Endothelium-dependent relaxation in response to acetylcholine in pregnant guinea-pig uterine artery

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Recently, strong evidence has suggested that nitric oxide (NO) synthesis is significantly increased in the uterine artery during pregnancy, which may mediate the increased blood flow to the uterus that is characteristic of pregnancy. We therefore investigated the nature of the mediators of acetylcholine (ACh)-induced relaxation in pregnant guinea-pig uterine arterial rings. ACh (0.1 nM to 60 µM) induced endothelium-dependent relaxation of phenylephrine-precontracted pregnant guinea-pig uterine artery. N⁶-monomethyl-L-arginine (3–30 µM) antagonized the effect of ACh, with suppression of maximal ACh-induced relaxation, in a concentration-dependent manner. The inhibition of relaxation by N⁶-monomethyl-L-arginine (10 µM) was significantly overcome by L-arginine (10 µM), but not by D-arginine (100 µM). On the contrary, the administration of indomethacin (10 µM) and diethylcarbamazine (100 µM) did not modify the relaxation of pregnant guinea-pig uterine artery induced by ACh. The ACh-evoked relaxation was unaltered when K⁺-rich Krebs–Ringer bicarbonate solution was used to induce tone instead of phenylephrine, or when a non-selective blocker of K⁺ channels, 4-aminopyridine (6 mM), was applied to phenylephrine-precontracted segments. It is concluded that the relaxation induced by ACh in pregnant guinea-pig uterine artery can be explained entirely by the release of NO from vascular endothelial cells, without involvement of other endothelium-derived relaxing factors, similar to that previously reported for non-pregnant guinea-pig uterine artery. Thus, it seems that increased activity of NO synthase during pregnancy is without significant influence on the ACh action on uterine artery.

Key words: acetylcholine/endothelium/nitric oxide/pregnancy/uterine artery

Introduction

Recently, strong evidence has suggested that nitric oxide (NO) synthesis is significantly increased in uterine artery during pregnancy (Weiner et al., 1994), which may mediate the increased blood flow to the uterus that is characteristic of pregnancy (Peeters et al., 1980). However, it is surprising that the induction of NO synthesis during pregnancy was not accompanied by increased relaxation of uterine artery in response to acetylcholine (ACh) (Jovanović et al., 1994a). Recent studies have suggested that, depending on the stimulus used, during pregnancy, vascular endothelium of uterine artery increasingly releases vasodilator-related prostaglandins (Grbović and Jovanović, 1996), unidentified relaxing factor(s) (Jovanović et al., 1995b) or remains unchanged (Jovanović et al., 1995c). Thus, the role of increased NO production in uterine artery remains unclear.

Previously, we have shown that the endothelium-dependent relaxation evoked by ACh in both non-pregnant human and guinea-pig uterine artery is mediated through production of NO from the vascular endothelium, without involvement of other endothelium-derived relaxing factors (Jovanović et al., 1994b, 1995a). However, none of the previous studies directly and clearly addressed the nature of the mediators in ACh-induced relaxation in uterine artery in pregnancy. The observed lack of difference between ACh potency and efficacy in relaxation of non-pregnant and pregnant guinea-pig uterine artery (Jovanović et al., 1994a) does not necessarily imply that the mechanism of action is the same, since it has been previously reported that, under various conditions, different pathways of endothelium-dependent vasorelaxation can be activated, but leading to the same result of ACh action (Yang et al., 1991).

Therefore, the purpose of this study was to determine the nature of the mediators of ACh-induced relaxation in pregnant guinea-pig uterine artery, and to examine the possibility that during pregnancy the release of relaxing factor(s) derived from endothelium in response to ACh is altered.

