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The effect of ovariectomy on depressed contractions to phenylephrine and KCl and increased relaxation to acetylcholine in isolated aortic rings of female compared to male rabbits

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1 Differences in vascular responses to phenylephrine, acetylcholine (ACh) and potassium chloride (KCl) were studied in rabbit aorta from female and male rabbits, in the absence and presence of an inhibitor of nitric oxide (NO) production, N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM).

2 Phenylephrine and KCl-induced contractions, were significantly reduced in amplitude ($P<0.01$) in the rings from female rabbits compared to those from male rabbits.

3 ACh-induced relaxation was greater ($P<0.01$) in aortic rings from females than from males.

4 Incubation of the rings with L-NAME abolished the phenylephrine-induced contraction differences between rings from male and female rabbits.

5 Ovariectomy eliminated the differences in vascular responses to phenylephrine, KCl and ACh of aortic rings from the female rabbits.

6 Both basal and ACh-stimulated release of nitrites from aortic rings was greater ($P<0.01$) in vascular tissue from female than male rabbits.

7 These results indicate that differences in vascular reactivity in aortic rings from male and female rabbits may be associated with a higher release of NO, resulting in an increased vasodilator response in the female rabbits.

Keywords: Nitric oxide; oestrogens; vascular reactivity; acetylcholine; N^G-nitro-L-arginine methyl ester (L-NAME)

Introduction

Sex and sex hormone levels have been associated with important differences in vascular reactivity. Altura (1972) demonstrated that intact mesenteric arterioles of female rats are much more sensitive to the constrictor actions of catecholamines, than those of male rats. Other vasoactive peptides, e.g. arginine-vasotocin, produced higher contractile responses to terminal arterioles in female rats than in males (Altura, 1975). Administration of oestrogen to male rats potentiated the concentration-response curves to arginine-vasotocin in terminal arterioles, abolishing the sex differences (Altura, 1975). These observations suggest that oestrogens may be responsible for stimulating vasoconstrictor mechanisms and the authors postulated the release of catecholamines, changes in intracellular calcium or membrane potentials as possible mechanisms. Moreover, in mesenteric artery segments, treatment with 17β-oestradiol decreased the EC_{50} to noradrenaline (Colucci *et al.*, 1982) further supporting the provasoconstrictor action of oestrogens. However, recent data contradict this hypothesis. In oestrogen treated ovariectomised monkeys, acetylcholine (ACh) induced small increments in the coronary artery diameter, suggesting that the effect of ACh under those conditions was vasodilatation. Whereas in oestrogen-deficient monkeys, the same concentration of ACh resulted in a significant decrease in coronary artery diameter, suggesting a clear constrictor effect of ACh on the coronary artery (Williams *et al.*, 1990). These results suggest that oestrogens have a provasodilator effect. On the other hand, male hormones also play a role in the regulation of vascular reactivity. It has been found that testosterone increases vascular reactivity to noradrenaline in pithed cats and perfused hind limbs of dogs (Bhargana *et al.*, 1967). These sex-related differences in vascular reactivity have been associated with important effects on vascular function. Thus, under standard environmental conditions, hand blood-flow, finger blood-flow, and skin perfusion in women were half those of men (Greenberg *et al.*, 1974; Cooke *et al.*, 1990). These differences appear to be due to an increase in sympathetic outflow to the cutaneous circulation in women, supporting the hypothesis that oestrogens are provasoconstrictors. Whereas, in contrast, Baker *et al.* (1978) demonstrated that injection of arachidonic acid lowered diastolic pressure more in females than in males, and castrated rats were more sensitive than the ovariectomised female rats to the depressor action of arachidonate (Baker *et al.*, 1978), supporting the role of oestrogen as a provasodilator.

