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The Possible Involvement of Nitric Oxide/Endothelium Derived Relaxing Factor in Atropine-Induced Vasorelaxation

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ABSTRACT

Atropine has been used to block cholinergic neurotransmission in basic research. Large doses of atropine cause vasodilation of the blood vessels in the skin. This effect is apparently unconnected with the antimuscarinic activity of atropine and seems to be due to a direct action on the blood vessels. It has been suggested that atropine blocks muscarinic receptors at low doses and it induces the release of endothelium derived relaxing factor (EDRF) at large doses. This study examined the effects of atropine on isolated rat pulmonary artery rings with or without endothelium intact in the absence and presence of nitric oxide synthase inhibitors, N-omega nitro-L-arginine methyl ester (L-NAME) or N-omega nitro-L-arginine (L-NOARG) that precontracted with phenylephrine (PHE), 5-hydroxy-tryptamine (5-HT) or KCl. Atropine (1 nM) blocked the vasorelaxant effect of acethylcholine (1 µM) in pulmonary artery rings precontracted with PHE (100 nM). Atropine (10 nM-5 µM) also produced concentration dependent relaxation in these rings precontracted with PHE or 5-HT, but did not relax rings precontracted with KCl. The vasorelaxant effects of atropine were partially inhibited by the mechanical remove of endothelium or pretreatment the rings with L-NAME or L-NOARG, although they were not statistically significant. These results suggest that the ability of atropine to relax pulmonary artery rings may be dependent upon the mechanism of action of the precontracting agonist and also, the vasorelaxant effect of atropine is not wholly mediated by the release of NO (nitric oxide)/EDRF. *Iran. Biomed. J. 5 (4): 133-139, 2001*

Keywords: Atropine, Nitric Oxide (NO)/Endothelium Derived Relaxing Factor (EDRF), Pulmonary artery

INTRODUCTION

There are many different types of agents that induce relaxation in pulmonary arteries. Cholinergic agonists, alpha-adrenergic antagonists, beta-adrenergic agonists, peptides, prostaglandins, histamine, calcium channel blocking and potassium channel opening agents, endothelium derived relaxing factor (EDRF) or nitric oxide (NO), and other non-specific vasodilators are some of the substances that induce vascular smooth muscle relaxation in pulmonary arteries [1-8]. Vasodilating agents can either act directly on the vascular smooth muscle to cause its relaxation, or they can act indirectly by stimulating the release of an endogenous dilator, such as EDRF, or by inhibiting the action of an endogenous vaso-constrictor [9]. In 1987, the demonstration of the formation of NO by an enzyme in vascular endothelial cells opened up a new area of biological research [10, 11]. NO, that accounts for the biological properties of EDRF, is the endogenous stimulator of the soluble guanylate cyclase. In addition, NO is a potent vasodilator that activates guanylate cyclase resulting in the generation of cGMP, which is presumed to be the principal effector of NO-induced vasorelaxation in various tissues. NO is synthesized from the amino acid L-arginine by an enzyme, the NO synthase [12]. Atropine is a competitive antagonist of the action of acetylcholine (ACh) and other muscarinic agonists [13]. Large doses of atropine cause vasodilation of the blood vessels in the skin [14]. This effect is apparently unconnected with the antimuscarinic activity of atropine and seems to be due to a direct action on the blood vessels. This direct vasodilator action of atropine gives rise to the characteristic "flush" which is caused by dilation of superficial blood vessels of the skin in the neck and face and a sign of atropine toxicity [14]. In pulmonary vessels,

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atropine blocks the action of acetylcholine and antagonizes the relaxation of pulmonary arteries. However, the effects of atropine on pulmonary artery rings are variable at high concentrations. It has been claimed that high concentrations of atropine enhance EDRF secretion from vascular endothelium [15]. We examined the effects of different concentrations of atropine in isolated rat pulmonary artery rings and evaluated the possible involvement of (nitric oxide) NO/EDRF in induced relaxant responses.

MATERIALS AND METHODS

Experimental rats. Male Wistar rats were kept in a control area. The food was obtained from Labsure CRM diet and ordinary tap water was used for drinking. Temperature and light of the animal-house were controlled. The temperature was maintained between 18-22°C and the lighting of the area was regulated in a cycle of twelve hours light and twelve hours dark (i.e. 06:30-18:30 h).