Materials and methods

Adult female pregnant (55–60 days gestation; term, 65–68 days gestation) guinea-pigs (700–900 g) were used in this study. The animals were stunned and decapitated. The right and left uterine arteries respectively were carefully dissected free from surrounding fat and connective tissue and cut into 3 mm long circular segments. All vessel segments were immediately placed in Krebs–Ringer bicarbonate solution (composition in mM: NaCl 118.3; KCl 4.7; CaCl₂ 2.5; MgSO₄ 1.2; KH₂PO₄ 1.2; NaHCO₃ 25.0; CaEDTA 0.026; glucose 11.1). The endothelium was removed from some rings by gently rubbing the intimal surface with stainless steel wire. Ring preparations were mounted between two stainless-steel triangles in an organ bath containing 10 ml Krebs–Ringer bicarbonate solution (37°C, pH 7.4), aerated with 95% O₂ and 5% CO₂. One of the
triangles was attached to a displacement unit allowing the fine adjustment of tension and the other was connected to a force-displacement transducer (Hugo Sachs K30). Isometric tension was recorded on a Hugo Sachs model MC 6621 recorder (Hugh Sachs, Freiburg, Germany).

Preparations were allowed to equilibrate for 60 min in Krebs–Ringer bicarbonate solution. Subsequently, each ring was gradually stretched to the optimal point of tension (6.4 mN; Jovanović et al., 1994a). Once at their optimal length, the segments were allowed to equilibrate for 30 min before experimentation. The experiments were performed as has been previously described in detail (Jovanović et al., 1994a, 1995a). Briefly, the following protocol was used: (i) contraction in response to EC\textsubscript{50} (the concentration that produces 80% of maximal response) phenylephrine (0.2–0.6 µM), addition of 1 µM Ca\textsuperscript{2+} ionophore A23187 (to confirm the presence or successful denudation of endothelium), followed by three washes and rinsing at 15 min intervals for the next 60 min; (ii) contraction in response to EC\textsubscript{50} phenylephrine (0.2–0.6 µM) and concentration–response curve with ACh (0.1 nM to 60 µM), followed by three washes, addition of the antagonists and a 15 min [N\textsuperscript{G}-monomethyl-L-arginine, L-arginine (L-NMMA) and \(\beta\)-arginine] 20 min (4-aminopyridine), 30 min (diethylenetriaminepentaacetic acid) and 40 min (indomethacin) equilibration period; (iii) contraction in response to EC\textsubscript{50} phenylephrine (0.2–2.0 µM) and the concentration–response curve with ACh (0.1 nM to 60 µM).

In a separate series of experiments, vascular rings were precontracted with K\textsuperscript{+}-rich Krebs–Ringer bicarbonate solution. The solution was prepared by direct replacement of 65 mM NaCl with 65 mM KCl (Plane and Garland, 1993).

At the end of each experiment, papaverine (300 µM) was added to the organ bath to determine the maximal relaxation of preparations (Toda et al., 1985; Mellemkjaer et al., 1989; Jovanović et al., 1994a,b,c, 1995a). The relaxation achieved by papaverine was 108 ± 6% of the initial contraction induced by phenylephrine (\(n = 44\)) and 106.4 ± 11.2% of the initial contraction induced by K\textsuperscript{+}-rich Krebs–Ringer bicarbonate solution (\(n = 5, P > 0.05\)). Additionally, at the end of some experiments (10 of each), the condition of the endothelium was verified by Van Gieson’s staining with iron haematoxylin and light microscopic examination of the intimal surface (Disbrey and Rack, 1970).

All experiments were carried out in tissues with a functionally intact endothelium precontracted with phenylephrine unless otherwise stated.

**Treatment of data and statistics**

The relaxation induced by each concentration of ACh was expressed as a percentage of the maximum relaxation achieved by papaverine and used in the construction of the concentration–response curves. The concentration of ACh eliciting 50% of its own maximum response (EC\textsubscript{50}) was determined graphically for each curve by linear interpolation. EC\textsubscript{50} values are presented as pEC\textsubscript{50} (pEC\textsubscript{50} = –log EC\textsubscript{50}).

