to 45 min was elapsed for equilibration, after the agonist was added at EC_{50} level (PE 10^{-5}M, U-46619 10^{-7}M) to the medium. U-46619 was used only in experiment as to whether alpha adrenoceptor activation releases EDRF. Tension was measured isometrically using Narco F-60 transducers and was displayed on Narco physiographs.

Histological examination

To ascertain that the mechanical rubbing applied to the canine renal rings had successfully removed the endothelium, control and rubbed rings were incubated for 2 hour in Krebbicarbonate solution at 37℃. They were sectioned horizontally and stained in vitro with Hematoxylin and Eosin. Light microscopic examination of the intimal layer of the control rings revealed endothelial cells (Fig. 1 A). However, endothelial cells was not seen in arteries where the intimal layers has been rubbed mechanically (endothelium-denuded ring Fig. 1 B) indicating the procedure had removed the endothelial layer.

Drug

Phenylephrine hydrochloride, Acetylcholine chloride, Atropine sulfate, Indomethacin, Prostacyclin, Propranolol hydrochloride, Sodium nitroprusside, Clonidine were purchased from Sigma Chemical Co. U-46619 was kindly gifted from the Upjohn Company (Kalamazoo, MI). Methylene blue was obtained Fluka AG (Switzerland). All other solutions were freshly prepared immidiately prior to use. All working solutions were stored on ice throughout the experiment.

RESULTS

Endothelium dependent vasodilatation

In order to investigate action of Ach on the endothelium preserved CRA rings precontracted with 10^{-5}M phenylephrine, their rings were cumlatively exposed to Ach 10^{-8} to 10^{-5} M, which results in concentration dependent relaxation. On the other hand, in endothelium depleted CRA rings of contracted with phenylephrine, cumulative addition of Ach (10^{-8} M～10^{-5} M) causes no change or rather increases tension. The IC_{50} of Ach on the endothelium preserved CRA rings was 34.5 nM (Fig. 2).

Ach stimulate release of EDRF via muscarinic receptor

As to whether relaxation induced by Ach is an event through muscarinic receptor, after preincubating of 10^{-7} M of atropine for 15 min, expo-