Tissue preparation. Male Wistar rats (obtained from animal-house of the Pharmacology Department of Glasgow University) weighing 250-300 g were killed by stunning and exsanguinations. The heart and lungs were excised together and pinned on silicon rubber in a Petri dish containing Krebs-Henseleit (0°C) in an orientation that facilitated identification of the pulmonary arteries. The pulmonary arterial tree was rapidly dissected from the lung parenchyma with a small scissors and forceps. Segments of the main pulmonary artery (MPA) was carefully cleared of adhering tissue and cut to obtain a ring of approximately 3 millimeter in diameter. Then, it was transferred to fresh ice-cold Krebs-Henseleit. Endothelial cells were removed from some rings by gently rubbing the intimal surface with a moist wooden stick for 30-60 seconds [16]. The effectiveness of this procedure was subsequently investigated using acetylcholine (ACh, 1 mM), which normally relaxed artery rings but had no such effect in the rubbed rings precontracted with phenylephrine [16].

Organ bath experiment. Artery rings were mounted under 1 gram resting tension onto two 0.2 millimeter shaped stainless steel wire hooks gently inserted into the lumen to avoid damage to the endothelium in 25 milliliter organ baths containing Krebs-Henseleit solution maintained at 37°C, and containing NaCl, 118 mM; KCl, 4.8 mM; $CaCl_2$, 2.5 mM; $MgSO_4$, 1.2 mM; KH_2PO_4, 1.2 mM; $NaHCO_3$, 24 mM; Glucose, 11 mM; gassed with mixture of 95% O_2 and 5% CO_2, which gave a pH of 7.3-7.4. Tension responses were recorded isometrically with Grass FTO3 transducers and were displayed on a Grass model 7 polygraph. Before commencing each experiment, artery rings were allowed to equilibrate for 1 hour, during that time a steady resting tension was achieved. Also, the intact endothelium rings precontracted with PHE were confirmed with ACh, which relaxed artery rings. After each experiment, the tissues were then washed by changing the bathing Krebs solution (37°C) three times over a 15 minutes period before further increasing the degree of stretch of the tissue. At the end of each day's experiment, the artery strip preparations were gently blotted, dried and weighed on a microbalance. The contractile force was expressed in terms of milligram tension per milligram weight of tissue.

Drugs. Atropine sulphate, L-phenylephrine hydrochloride, 5-hydroxytryptamine creatine sulphate, acetylcholine chloride, N-omega nitro-L-arginine methyl ester (L-NAME), N-omega nitro-L-arginine (L-NOARG), L-arginine hydrochloride, DL-propranolol hydrochloride, 3-isobutyl-1-methyl -xanthine, haemoglobin and indomethacin were obtained from Sigma (Dorset, U.K.), and potassium chloride from Fisons (U.K). All drugs were dissolved in saline (0.9%) and were diluted in Krebs buffer.

Statistical analysis. Repeated analysis of variance (ANOVA) with a two-tailed Tukey mean comparison test was performed on the responses induced to all drugs. All results are expressed as mean ± S.E.M. and considered to be significant when P value was < 0.05.

RESULTS

Atropine (AT, 10 nM-5 μM) induced concentration-dependent relaxation in main pulmonary artery rings (MPAR) precontracted with either phenylephrine (PHE, 100 nM, EC_{75}) or 5-hydroxytryptamine (5-HT, 10 μM, EC_{75}), but did not produce relaxation in artery rings precontracted with potassium chloride (KCl, 20 mM, EC_{75}) (Fig.1 a, b and c, respectively). The maximum inhibitions produced by atropine (AT) in MPAR precontracted

