

Acetylcholine-induced aortic relaxation studied in salbutamol treated rats

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Abstract

It has been proposed that the acetylcholine (ACh)-induced relaxation of the rat aorta is entirely mediated by endothelium derived-nitric oxide (NO). However, some authors have reported that indomethacin pretreatment attenuates ACh-induced relaxation of rat aortic ring preparations. Moreover, it has also been suggested that cAMP accumulation may regulate either nitric oxide synthase (NOS) or cyclooxygenase (COX) expression in different tissues. Thus, in this *in vitro* study we have investigated the endothelial mechanisms involved in the ACh-induced relaxation of ring preparations of the rat thoracic aorta, as well as the influence chronic treatment with the selective β_2 -agonist salbutamol had upon such mechanisms. Results of functional experiments show that N^G-monomethyl-L-arginine (L-NMMA, 3×10^{-4} M) considerably inhibited the ACh-induced relaxation of rat aortic ring preparations. However, indomethacin (10^{-5} M) was also found to partially attenuate this ACh response, suggesting that although NO is the most important mediator of the ACh-induced relaxation of the rat aortic ring preparations, vasorelaxation may also involve prostanoids. Moreover, the results suggest that treatment with salbutamol failed to produce any change in the ACh-induced relaxation of rat aortic ring preparations.

Key words: acetylcholine, endothelium, nitric oxide, prostanoids, salbutamol

Introduction

The involvement of the vascular endothelium in the relaxation of arterial smooth muscle caused by acetylcholine (ACh) has been well demonstrated (Furchgott and Zawadzki, 1980; Ignarro *et al.*, 1986). Furthermore, it has been shown that different endothelium derived relaxing substances such as nitric oxide (NO), endothelium derived hyperpolarizing factor (EDHF) and prostanoids could also be involved in the mediation of the ACh-induced

vasorelaxation, depending on the vessel being studied (Komori and Vanhoutte, 1990; Franci-Micheli *et al.*, 2000; Matsumoto *et al.*, 2003; Colle *et al.*, 2004; De Moraes *et al.*, 2004; Woodman and Boujaoude, 2004).

ACh-induced relaxation in the rat aorta has been attributed entirely to endothelium derived-NO (Shimokawa *et al.*, 1996; Tomioka *et al.*, 1999; Sekiguchi *et al.*, 2001; Freitas *et al.*, 2003; Woodman and Boujaoude, 2004). Pretreatment with the cyclooxygenase (COX) inhibitor indomethacin was reported not to affect the ACh-induced relaxation of rat aortic rings (Bobadilla *et al.*, 1997; Tomioka *et al.*, 1999), suggesting that prostanoids are not involved in ACh-induced relaxation of the rat aorta. But some workers have shown that indomethacin does increase the ACh-induced vasorelaxation of rat aortic rings (Heymes *et al.*, 2000; Matz *et al.*, 2000; De Angelis *et al.*, 2004). However, indomethacin pretreatment has also been reported to attenuate ACh-induced relaxation of rat aortic rings (Yang *et al.*, 1992; Callera *et al.*, 2000). Consequently, the role of endothelial prostanoids in the ACh-induced relaxation of the rat aorta is still unclear.

ACh-induced relaxation of the aorta may be influenced by several factors. Intracellular accumulation of cAMP in cultured mesangial cells may increase the expression of the inducible nitric oxide synthase (iNOS) (Kunz *et al.*, 1994; Kunz *et al.*, 1996; Nüsing *et al.*, 1996; Kunz *et al.*, 1997; Eberhardt *et al.*, 1998; Klein *et al.*, 1998), and activation of the AMP-activated protein kinase stimulates NO synthesis in human aortic endothelial cells (Morrow *et al.*, 2003). Similarly, cAMP accumulation in cultures of rat mesangial cells has been shown to induce COX expression and activity (Nüsing *et al.*, 1996). On the other hand, intracellular cAMP accumulation inhibited iNOS expression in both cultured rat hepatocytes (Harbrecht *et al.*, 2001; Harbrecht *et al.*, 2004) and in aortae obtained from rats submitted to endotoxic shock (Szabó *et al.*, 1997). Stimulation of vascular β_2 -adrenoceptors was also shown to cause cAMP accumulation (Priest *et al.*, 1999; Nakamura *et al.*, 2000).