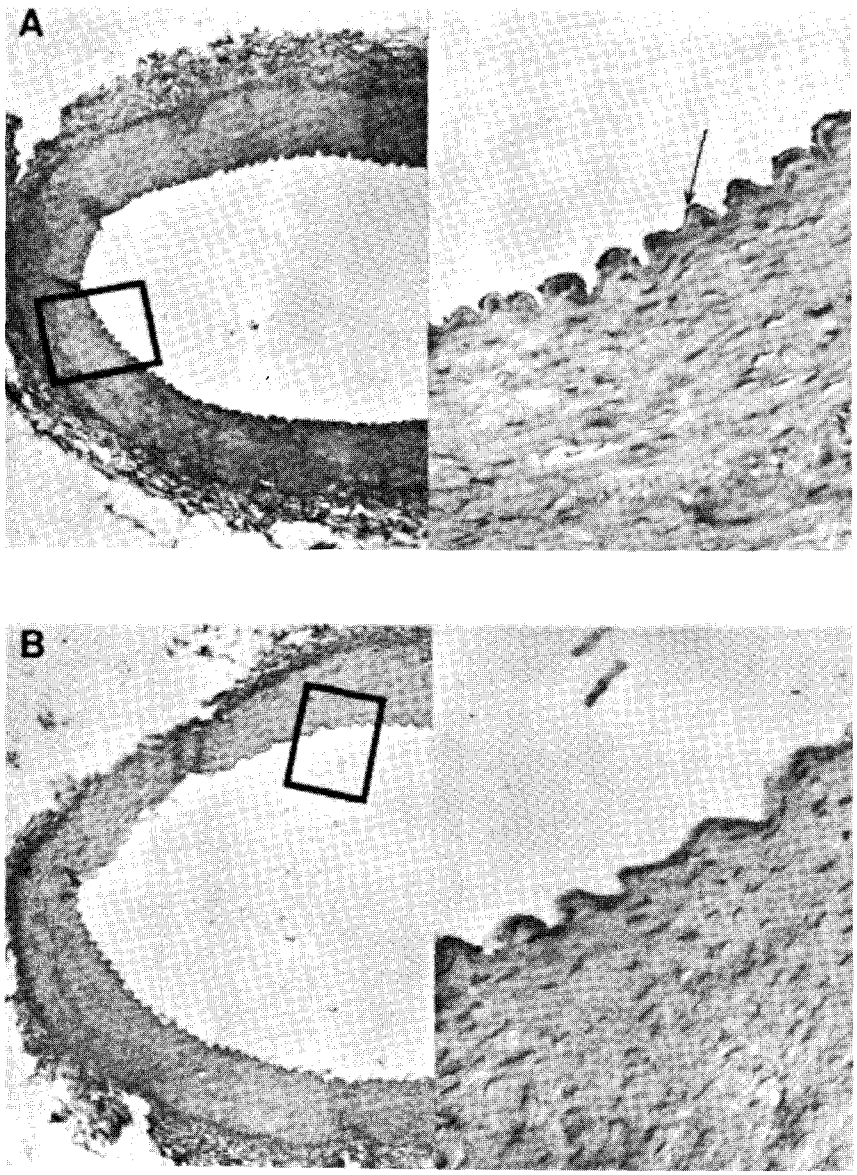

Fig. 1. Histological examination of the intimal surface (Hematoxylin-Eosin staining), Light microscopic examination of the intimal layer of the control rings revealed endothelial cells (arrow, A). However, endothelial cells was not seen in arteries where the intimal layers has been rubbed mechanically (B), indicating the procedure had removed the endothelial layer. Left (X 10) Right (X 200)

sure of phenylephrine precontracted endothelium preserved CRA ring to 10^{-5} M Ach resulted totally blocking of relaxation effect (Fig. 3A). These results confirm the relaxation of isolated CRA rings to Ach is endothelium dependent, involves a muscarinic receptor.

EDRF are not inhibited by cyclooxygenase inhibitor

There are some reports that intact endothelium releases vasodilating substance such as PG I_2 after

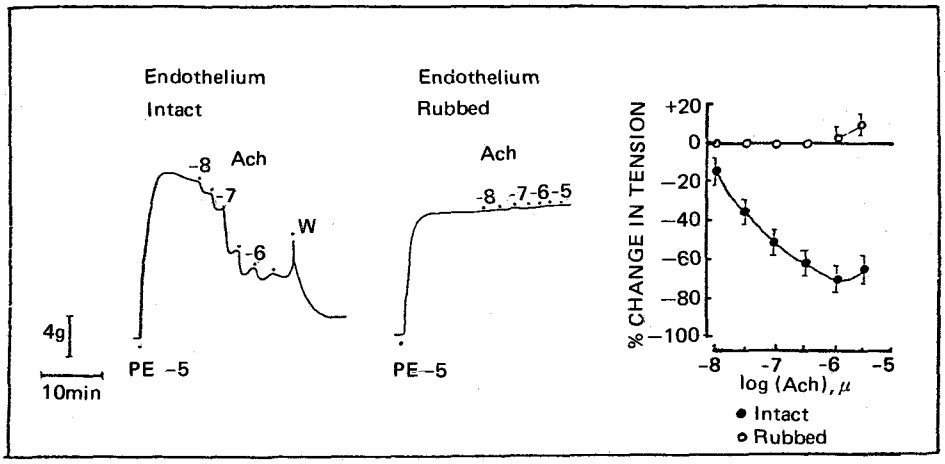

Fig. 2. Tracing of isometric tension recording of isolated canine renal artery. Phenylephrine (PE) added to the organ bath causes a contraction. In rings that have been rubbed of endothelium, cumulative addition of acetylcholine (Ach) causes no change in tension, whereas in rings with endothelium, concentration dependent relaxation results. Summary data of experiments are presented in the right side of tracing. W indicates wash out of preparation. All concentrations are expressed as logarithms of molar concentrations.