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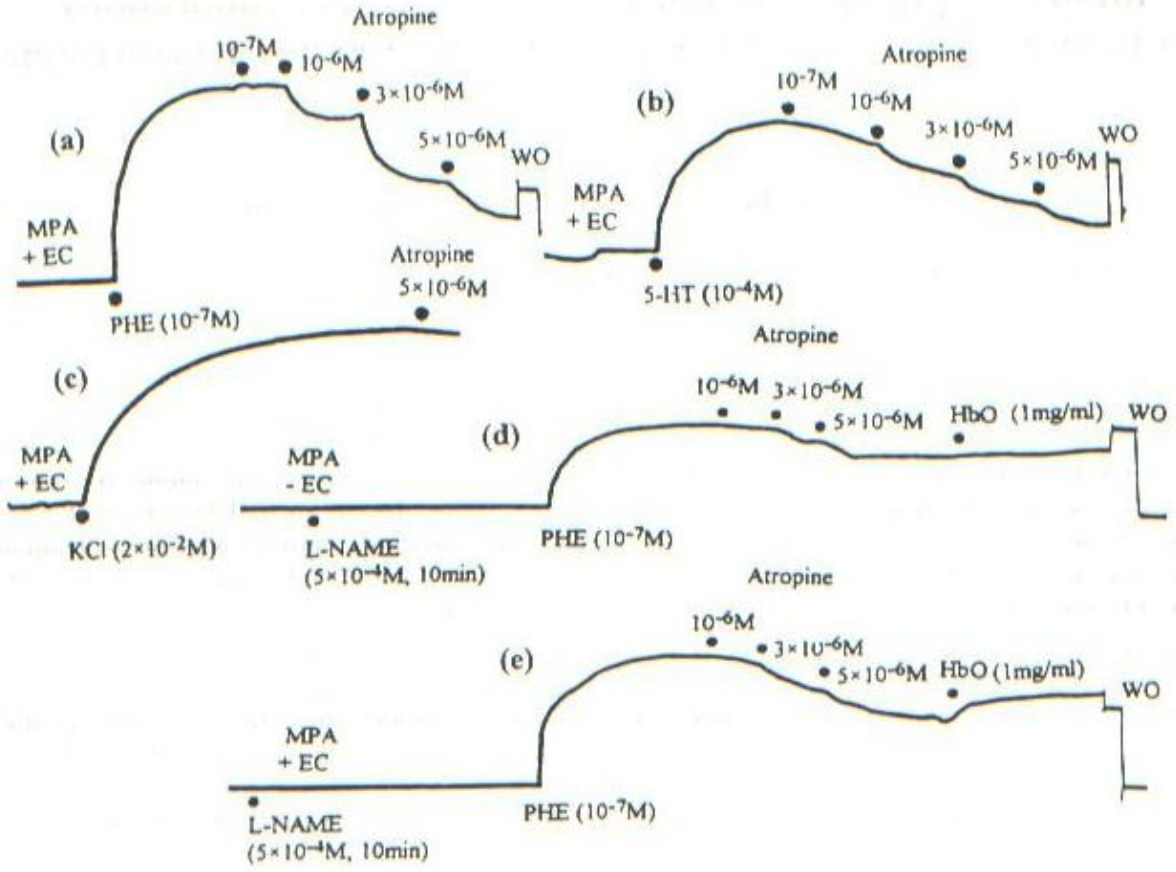

Fig. 1. Individual traces showing the inhibitory effects of atropine (AT, 100 nM to 5 µM) on contractile responses to phenylephrine (PHE, 100 nM, a), 5-hydroxytryptamine (5-HT, 100 µM) and KCl (20 mM) in intact main pulmonary artery rings (+EC, MPARs). Also, the inhibitory effects of atropine in denuded (-EC, d) and intact (+EC, e) endothelium artery rings precontracted with PHE in presence of L-NAME. Symbol (•) indicates drug addition and (WO) indicates wash off.

with PHE and 5-HT, were respectively 65.87 ± 3.72% (n = 6, Fig. 2A) and 62.70 ± 6.2% (n = 6, Fig. 2B). There were no significant differences between responses to AT in MPAR precontracted either with PHE or 5-HT. Pretreatment of pulmonary artery rings with or without the endothelium (n = 6, each) with the nitric oxide synthase inhibitors, L-NAME (500 µM, for 10 min, fig. 1 d and e), or L-NOARG (100 µM, for 10 min) and also, oxy-haemoglobin (HbO, 1 mg/ml) did not abolish atropine-induced relaxations, but reduced partially relaxant responses to atropine that were not significant statistically (Figs. 2A and 3B). Paradoxically, the inhibitory effects of atropine on the contractile responses to PHE in MPAR with intact endothelium were partially inhibited by L-NAME (n = 6). However, this effect did not occur in denuded endothelium rings (n = 6). This effect of L-NAME was reversed by L-arginine (L-Arg, 1 mM, n = 6; F = 31.885, $P<0.0001$). The ability of atropine to inhibit PHE-induced tone in MPAR, with or without the endothelium, was unaffected with the phosphodiestrase inhibitor, 3-isobutyl-1-methylxanthine (IBMX, 10 nM, for 3 min, or by beta-adrenoceptor antagonist, propranolol (PROP, 1 µM, for 10 min, n = 6; Fig. 3A, Table 1) and or prostacycline inhibitor, indomethacin (1 µM, for 10 min, n = 6; Fig. 3B, Table 1). The inhibitory effects of atropine on contractile responses to PHE persisted in MPAR in which the endothelium had been removed and in the presence of ACh (1 µM, data not shown). The ACh-induced relaxations (1 nM-10 µM) were obtained in intact MPARs that had been precontracted with PHE (n = 10; Fig. 4), and these relaxations were antagonized by atropine (10 nM).