The role of the endothelium as the source of endothelium-derived relaxing factor (EDRF) was first demonstrated by Furchgott & Zawadzki (1980). However, it was not until 1987 that the biological properties of EDRF were shown to be a consequence of the release of NO from vascular endothelial cells (Moncada *et al.*, 1991). It has been demonstrated that vascular relaxation induced by a number of pharmacological agents is mediated by NO (Furchgott, 1984). However, it has recently been found that hyporesponsiveness to the pressor effects of catecholamines and other agonists (Schaller *et al.*, 1985), as well as hyporeactivity in arteries studied *ex vivo* or *in vitro* (McKenna, 1988; Julous-Schaeffer *et al.*, 1990), can be prevented by inhibitors of NO (Julous-Schaeffer *et al.*, 1990). This strongly suggests that basal or stimulated release of NO may play an important role in regulating the effects of vaso-

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constrictor agonists. These observations raise the possibility that differences in vascular responses of arteries from male and female animals may be due to a difference in the release of NO by the endothelium. Indeed, several observations have suggested that nitric oxide synthase (NOS) might be regulated by sex hormones. For example, the aorta of female rabbits has a higher basal release of NO than that of males and castration eliminates this difference (Hayashi *et al.*, 1992), while the administration of oestradiol increased endothelium-dependent relaxations (Gisclard *et al.*, 1988; Miller & Vanhoutte, 1988) in rabbit aorta.

These results suggest that sex hormones could play an important role in the vascular responsiveness to different agonists through modulation of NOS. In the present study, we therefore evaluated the role of NO in the differences in vascular reactivity between aortic rings from male and female rabbits. The effect of ovariectomy on the vascular responses was also evaluated.

Methods

New Zealand white rabbits (2.5 to 3.0 kg) of either sex were divided into two groups (control and ovariectomised). One group of animals was subjected to a surgical procedure to extirpate the gonads (ovaries). Two weeks after surgery, aortic rings were obtained from control or ovariectomised animals. At the same time blood samples were taken to determine oestrogen and testosterone blood levels. Experiments were conducted by pairing female and male aortic rings on the day of the experiment.

Preparation of rabbit aortic rings

Control or ovariectomised rabbits were killed by cervical dislocation and the thoracic aorta was carefully removed and placed into cold Krebs-bicarbonate buffer. Periadventitial fat was removed and the artery was cut into 3 to 4 mm wide rings. The ionic composition of the Krebs-bicarbonate buffer was (in gl^{-1}): NaCl 6.92, KCl 0.354, $CaCl_2$ 0.280, KH_2PO_4 0.162, $MgSO_4.7H_2O$ 0.294, $NaHCO_3$ 2.1 and dextrose 2.0. The solution was gassed constantly with 95% O_2 and 5% CO_2. Care was taken during the dissection to avoid unnecessary stretching or contact of intruments with the luminal surface of the ring to ensure the integrity of the vascular endothelium. The aortic rings were mounted in 5 ml water-jacketed organ baths maintained at 37°C, and equilibrated for 1.5 to 2.0 h. A maximum of four rings were used for each experiment (2 male and 2 female, 2 control and 2 ovariectomised or 2 control and 2 N^G-nitro-L-arginine methyl ester (L-NAME; 100 μM)-treated rings). Basal tone was set at 2.0 g and adjusted accordingly over the equilibration period. Changes in tension were measured by Grass model FT 03C force transducers coupled to a Grass polygraph model 7C 8A. This procedure was found to produce optimal conditions for reproducible isometric force development. Concentration-response curves to phenylephrine, ACh and KCl were obtained by the cumulative addition of drugs to the organ bath, the concentration being increased only after the maximal response to the previous concentration had been attained. The presence of endothelium was confirmed by assessing the effectiveness of ACh (0.1 μM) to relax aortic rings precontracted with phenylephrine (0.1 μM). ACh concentration-response curves were done in aortic rings precontracted with phenylephrine (0.1 μM). A control concentration-response curve to phenylephrine was obtained; following 30 min incubation of the rings with L-NAME, the concentration-response curve to phenylephrine was repeated.

Assay of nitrites

Aortae were obtained from each group of rabbits ($n=5$) and cleaned of any loose connective tissue as described. Each aorta was divided in two segments of the same length, and each segment was cut into rings of 3–4 mm width (approximately 15 rings per segment). Each group of rings was incubated in 5 ml of Krebs solution at 37°C in a shaking water bath. The solution was changed every 10 min, until all blood elements were eliminated from the rings, approximately 30–45 min. At the end of this period, the rings were transferred into 1 ml of Krebs solution and 100 μl of Krebs solution was added to control group and 100 μl of ACh (1 μM final concentration) was added to the other group. The rings were incubated for 30 min at 37°C in a shaking water bath, at the end of which, two 500 μl aliquots of the solution were removed and treated with *Escherichia coli* nitrate reductase as described previously (Schultz & Raij, 1992), in order to reduce NO_3^- in the sample to NO_2^-. After reduction, Griess reagent (Green *et al.*, 1982) was added and total nitrites (representing NO_2^- and reduced NO_3^-) were measured from a standard curve. This assay detects nitrites in the range 0.5 to 100 μM. Rings were dried at 60°C in an oven for 1 h and the dry weight was calculated. Data obtained are expressed as mol mg^{-1} 30 min^{-1}.