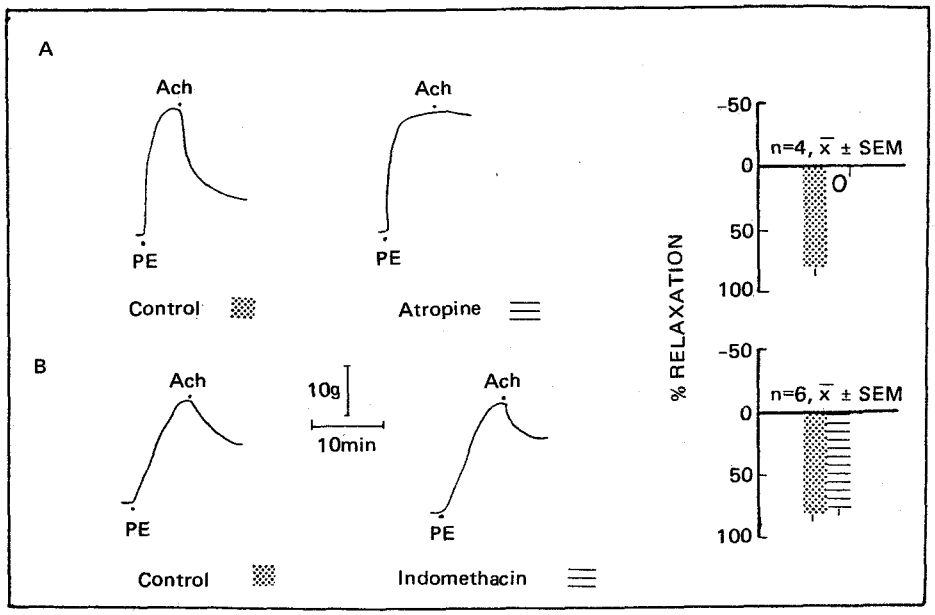

Fig. 3. Tracing of isometric tension of isolated rings of canine renal artery with before and after addition of atropine and indomethacin. Panel A illustrates acetylcholine (Ach) induced relaxation was competitively inhibited by pretreatment of atropine. Panel B illustrates acetylcholine (Ach) induced relaxation was not attenuated with presence of indomethacin. Summary data of experiments are presented in the right side of tracing.

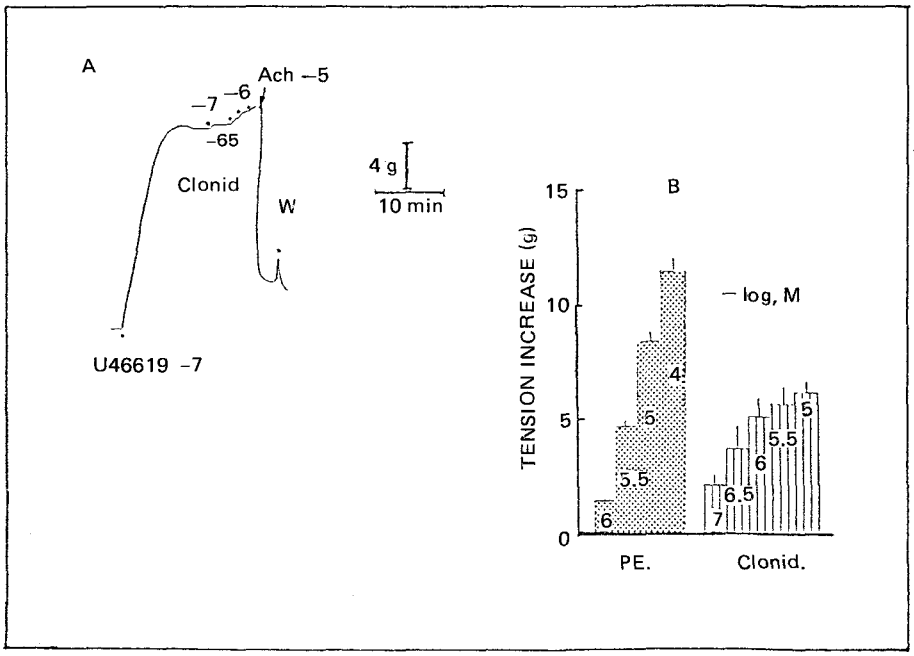

Fig. 4. Tracing of isometric tension of isolated rings of canine renal artery. Panel A shows clonidine fails to relax endothelial intact renal artery. Rings were contracted by U-46619 (100 nM) in the presence of propranolol (3 nM). Endothelial integrity was checked by adding acetylcholine (10 nM). Panel B illustrates dose-dependent tension increment of alpha-adrenergic receptor mediated response in canine renal artery with endothelium. Data represent mean s.e.m of 4 to 6 preparations.