Since L-NMMA produced suppression of the maxima of the ACh concentration-response curve, to analyse non-competitive antagonism by L-NMMA, the IC\textsubscript{50} value (the molar concentration of antagonist that reduces the maximal response to an agonist by 50%) was calculated. Thus, the percentages of inhibition of maximal response to ACh in the presence of different concentrations of L-NMMA were calculated (Emax – Emax\textsubscript{B}); Emax is a maximal response to ACh in the absence of L-NMMA taken as 100%, Emax\textsubscript{B} is a maximal response to ACh in the presence of L-NMMA calculated as a percentage of Emax value) and a plot of (Emax – Emax\textsubscript{B}) against −log concentration L-NMMA was constructed using regression analysis. The pIC\textsubscript{50} (−log IC\textsubscript{50}) value was determined graphically by linear interpolation. The least squares method was used for calculating linear regressions.

The results are expressed as means ± SEM; n refers to the number of experiments. Statistical significance of differences between two means was determined with Student’s t-test for paired or unpaired observations where appropriate. One-way analysis of variance (ANOVA) followed by Dunnett’s test was used when more than two groups were analysed. A value of \(P < 0.05\) was considered to be statistically significant.

**Drugs used**

The following drugs were used: phenylephrine hydrochloride, acetylcholine chloride, indomethacin, diethylenetriaminepentaacetic acid, Cu\textsuperscript{2+} ionophore A23187, L-arginine hydrochloride, \(\beta\)-arginine hydrochloride, 4-aminopyridine (4-AP, Sigma, St Louis, MO); N\textsuperscript{G}-monomethyl-L-arginine acetate (L-NMMA; Wellcome, London, UK), papaverine hydrochloride (Merck, Whitehouse Station, NJ, USA). All agents were dissolved in distilled water and diluted to the desired concentration. A solution of N\textsuperscript{G}-monomethyl-L-arginine [L-NMMA] 20 min (4-aminopyridine), 30 min papaverine (300 µM).

**Results**

ACh (0.1 nM to 60 µM) induced a concentration-dependent relaxation of the precontracted uterine arterial rings with intact endothelium (pEC\textsubscript{50} = 7.48 ± 0.02, maximal response = 92.5 ± 5.1%, \(n = 44\), \(P > 0.05\)) (Figure 1). After the removal of the vascular endothelium the relaxation induced by ACh was almost completely abolished (maximal response = 8.0 ± 1.7%, \(n = 10\)) (Figure 1).

L-NMMA (3–30 µM), an inhibitor of NO-synthase, produced a rightward shift of the concentration-response curves to ACh, with suppression of maximal ACh-induced relaxation in a concentration-dependent manner (\(P < 0.001\)) (Figure 2A).
Acetylcholine response of pregnant uterine artery

Figure 2. The antagonism of the relaxant effects of acetylcholine by L-NMMA. (A) Concentration-response curves for acetylcholine in pregnant guinea-pig uterine artery with intact endothelium in the absence (○) and presence of 3 µM (▼), 10 µM (□) and 30 µM (■) L-NMMA. Each point represents the mean ± SEM (n = 7–21). Responses are expressed as percentages of the maximal relaxation induced by papaverine (300 µM). The inset represents a replot of the data from (A) showing the percentage inhibition of maximal response to acetylcholine as a function of -log conc. L-NMMA (M) (y = -43.06x + 251.37, r = 0.998). The IC₅₀ value is identical to inverse –log conc. L-NMMA (M) corresponding to 50% of inhibition of acetylcholine-induced relaxation. (B) Concentration-response curves for acetylcholine in pregnant guinea-pig uterine artery with intact endothelium in the absence (○) and presence of 10 µM L-NMMA + 10 µM L-arginine (▼), and 10 µM L-NMMA ( ■). Each point represents the mean ± SEM (n = 5–14). Responses are expressed as percentages of the maximal relaxation induced by papaverine (300 µM).