There is no evidence concerning the functional effects of cAMP accumulation on the NO-dependent ACh-induced vasorelaxation in rat aortic rings, so chronic treatment with salbutamol (β_2 -adrenoceptor agonist) was used to investigate the functional effect of cAMP accumulation on ACh-induced vasorelaxation. The present study should improve our understanding of endothelial physiology, especially during treatment with β -adrenoceptor agonists. This is an important concern as such agonists are clinically used as vasoactive drugs in intensive care units or as bronchodilators in respiratory disease care.

Thus, the present study investigated the endothelial mechanisms involved in the ACh-induced relaxation of rat thoracic aorta ring preparations, to determine whether they were entirely related to NOS or not. It also examined what influence chronic treatment with the selective β_2 -agonist salbutamol had upon such mechanisms.

Materials and Methods

Animals

Male Wistar rats weighing 330 ± 25 g at the beginning of salbutamol treatment and 390 ± 30 g at the end of it, together with weight-matched untreated controls, were used in the

experiments. Rats were housed at room temperature (25°C) in plastic cages (50 × 40 × 20 cm), with 5 animals per cage in an animal room under a 12 h light-dark cycle beginning at 0700 h. Animals received food and water ad libitum. The experiments were carried out according to the Guide for the Care and Use of Laboratory Animals, National Academy of Sciences (1996).

Salbutamol treatment

The animals were divided into 3 groups: untreated, treated for 24 h and treated for 5 weeks with 5 mg/100 ml salbutamol sulfate (Aerolin®) in their drinking water, as adapted from the treatment protocol described by Dusseau and Hutchins (1979). Daily drinking water consumption for each animal was estimated at 62.93 ± 2.22 ml (with salbutamol) and 63.62 ± 3.18 ml (control). Treatment was interrupted 24 h before the experiments to allow salbutamol to wash out of the animal tissues.

Organ Bath studies

Animals were anesthetized with tribromoethanol (250 mg/kg, *i.p.*) and exsanguinated. The thoracic aorta was carefully removed, cleaned of all fat and connective tissue and cut into 3–4 mm rings. Each ring was suspended in a 25 ml organ bath using two stainless steel stirrup hooks placed vertically through the lumen. The lower hooks were fixed, while the upper ones were connected to isometric force recording transducers (GRASS FT03, Grass Instrument Company, Quincy, MA, USA). Contractions were recorded using a digital acquisition system (GOULD, Cleveland, Ohio, USA). Each organ bath contained Krebs solution with the following composition in mM: NaCl 130; KCl 4.7; CaCl₂ 2.5; KH₂PO₄ 1.2; MgSO₄ 1.2; EDTA 0.03; NaHCO₃ 15 and glucose 5.5. The nutrient solution was maintained at 37°C and continuously bubbled with a 95% O₂ and 5% CO₂ mixture at a pH of 7.4. Prior to drug addition, rings were equilibrated for 60 min at a resting tension of 10 mN. Then, ring endothelial integrity was confirmed by a characteristic 70 to 80% ACh (10^{-6} M)-induced relaxation in preparations pre-contracted with phenylephrine (3×10^{-6} M).

Cumulative concentration-effect (relaxation) curves were generated by adding ACh (10^{-10} M to 10^{-4} M) to each phenylephrine 3×10^{-6} M pre-contracted ring. Since residual salbutamol molecules may remain in the preparations obtained from treated animals, all experiments were performed in the presence of propranolol 3×10^{-5} M. COX and NOS product involvement in the contractile effects of phenylephrine were evaluated by comparing the effects of ACh on rings pre-incubated with indomethacin (10^{-5} M), N^G-monomethyl-L-arginine (L-NMMA, 3×10^{-4} M) or 10^{-5} M indomethacin plus L-NMMA (3×10^{-4} M) for 20 min with those observed in untreated rings.