Ach treatment (Jaun, 1981, Moncada *et al.*, 1977). To test these possibilities after 25 min preincubation of cyclooxygenase inhibitor, indomethacin (10^{-5} M), Ach (10^{-5} M) introduced to the vessels precontracted with 10^{-5} M phenylephrine (Fig. 3B). Action of EDRF are not inhibited by cyclooxygenase inhibitor. These result confirm the hypothesis that EDRF is not a prostanoid substances.

Effects of alpha adrenoceptor activation on EDRF release

After testing with acetylcholine for relaxation as proof of intact endothelium, CRA rings were washed with normal Krebs solution 4 to 5 times and allowed to re-equilibrate for 45 minute. Rings were then preincubated with 10^{-7} M U-46619, a stable analog of PGH_2 and mimic of the activity of TXA_2, and at peak contraction, exposed to clonidine, selective alpha-2 adrenergic agonist, from 10^{-7} M to 10^{-5} M. Clonidine caused a dose-dependent constriction (Fig. 4A). Phenylephrine, selective alpha-1 adrenergic agonist, also increased in tension dose dependently in endothelium intact CRA rings precontracted with U-46619. Fig. 4B shows tension increment of alpha-adrenceptor mediated responses in CRA rings with intact endotelium. However, maximum developed tension to clonidine was 47.6% to that of phenylephrine.

Action mechanism of EDRF

Methylene blue is known to an inhibitor of soluble guanylate cyclase (Cherry *et al.*, 1982, Miller *et al.*, 1984, Rapopport *et al.*, 1982) and Ignarro *et al.* (1987) have postulated that it is the inhibition of this enzyme which antagonizes endothelium dependent vasodilator responses of Ach. Preincubation with 10^{-5} M methylene blue completely blocked the action of Ach in relaxation of endothelium preserved CRA rings which was precontracted with 10^{-5} M phenylephrine (Fig. 5A). Preincubation with methylene blue (10^{-5} M -10^{-4} M) also antagonized sodium nitroprusside-induced relaxation in the present experiment (data not shown). It is implying that relaxing effect of