The IC₅₀ value was 21.0 ± 1.5 µM, n = 7 (inset in Figure 2A). L-Arginine (100 µM) did not affect endothelium-dependent relaxation in response to ACh (pEC₅₀ = 7.51 ± 0.05, maximal response = 90.7 ± 4.6%, n = 4, in the absence and pEC₅₀ = 7.53 ± 0.08, maximal response = 91.6 ± 4.3%, n = 4, in the presence of L-arginine, P > 0.05, data not shown), but concomitant addition of L-arginine (10 µM) significantly overcame the inhibition of ACh-induced relaxation produced by L-NMMA (10 µM) (Figure 2B). On the contrary, D-arginine (100 µM) did not alter the response to ACh (pEC₅₀ = 7.46 ± 0.07, maximal response = 92.8 ± 3.9%, n = 4, in the absence and pEC₅₀ = 7.49 ± 0.07, maximal response = 92.6 ± 5.1%, n = 4, in the presence of D-arginine, P > 0.05), nor the inhibition of ACh-induced relaxation produced by 10 µM L-NMMA (maximal response to ACh in the presence of L-NMMA was 54.7 ± 5.3% and 57.1 ± 5.9% in the presence of both L-NMMA and D-arginine, n = 5, P > 0.05) (data not shown).

The administration of indomethacin (10 µM) and diethylcarbamazine (100 µM), did not modify the relaxation of guinea-pig uterine artery induced by ACh (Figure 3).

Finally, the ACh-induced relaxations were unaltered when K⁺-rich Krebs–Ringer bicarbonate solution was used to induce tone instead phenylephrine (Figure 4A) or when a non-selective blocker of K⁺ channels, 4-AP (6 mM), was applied to phenylephrine-precontracted segments (Figure 4B).

Discussion

In the present study, we confirmed our previous findings that ACh induced endothelium-dependent relaxation of pregnant guinea-pig uterine artery (Jovanović et al., 1994a). Moreover, the pEC₅₀ and maximal response values for ACh were similar to those obtained in previous studies on non-pregnant human and guinea-pig uterine artery (Jovanović et al., 1994a,b,c, 1995a).

Figure 3. The antagonism of the relaxant effects of acetylcholine by indomethacin and diethylcarbamazine. Concentration-response curves for acetylcholine in pregnant guinea-pig uterine artery, precontracted with phenylephrine, with intact endothelium in the absence (○) and presence (▼) of 10 µM indomethacin and (□) 100 µM diethylcarbamazine. Each point represents the mean of four or five experiments. Standard errors are excluded for clarity and do not exceed 15% of the mean value for each point. Responses are expressed as percentages of the maximal relaxation induced by papaverine (300 µM).
Figure 4. The antagonism of the relaxant effects of acetylcholine by K⁺-rich Krebs–Ringer bicarbonate solution and 4-aminopyridine. (A) Concentration-response curves for acetylcholine in pregnant guinea-pig uterine artery with intact endothelium precontracted with phenylephrine (○) and with K⁺-rich Krebs–Ringer bicarbonate solution (■). (B) Concentration–response curves for acetylcholine in pregnant guinea-pig uterine artery with intact endothelium precontracted with phenylephrine in the absence (○) and presence (■) of 6 mM 4-aminopyridine. Each point represents the mean of five experiments. Standard errors are excluded for clarity and do not exceed 15% of the mean value for each point. Responses are expressed as percentages of the maximal relaxation induced by papaverine (300 µM).

Endothelium-dependent vasodilatation mediated by cyclooxygenase products has been observed in dog mesentery and aortic rings (Toda, 1984), cat cerebral arteries (Kontos et al., 1990), rat brain vessels (Haberl et al., 1990) and human pulmonary artery (Ortiz et al., 1992). Furthermore, it has been shown that in the isolated perfused rat aorta and the canine femoral artery, prostacyclin is released from endothelial cells after stimulation with ACh (Lüscher et al., 1986; Rubanyi et al., 1986). In the present study, the blockade of cyclooxygenase with indomethacin did not modify ACh effects, indicating that the relaxation was not due to prostanoid production.

It has been reported that the lipoxygenase pathway is involved in mediation of endothelium-dependent relaxation of the rat aorta, canine femoral artery and non-pregnant and pregnant canine uterine artery (De Mey et al., 1982; Uotila et al., 1987; Matsumoto et al., 1992). In this study, diethylcarbamazine, used at a concentration that inhibits lipoxygenase products of arachidonic acid (Mathews and Murphy, 1982), did not reverse the relaxant effect of ACh on preparations studied. Accordingly, these findings suggest that lipoxygenase cascade products of arachidonic acid are not involved in mediation of this effect of ACh.