The negative logarithm of the concentration evoking 50% of the maximal response (pD₂) was calculated from the concentration-response curves determined from control preparations and incubated with indomethacin as an indication of potency. It was not possible to calculate the pD₂ from preparations incubated with L-NMMA since import changes in the curves slope occurred.

Experimental design

First of all, ACh-induced aortic ring relaxation curves were repeated in the same

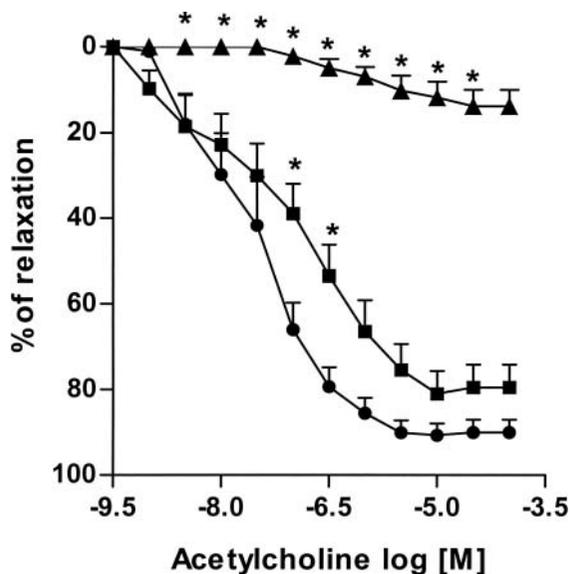


Fig. 1. Concentration-response curves for acetylcholine-induced relaxation determined in rat thoracic aortic rings pre-contracted with phenylephrine 3×10^{-6} M in the presence of saline (●), indomethacin 10^{-5} M (■) or indomethacin 10^{-5} M plus L-NMMA 3×10^{-4} M (▲). Points represent the mean \pm SEM of 8 independent determinations. *, $P < 0.05$ in relation to the saline control (two way ANOVA). #, $P < 0.05$ in relation to the indomethacin incubated preparations (two way ANOVA).

preparation, without COX or NOS inhibitors, to investigate whether curve displacement occurred as a consequence of the repetition. As no displacements were observed, parallel sets of aortic rings obtained from untreated and salbutamol treated animals were then studied in the absence and, subsequently, in the presence of indomethacin and indomethacin plus L-NMMA or L-NMMA and L-NMMA plus indomethacin.

Statistical analysis

Data are reported as the mean \pm standard error of the mean (S.E.M). The pD_2 values were compared using paired Student's *t* tests. Selected curves were compared using two-way ANOVA followed by a Bonferroni correction as a post hoc test. Values of $P < 0.05$ were considered to be statistically significant.

Results

Effect of pretreatment with indomethacin, L-NMMA or indomethacin plus L-NMMA on ACh-induced aortic ring relaxation

Treatment with indomethacin attenuated the ACh-induced relaxation of rat thoracic aortic rings as well as shifting the ACh-concentration-response curve significantly to the right (Fig. 1). In fact, the pD_2 values in the absence or presence of indomethacin were respectively 7.54 ± 0.18