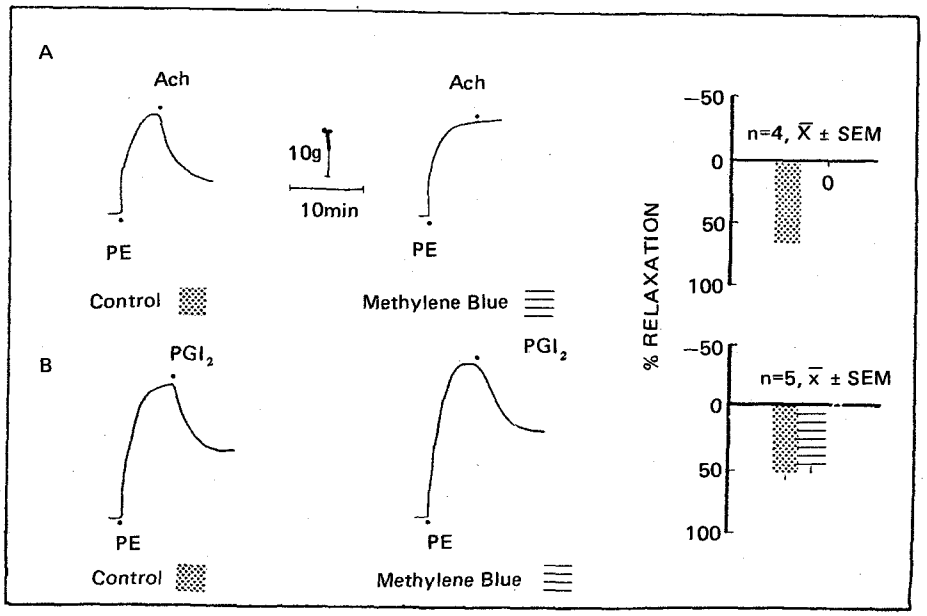

Fig. 5. Tracing of isometric tension of isolated rings of canine renal artery with endotelium before and after addition of methylene blue. Panel illustrates acetylcholine (Ach) induced relaxation was completely inhibited by pretreatment of methylene blue. Panel B illustrates Prostacyclin (PGI_2) induced relaxation was not attenuated with presence of methylene blue. Summary data of experiments are presented in the right side of tracing.

Ach in this experiment is through cGMP

Prostacyclin-induced relaxation are not inhibited by methylene blue

In other experiments, 10^{-6} M concentration of PGI_2 was found to induce relaxation in endothelial intact CRA rings precontracted with 10^{-5} M phenylephrine. These relaxation are not inhibited by preincubation for 15 min with 10^{-5} M methylene blue (Fig. 5B).

DISCUSSION

The present results provide further evidence for the hypothesis that relaxation of isolated CRA rings to Ach is endothelium-dependent, involves a muscarinic receptor and is antagonized by methylene blue. Furchgott and collegues (1980) pioneered to uncover the existence of an EDRF in intact vascular smooth muscles.

This factor is obviously not prostaglandin (Furchgott *et al.*, 1980) which is confirmed here by the ineffectiveness of indomethacin against the relaxation (Fig. 3B). Methylene blue has become a valuable pharmacological tool since it antagonized the increase in cGMP as well as the relaxant effect of various nitrosocompound (Holzmann *et al.*, 1982, Miller et al., 1984, Rapopport *et al.*, 1982). Juan (1981) reported that Ach dose dependently released PGI_2 in the isolated perfused rabbit ear (Moncada *et al.*, 1977) which, however, was not the case in the present experiment since indomethacin did not inhibit Ach-induced relaxation (Fig. 3B). The data from this experiment show that EDRF-mediated relaxation is inhibited by addition of methylene blue, providing further evidence that EDRF acts through a cGMP mechanism (Holzmann *et al.*, 1982, Ignarro *et al.*, 1984, Miller *et al.*, 1984, Rapopport *et al.*, 1982). The stimulus for the production of cGMP is probably EDRF. Since the relaxation of arterial preparations to prostacyclin was not inhibited by methylene blue, the antagonism of Ach induced relaxation by methylene blue was not nonspecific but more likely due to the inhibition of cGMP production.

On the other hand, it is now generally accepted that alpha-1 and alpha-2 adrenoceptors coexist postjunctionally in vascular beds of many mammalian species (Ruffolo *et al.,* 1984) and both subtypes mediate vasoconstriction (Scarborough *et al.,* 1984). The present experiment showed that both phenylephrine, alpha-1 adrenergic agonist and clonidine, alpha-2 adrenergic agonist caused vasoconstriction (Fig. 4) which confirms that alpha 1 and alpha 2 adrenergic receptors are present in canine renal arteries. In our experiment, alpha receptors mediated responses in CRA ring preparation were vasoconstriction regardless of its subtype. It should be noted, however, maximal constriction effect of phenylephrine was greater than that of clonidine. At the present time the underlying mechanism of this difference is not known. One possible explanation for this apparent difference is that alpha -2 selective agonists can be taken up to endothelium with high affinity more than alpha 1 selective agonists resulting in diminition of agonist concentration at the biophase. On the other hand, there is a possibility that alpha receptor of endothelium of CRA might be an alpha 2 subtype so that EDRF release via this receptor partly countracts contractile effect of clonidine. Further investigation is required to resolve this difference. Damage or loss of th endothelial layer in CRA is one of possible mechanism in increasing renal resistance, which may also contribute to renovascular disease such as renal hypertension. The present results clearly demonstrate that muscarinic receptor is responsible for releasing EDRF and stimulation of endothelial alpha receptor of CRA caused vasoconstriction rather than vasorelaxation. However, alpha 2 subtype is more susceptible than alpha 1 subtype receptor. Cyclic GMP increment might be the action mechanism of EDRF.