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Table 1. Comparison between test results of atropine-induced relaxant responses (control) in rat pulmonary artery rings with endothelium intact (+E) and in the presence L-NAME, or indomethacin (INDO), or propranolol (PROP), or isobutylmethylxanthine (IBMX), and or in endothelium denuded rings (-E) precontracted by phenylephrine.

Atropine concentrations	Control, +E+L-NAME and -E	Control, +E +INDO and -E	Control, PROP and IBMX
10 nM	[F (3, 17) = 1.66, P = 0.23]	[F (3, 17) = 2.29, P = 0.15]	[F (3, 17) = 2.19, P = 0.16]
100 nM	[F (3, 17) = 1.89, P = 0.20]	[F (3, 17) = 1.94, P = 0.19]	[F (3, 17) = 0.66, P = 0.53]
1 μM	[F (3, 17) = 2.30, P = 0.15]	[F (3, 17) = 1.40, P = 0.29]	[F (3, 17) = 2.48, P = 0.13]
3 μM	[F (3, 17) = 1.10, P = 0.36]	[F (3, 17) = 0.74, P = 0.50]	[F (3, 17) = 0.54, P = 0.59]
5 μM	[F (3, 17) = 0.45, P = 0.64]	[F (3, 17) = 0.42, P = 0.66]	[F (3, 17) = 0.95, P = 0.41]

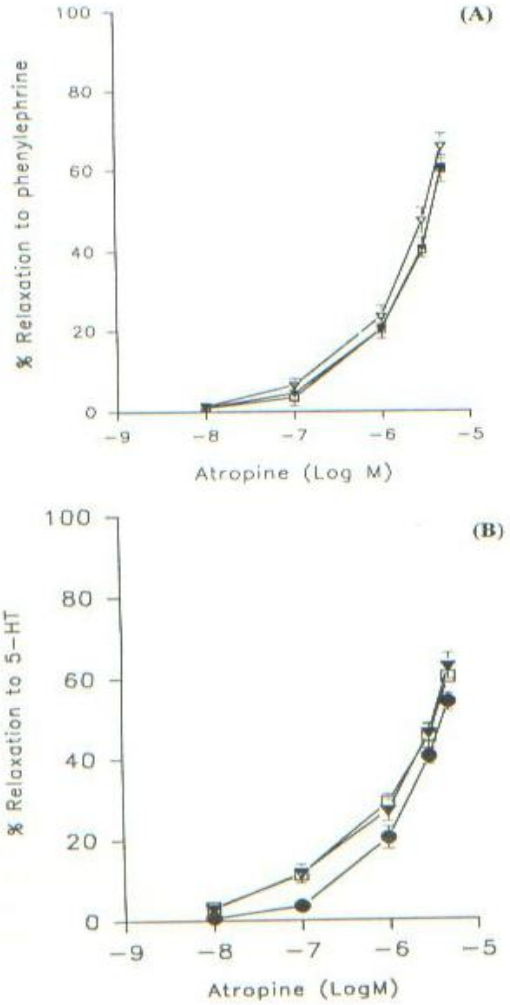

Fig. 2. (A) Comparison between cumulative concentration-response curves to atropine (10 nM to 5 μM) in rat main pulmonary rings with endothelium intact, in the absence (closed square, n = 6) and presence (open triangle, n = 6) of L-NAME (500 μM) and in endothelium denuded (closed triangle, n = 6) rings precontracted with phenylephrine (100 nM). **(B)** Comparison between cumulative concentration-response curves to atropine (10 nM to 5 μM) in rat main pulmonary artery rings with endothelium intact, in the absence (closed triangle, n = 6) and presence (closed circle, n = 6) of L-NAME (500 μM) and in endothelium denuded (open square, n = 6) rings precontracted with 5-hydroxytryptamine (100 μM).