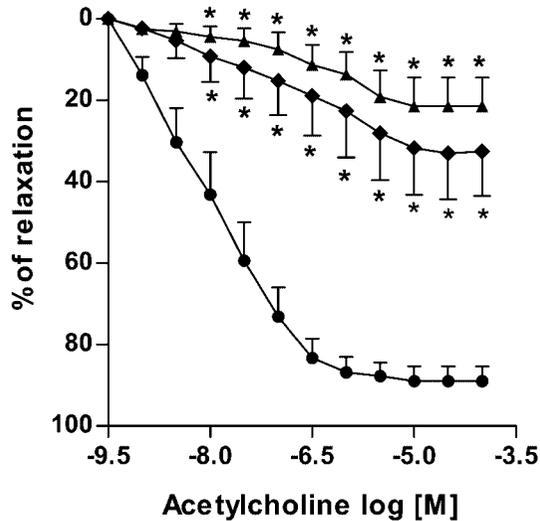


Fig. 2. Concentration-response curves for acetylcholine-induced relaxation determined in rat thoracic aortic rings pre-contracted with phenylephrine 3×10^{-6} M in the presence of saline (●), L-NMMA 3×10^{-4} M (◆) or L-NMMA 3×10^{-4} M plus indomethacin 10^{-5} M (▲). Points represent the mean \pm SEM of 6–8 independent determinations. *, $P < 0.05$ in relation to the saline control (two way ANOVA).

and 6.70 ± 0.12 ($n=8$; $t=3.91$, $P < 0.002$, paired t -test). The ACh-induced relaxation of the rat thoracic aortic rings was almost completely abolished when preparations were incubated with indomethacin plus L-NMMA (Fig. 1). ACh-induced relaxation of rat thoracic aortic rings was also markedly reduced in the presence of L-NMMA (Fig. 2). However, no further reduction of ACh-induced relaxation was observed when preparations were incubated with indomethacin plus L-NMMA (Fig. 2).

Effect of salbutamol pretreatment on the effects of indomethacin, L-NMMA or indomethacin plus L-NMMA on aortic ring vasorelaxation caused by ACh

Pretreatment of rats with salbutamol for a 5-week period did not change either the phenylephrine-induced pre-contraction (Fig. 3) or the ACh-induced relaxation of thoracic rat aortic rings (Fig. 4) when compared with untreated control animals. No significant difference was observed between the control and salbutamol-treated groups for ACh-induced relaxation in preparations preincubated with indomethacin (Fig. 4A), L-NMMA (Fig. 4B) or indomethacin plus L-NMMA (Fig. 4A, Fig. 4B).

Discussion

The present results suggest that NO is, in fact, the most important endothelium-derived mediator involved in ACh-induced relaxation of rat thoracic aortic rings. NO's pivotal role in ACh-induced relaxation of the rat aorta has already been proposed (Shimokawa *et al.*, 1996; Tomioka *et al.*, 1999; Callera *et al.*, 2000; Sekiguchi *et al.*, 2001; Freitas *et al.*, 2003; Woodman

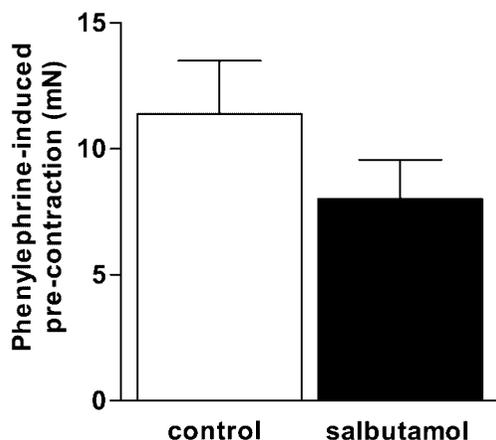


Fig. 3. Phenylephrine 3×10^{-6} M-induced pre-contraction determined in thoracic aortic rings obtained from untreated control animals (open bar) or rats treated with salbutamol for 5 weeks (filled bar). Bars represent the mean \pm SEM of 6–8 independent determinations.