Acknowledgements: The authors express their appreciation to the Upjohn Comany for providing a sample of research compound U-46619.

REFERENCES

Angus JA: *Alpha-2 adrenoceptors and endothelium dependent relaxation in canine large arteries. BJ Pharmacol 88:767-777, 1986*

Catravas JD and White Re: *Kinetics of pulmonary angiotensin-converting enzyme and 5-nucleotidase in vivo. J Appl Physiol 57:1173, 1984*

Chand N and Altura BM: *Inhibition of endothelial cell dependent relaxation to acetylcholine and bradykinin by lipoxygenase inhibitors in canine isolated renal arteries. Microcirculation 1:211-223, 1981*

Cherry PD, et al: *The role of endothelial cells in the relaxation of isolated arteries by bradykinin. Proc Nati Acad Sci USA 79:2106-2110, 1982*

Cocks TM and Angus JA: *Endothelium dependent relaxation of coronary arteries by noradrenaline and serotonin. Nature 305:627-630, 1983*

Cohen RA, Shepherd JT Vanhoutte PM: *5-Hydroxytrytamine released from aggregating platelets can mediate endothelium dependent inhibition of coronary smooth muscle. Am J Physiol 245: H1077-1080, 1983*

De May JG and Vanhotte PM: *Role of the intima in cholinergic and purinergic relaxation of isolated canine femoral arteries. J Physiol 316:347-355, 1981*

De May JG and Vanhotte PM: *Endothelium-dependent inhibitory effects of Ach, ATP, throumbin and arachidonic acid in the canine femoral artery. J Pharmacol Exp Therapeut 222:166-173, 1983*

Egleme C, Godfraind T and Miller RC: *Enhanced responsiveness of rat isolated aorta to clonidine after removal of the endothelial cells. Br J Physiol 81:16-18, 1984*

Forstermann U and Neufang B: *The endothelial mediated relaxation of rabbit aorta: Effects of antioxidants and hydroxylated eicosatetranoic acids. B J Pharmacol 82:765-767, 1984*

Forstermann U, Trogisch G and Busse R: *Species dependent differences in the nature of endotheliumdervied relaxing factor. Eur J Pharmacol 106:639-643, 1984*

Furchgott RF and Zawadzki JV: *The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288:373-376, 1980*

Griffith TM, et al: *The nature of endothelium derived vascular relaxant factor. Nature 308:645-647, 1984*

Holzmann S: *Endothelium induced relaxation by acetylcholine associated with larger rises in cyclic GMP in coronary arterial strips. Cyc Nucl Res 8:409-419, 1982*

Ignarro LJ, Burke TM and Wood KS: *Association between cGMP accumlation and acetylcholine-elicited relaxation of bovine intrapulmonary artery. J Pharmacol Exp Therapeut 228:682-690, 1984*

Ignarro LJ and Byrns RE: *Pharmacolgical evidence that EDRF is nitric oxide. Use of Pyrogallol and superoxide dismutase to study endothelium dependent and nitric oxide-elicited vascular smmoth muscle relaxation. J Pharmacol Exp Therapeut 244:181-189, 1987*

Jaun H: *Influence of nicotine basal and cumulative PG biosynthesis in perfused vascular tissue of rabbit. Naunyn-Schmiedeberg's Arch Pharmacol 317:345-350, 1981*

Lues I and Schumann H: *Effect of removing the endothelial cells on the reactivity of rat aortic segments to different alpha-adrenoceptor agonists. Arch Pharmacol 328:160-163, 1984*

Miller RC and Monu M: *Endothelial mediated inhibition of contraction and increase in cyclic GMP levels evoked by the alpha-adrenoceptor agonist B-HT 920 in rat isolated aorta. Br J Pharmacol 83:903-918, 1984*