DISCUSSION

The mechanism of the vascular smooth muscle relaxation by Ach and other muscarinic agents has been studied extensively [17-20] and shown to be dependent upon the presence of the endothelium [17]. ACh and other muscarinic agents cause a diffusible factor namely EDRF [21] that is released from endothelial cells following the binding of ACh to muscarinic receptors [13].

EDRF is thought to be NO [10, 22], which is formed from the conversion of L-arginine to L-citrulline [23], or an its derivative, since relaxation to ACh is blocked by compounds including haemoglobin [21], or the L-arginine analogues, such as L-NAME [24-26]. Atropine selectively blocks the relaxation effect of ACh, resulting in inhibition of pulmonary vascular relaxation [13]. However, the action of atropine is not simple, but conflicting at different concentrations. This study investigated the effects of atropine in MPAR with or without endothelium-intact. We have observed the Ach-induced relaxation (10 μ M) was antagonized by atropine in intact rings that had been precontracted with PHE or 5-HT. This confirms the antimuscarinic action of atropine that is consistent with the results obtained by other researchers [13, 27]. When atropine was directly administered to MPAR precontracted with PHE or 5-HT, conflicting results were observed. Atropine induced concentration-dependent relaxations in these preparations.

These results are consistent with data obtained from others that large doses of atropine induce relaxations in isolated rabbit corpus cavernosum [28]. EDRF is known to mediate the relaxation of pulmonary smooth muscle [27]. A relaxing substance exerts its action by binding to the specific receptors located on the endothelial membrane.

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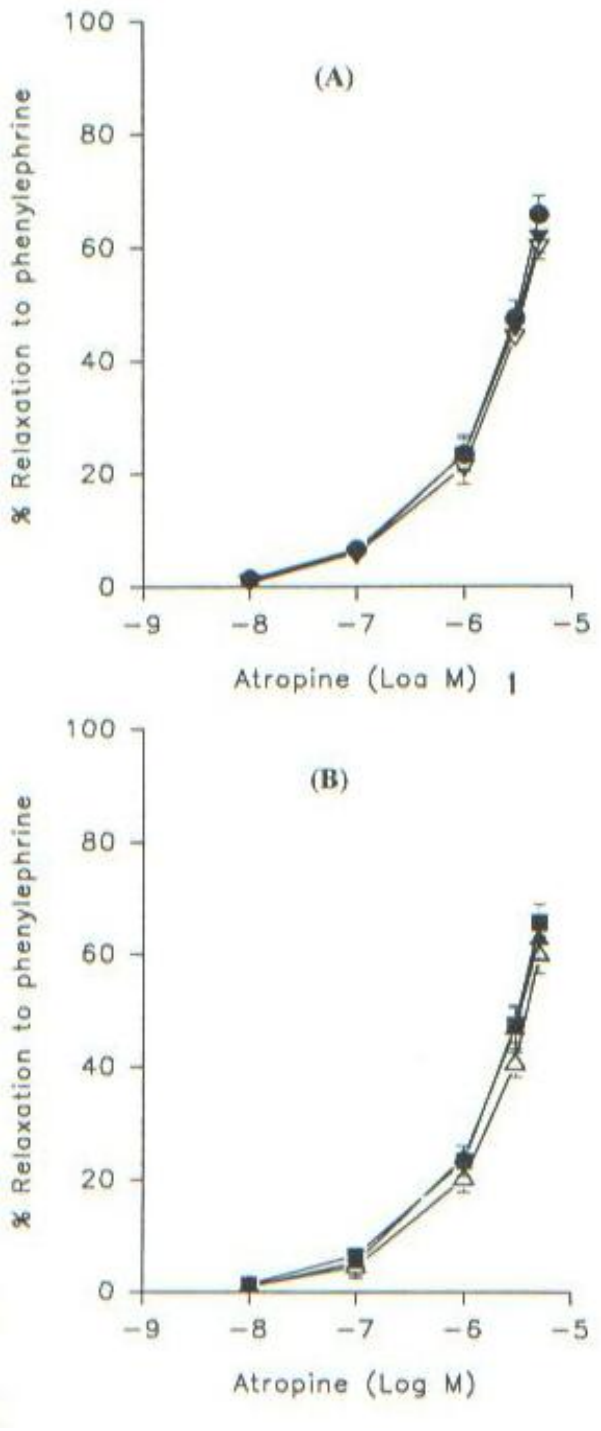