In 1980 it was reported that ACh dilated certain blood vessels by releasing a non-prostanoid diffusible substance from the endothelium, which was designated endothelium-derived relaxing factor (EDRF) (Furchgott and Zawadzki, 1980). EDRF is believed to be nitric oxide (NO) or a labile nitroso compound synthesized from the terminal guanidine nitrogen atom of the amino acid l-arginine in the vascular endothelial cells, probably followed by the metabolism of l-arginine to citrulline (Palmer et al., 1987, 1988). In order to analyse the involvement of NO in ACh-induced responses in pregnant guinea-pig uterine artery, the inhibitor of NO synthesis, l-NMMA (Palmer et al., 1988; Rees et al., 1989), was tested. l-NMMA antagonized the effects of ACh on uterine arteries in a concentration-dependent manner. The applied concentrations of l-NMMA brought about non-competitive inhibition of ACh-induced vasodilatation as shown by depression of the maximum response and the rightward shift of the concentration-response curve. The IC₅₀ values for l-NMMA obtained with pregnant guinea-pig uterine arteries were similar to those observed on rabbit aorta (10.0 µM, Rees et al., 1989), and non-pregnant guinea-pig and human uterine artery (12.8 µM, Jovanović et al., 1994b; 17.2 µM, Jovanović et al., 1995a). In addition, l-arginine, but not d-arginine, antagonized effects of l-NMMA on ACh-induced relaxation. These results suggest that both l-NMMA and l-arginine are competing specifically for the same mechanisms, which, since the effect was enantiomerically specific, is probably mediated through NO synthesis (Palmer et al., 1988). On the basis of these results, it seems reasonable to suggest that endothelial NO production is involved in the ACh-induced relaxation of pregnant guinea-pig uterine artery.

It has been shown that ACh releases from the endothelium of some blood vessels a factor independent of NO (Chen et al., 1988, 1991), which is able to hyperpolarize vascular smooth muscle (Chen et al., 1988, 1991) and has been named as an endothelium-derived hyperpolarizing factor (EDHF). Moreover, in non-pregnant guinea-pig uterine artery hyperpolarization produced by NO itself has been reported (Tare et al., 1990), but this finding remains controversial (Jovanović et al., 1995a). The mechanism proposed for the endothelium-dependent hyperpolarization involves an increase in potassium conductance through the opening of potassium channels (Chen et al., 1994).
et al., 1988, 1991; Tare et al., 1990). On the other hand, it has been shown that precontraction with a K⁺-rich Krebs–Ringer bicarbonate solution inhibits smooth muscle hyperpolarization to ACh (Plane and Garland, 1993). In the present study, smooth muscle relaxation in the uterine artery was unaffected by precontraction with a K⁺-rich Krebs–Ringer bicarbonate solution, suggesting that smooth muscle hyperpolarization is not involved in ACh action in pregnant guinea-pig uterine artery. In agreement with this conclusion is the fact that a high concentration of 4-AP, a potential and non-selective K⁺ channels blocker (Cook and Quast, 1990), did not affect the action of ACh.

Thus, our experiments suggest that endothelium-dependent relaxation induced by ACh in pregnant guinea-pig uterine artery can be entirely explained by the release of NO from vascular endothelial cells, similar to the non-pregnant guinea-pig uterine artery (Jovanović et al., 1995a). Consequently, the role of increased NO synthesis in uterine artery remains unclear. It is, however, possible that the increased NO synthesis is not of vascular endothelium origin. It has been postulated that the vascular smooth muscle of human uterine artery is able to synthesize NO from l-arginine (Jovanović et al., 1994d). Therefore, the possibility exists that pregnancy induces NO-synthase activity in uterine vascular smooth muscle, but not in vascular endothelium.

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References