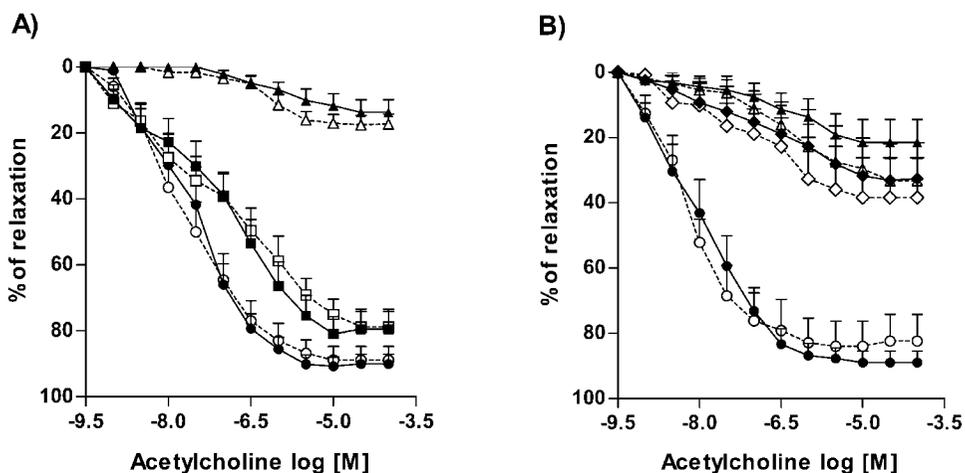


Fig. 4. Concentration-response curves for acetylcholine-induced relaxation determined in thoracic aortic rings pre-contracted with phenylephrine 3×10^{-6} M, in the presence of saline (●) in A) and B), indomethacin 10^{-5} M (■) in A), L-NMMA 3×10^{-4} M (▲) in A) and B) or indomethacin 10^{-5} M plus L-NMMA 3×10^{-4} M (◆) in B). Preparations were obtained from untreated control animals (filled symbols and continuous line) or rats treated with salbutamol for 5 weeks (open symbols and dotted lines). Points represent the mean \pm SEM of 6–8 independent determinations.

and Boujaoude, 2004). However, the question remains as to whether ACh-induced relaxation of the rat thoracic aorta also involves other endothelial derived mediators.

ACh-induced relaxation of rat thoracic aortic rings was attenuated in the presence of indomethacin and the ACh concentration-response curve was shifted significantly to the right. This suggests that prostanoids play a role in the vascular relaxation induced by ACh. It is

noteworthy that the effect of indomethacin demonstrated in the present study was observed only in the absence of the NOS inhibitor, suggesting a closed interrelationship between endothelium-derived NO and prostanoids. Our results are in agreement with those of Yang *et al.* (1992) and Callera *et al.* (2000), who suggested the participation of endothelium-derived vasodilator prostanoids in ACh-induced relaxation and are at difference with reports indicating negligible prostanoid involvement (Bobadilla *et al.*, 1997; Tomioka *et al.*, 1999). Our results also indicate that prostanoids may account for about 20% of the ACh-induced thoracic aortic ring relaxation.

Some studies have found that vasoconstrictor prostanoids may attenuate ACh-induced aortic relaxation in old rats (Heymes *et al.*, 2000; Matz *et al.*, 2000; De Angelis *et al.*, 2004), suggesting that prostanoid participation in ACh-induced rat aortic relaxation changes with age. In the present study, we used 12–14 week-old animals and our results suggest vasodilator instead of vasoconstrictor prostanoid involvement.

There is also evidence that responses change according to the resting tension applied to the preparation. It was proposed that in helical strips of the rat aorta with a resting tension of lower than 12 mN, prostanoids act synergistically with NO to evoke ACh-induced relaxation, while at higher resting tensions prostanoids only marginally influence ACh-induced relaxation (Franci-Micheli *et al.*, 2000). In that light, lower resting tensions are indicated for studying the interaction of NOS-related and prostanoid-related mechanisms in ACh-induced relaxation. The resting tension of 10 mN used in the present study and in the previous study by Callera *et al.* (2000) may have allowed the expression of prostanoid involvement. In fact, some results indicating the absence of an indomethacin effect on ACh-induced aorta relaxation were obtained in preparations with a resting tension equal to or higher than 15 mN (Tomioka *et al.*, 1999; Freitas *et al.*, 2003). On the other hand, indomethacin has also failed to inhibit ACh-induced aortic relaxation even in preparations with a resting tension of 5 mN (Woodman and Boujaoude, 2004). Moreover, Yang *et al.* (1992) provided evidence of prostanoid participation in the ACh-induced rat aortic relaxation with a resting tension of 50 mN, suggesting the possible involvement of other methodological variables.