Fig. 3. **(A)** Comparison between cumulative concentration-response curves to atropine (10 nM to 5 µM) in endothelium intact rings precontracted with phenylephrine (100 nM, closed circle, n = 6) and either in the presence phosphodiestrase inhibitor, 3-isobutyl-1-methylxanthine (IBMX, 10 nM, closed triangle, n = 6) or beta-adrenoceptor antagonist, propranolol (PROP, 1 µM, open triangle, n = 6). (**B)** Comparison between cumulative concentration-response curves to atropine (10 nM to 5 µM) in endothelium intact rings precontracted with phenylephrine (100 nM, open triangle, n = 6) and either in the presence prostacycline inhibitor, indomethacin (INDO, 1 µM, closed triangle, n = 6) or L-NAME (500 µM, open square, n = 6).

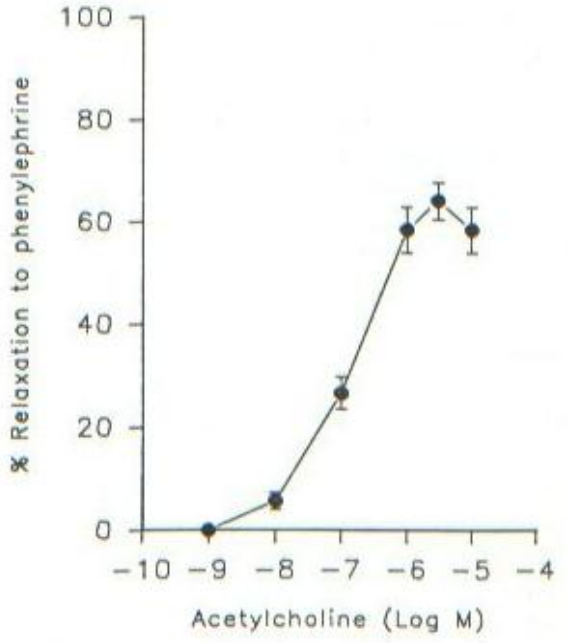

Fig. 4. Histogram showing cumulative concentration-response curves to acetylcholine (ACh, 1 nM-10 µM, n = 10) in rat main pulmonary artery rings precontracted by phenylephrine (PHE).

Then, the concentration of ionized calcium in the cytoplasm of endothelial cell is increased. The next step is the increased cellular production and release of EDRF. EDRF diffuses into the adjacent vascular smooth muscle cells where it causes activation of guanylate cyclase, the enzyme that catalyzes the production of cGMP from GTP within the smooth muscle cell [12, 22, 29, 30]. In turn, it probably inhibits contraction of vascular smooth muscle through activation of cGMP-dependent protein kinase (PKG). In our study, atropine (AT, 10 nM-5 µM) induced concentration-dependent relaxations in MPAR precontracted with either PHE or 5-HT, but did not produce relaxations in artery rings precontracted with potassium chloride.

These results indicate that the ability of atropine to relax MPAR may be dependent upon the mechanism of action of the precontracting agonist and suggest may the agonist-induced Ca^{2+} influx is more effectively blocked by atropine than depolarization-induced Ca^{2+} influx. NO synthase inhibitors, L-NAME (500 µM) or L-NOARG (100 µM) or de-endothelialization partially reduced relaxant responses to atropine, whereas relaxant responses to acetylcholine were abolished. These results indicate that relaxant responses of the rat MPAR to atropine do not require the presence of NO. However, presence of endothelium may

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facilitate vasorelaxant responses of atropine by releasing basal NO.

Paradoxically, when oxidized haemoglobin (1 mg/ml) or L-NAME (500 μM) was added during maximum relaxation to atropine, the relaxant response to atropine was partially reversed in rings with intact endothelium; but not without intact endothelium. This effect of L-NAME was inhibited by L-arginine (1 mM). These results indicate that the vasorelaxant effect of atropine is not wholly mediated by the release of nitric oxide and may be mediated by direct reduction of calcium transport via voltage dependent calcium channel or sarcoplasmic reticulum. The vasorelaxant effect of atropine was neither blocked by propranolol (1 μM) nor enhanced isobutylmethylxanthine (IBMX, 100 nM).

These results indicate that vasorelaxant effect of atropine was not mediated by beta-adrenoceptors and not by enhancing cGMP. Also, prostacycline inhibitor, indomethacin was not blocked relaxant responses to atropine. This result indicates that relaxant responses to atropine were not mediated by endothelial PGI2 release.

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