Moncada S and Herran AG: *Differential formation of prostacyclin by layers of the arterial wall. An explanation for the anti-thrombic properties of vascular endothelium. Thromb Res 11:323-344, 1977*

Ruffolo RR Jr, Morgan ER and Messick K: *Possible relationship between receptor reserve and the differential antagonism of alpha-1 and alpha-2 adrenoceptor mediated pressor response by calcium channel antagonists in the pithed rat. J Pharmacol Exp Therapeut 230:587-594, 1984*

Rapopport RM and Murad F: *Sodium nitroprussside-induced protein phosphorylation in intact rat aorta is mimicked by 8-bromo cyclic GMP. Proc Nati Acad Sci USA 79:6470-6474, 1982*

Scarborough NL, Carrier GO: *Nifedipine and alpha adrenoceptors in rat aorta. 1. Role of extracellular calcium in alpha-1 and alpha-2 adrenoceptor-mediated contraction. J Pharmacol Exp Therapeut 231:597-602, 1984*

Singer HA, Peach MJ: *Endothelium dependent relaxation of rabbit aorta. 2. Inhibition of relaxation stimulated by methacholine and A23187 with antagonists of arachidonic acid metabolism. J Pharmacol Therapeut 227:796-801, 1983*

Singer HA, Peach MJ: *Endothelium dependent relaxation of rabbit aorta 1. Relaxation stimulated by arachidonic acid. J Pharmacol Exp Therapeut 227:790-795, 1983*

Singer HA, Saye JA, Peach MJ: *Effects of cytochrome p-450 inhibitors on endothelium dependent relaxation in rabbit aorta. Blood Vessels 21: 223-230, 1984*

Stephen CT: *Pathophysiology of renovascular hypertension. Symposium on Renal Vascular Disease. 373-381, 1984*

= 국문초록 =

개 신동맥 내피세포의 무스카린성 및 알파 아드레날린성 수용체에 대한 작용

경상대학교 의과대학 약리학교실, 서울대학교 의과대학 약리학교실*

정 수 연·장 기 철·임 정 규*

내피세포가 잘 보존된 개 신동맥에서 Phenylephrine으로 전 수축시킨 후 Actylcholine을 투여하면 혈관 이완을 관찰할 수 있었지만 내피세포를 파괴시킨 개 신동맥에서는 혈관 이완을 관찰할 수 없었으므로 내피세포가 혈관 이완에 관여함을 알 수 있었다. 그래서 내피세포에서 분비되어 혈관 이완을 촉진하는 물질을 Endothelium Dependent Relaxation Factor (EDRF)라고 한다. Acetylcholine에 의한 혈관 이완은 Atropine에 의해 경쟁적으로 억제되었으므로 Muscarinc 수용체의 자극을 받아 EDRF가 분비되고 이것이 평활근에 작용하여 이완을 일으킬 것으로 생각되었다.

EDRF의 작용기전을 알아보기 위해 용해성 Guanylate cyclase 억제제인 Methylene blue를 전처치 했을 때는 Acetylcholine 뿐 만 아니라 Sodium nitroprusside에 의한 이완도 억제되었다. 그러나 Prostacyclin에 의한 이완은 억제되지 않았다. 이 결과로 판단 해 보면 EDRF에 의한 이완효과가 Cyclic GMP와 관련이 있을 것으로 생각되었다.

한편 Thromboxane A_2 유사체인 U-46619로 전수축 시킨 후 Alpha-2 아드레날린성 항진제인 Clondine을 투여했을 때 혈관 이완은 일어나지 않았다. 수축의 억제 양상에 있어서 Alpha-1 아드레날린성 항진제인 Phenylephrine보다 Clonidine이 더 효과적 이었다. 이상의 결과를 기초로 하여 다음과 같은 결론을 얻을 수 있었다.

1) Muscarinic 수용체의 자극을 받아 EDRF의 분비가 촉진된다.
2) Alpha-1 아드레날린성 수용체와 Alpha-2 아드레날린성 수용체가 개 신동맥에 존재한다.
3) EDRF에 의한 이완이 Methylene blue에 의해서 억제되므로 cGMP를 이용한 기전 임을 알 수 있었다.