The present data also indicates the existence of a residual ACh effect after the combined treatment with indomethacin plus L-NMMA. Similar results were reported by Callera *et al.* (2000), which may indicate that other vasodilator mechanisms, such as endothelium derived EDHF, participate in the ACh-induced relaxation of the rat aorta. There is evidence that ACh releases EDHF from the endothelium of the aorta (Tomioka *et al.*, 1999), however, further studies are necessary to investigate this residual ACh-induced relaxation.

Another finding from this study is that 5 weeks of treatment with salbutamol did not change either the phenylephrine-induced pre-contraction or the ACh-induced aortic ring relaxation. Similarly, the acute 24 h treatment with salbutamol did not influence the effects of phenylephrine or ACh in rat aortic rings (data not shown). We had expected that salbutamol activation of endothelial β -adrenoceptors, with the consequent intracellular cAMP accumulation, would affect NOS expression in the thoracic aortic ring preparations. In fact, cAMP elevation with forskolin, cholera toxin, salbutamol, or dibutyryl-cAMP for 24 h resulted in a 2- to 12-fold increase in NOS activity in rat mesangial cell cultures (Kunz *et al.*, 1994). Moreover, cultured

mesangial cells also revealed that intracellular cAMP accumulation increases iNOS expression (Kunz *et al.*, 1994; Kunz *et al.*, 1996; Nüsing *et al.*, 1996; Kunz *et al.*, 1997; Eberhardt *et al.*, 1998; Klein *et al.*, 1998). On the other hand, inhibition of iNOS expression has been observed in cultured cytokine-stimulated rat hepatocytes (Harbrecht *et al.*, 2001; Zhang *et al.*, 2004) and in the aorta obtained from rats under endotoxic shock (Szabó *et al.*, 1997) following intracellular cAMP accumulation.

There are also reports that cAMP accumulation induces COX expression in cultured rat mesangial cells, as well as its activity (Nüsing *et al.*, 1996). Moreover, cAMP accumulation was shown to induce NOS expression in rat mesangial cells, with consequent enhancement of NO production and NO activated COX enzymes (Tetsuka *et al.*, 1994). Cross-talk between COX and NOS pathways could also modify the effects of the ACh relaxation of the aorta.

The present study verified the effect of chronic salbutamol treatment on preparations treated with either indomethacin or L-NMMA. This methodological approach permitted us to individually investigate the participation of either COX or NOS pathways in ACh-induced aortic ring relaxation. The present results indicate that both COX and NOS pathways are unchanged in salbutamol treated animals. They also suggest that salbutamol treatment did not change ACh-induced aortic ring relaxation resistance to incubation with indomethacin plus L-NMMA.

As previous studies have suggested low oral bioavailability of salbutamol in rats (Montrade *et al.*, 1995; Smith, 1998), we treated our animals with a highly concentrated solution of salbutamol (5 mg/100 ml of drinking water). Considering that treated animals consumed an average of 63 ml/day of drinking water, we estimated that the animals received a daily dose of 3.15 mg salbutamol per animal, or between about 8 and 9.5 mg/kg. It is noteworthy that a single oral dose of salbutamol 300 µg/kg (by gavage), which is 1/30 of our treatment, produced important but transitory tachycardia in rats (Muacevic, 1985). Therefore, even though other routes and dosages could be tested, we conclude that treatment with salbutamol does not change ACh-induced thoracic aortic ring relaxation.

Our findings suggest that salbutamol treatment either failed to induce intracellular cAMP enhancement or that it does increase intracellular cAMP but without having an effect on the ACh-induced aortic ring relaxation. Alternatively, it has been suggested salbutamol along with other β -adrenoceptor agonists induce cAMP accumulation in endothelial and/or vascular smooth muscle cells of rats (Dusseau and Hutchins, 1982; Toyoshima *et al.*, 1998). However, it is proposed that while cAMP accumulation may have occurred in the cells of salbutamol-treated animals, this did not influence the ACh-induced thoracic aortic ring relaxation.

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