Blood levels of sexual hormones

Serum concentrations of testosterone and 17β-oestradiol were measured by radioimmunossay by use of polyclonal antibodies and ^{3}H-labelled hormones. The antisera were raised in New Zealand rabbits with testosterone-3-carboxymethyloxyme and oestradiol-6-keto-hemisuccinate coupled to bovine serum albumin as immunogens. Within and between the batches, coefficients of variations were lower than 10 and 15% for testosterone and oestrogens, respectively.

Materials

Phenylephrine, acetylcholine, KCl and L-NAME were stored as 10 mM stock solutions in Krebs solution and diluted as required on the day of the experiment. Chemicals were purchased from Sigma Chemical Co. (U.S.A.). All other substances were freshly prepared before each experiment.

Statistical analysis

Changes in vascular tone are expressed in grams (g) of tension developed (contraction) or % relaxation. Data are expressed as the mean $\pm$ s.e.mean. Concentration-response curves were compared by two-way analysis of variance, and specific differences between each concentration and treatment were compared by a modified t test. A P value <0.05 was considered significant.

Results

Vascular reactivity of aortic rings isolated from male and female rabbits

Phenylephrine induced a concentration-dependent increment in the vascular tension of aortic rings obtained from male and female rabbits. However, the increase in the vascular tension was higher in the aortic rings from male rabbits when compared to that developed in female rabbits (Figure 1a) (EC_{50} 0.12 ± 0.003 and 0.15 ± 0.015 μM for rings from male and female rabbits, respectively). The maximal tension developed in the rings, upon stimulation with phenylephrine (1 μM) from male rabbits was 4.2 ± 0.4 g, compared with 2.6 ± 0.5 g developed in rings from female rabbits ($P<0.05$).

KCl a non-receptor-mediated vasoconstrictor, produced an increment of the vascular tension of aortic rings, which was also found to be concentration-dependent in aortic rings from either sex. Higher tension was developed in the rings from male rabbits when compared with the rings from female rabbits (Figure 1b) (EC_{50} 31.8 ± 2.8 and 47.2 ± 3.05 mM for rings from male and female rabbits, respectively). The maximal tension developed by the rings after stimulation with KCl (90 mM) was

4.0±0.2 g, in the rings from male rabbits compared to 2.9±0.3 g in the rings from female rabbits ($P<0.05$). ACh produced a concentration-dependent relaxation in rings from the male and female rabbits. However, the degree of relaxation was higher in the arteries from female rabbits than the arteries from male rabbits (Figure 1c).

Effect of nitric oxide inhibition on phenylephrine-induced contractions

Incubation of the aortic rings with L-NAME (100 μM), produced a small increment in the vascular tension of 0.2±0.01 g and 0.4±0.01 g ($n=9$, $P<0.05$) for rings from male and female rabbits, respectively. Moreover, L-arginine (10 mM) reversed this contraction. Inhibition of the nitric oxide synthase with L-NAME (100 μM) did not affect the concentration-response curve to phenylephrine in the rings from male rabbits (Figure 2a), in this case the maximal tension developed was 5.9±0.2 g and 6.7±0.2 g for control and L-NAME-treated rings, respectively (EC_{50} 0.06±0.004 and 0.07±0.01 μM for rings without and with L-NAME, respectively). However, in the rings from female rabbits L-NAME (100 μM) treatment significantly potentiated the vasoconstrictor effect of phenylephrine displacing the concentration-response curve to the left (Figure 2b) (EC_{50} 0.07±0.007 and 0.04±0.004 μM for rings without and with L-NAME, respectively). The maximal tension developed was 3.9±0.3 g and 5.8±0.3 g for control and L-NAME-treated rings, respectively. No significant differences

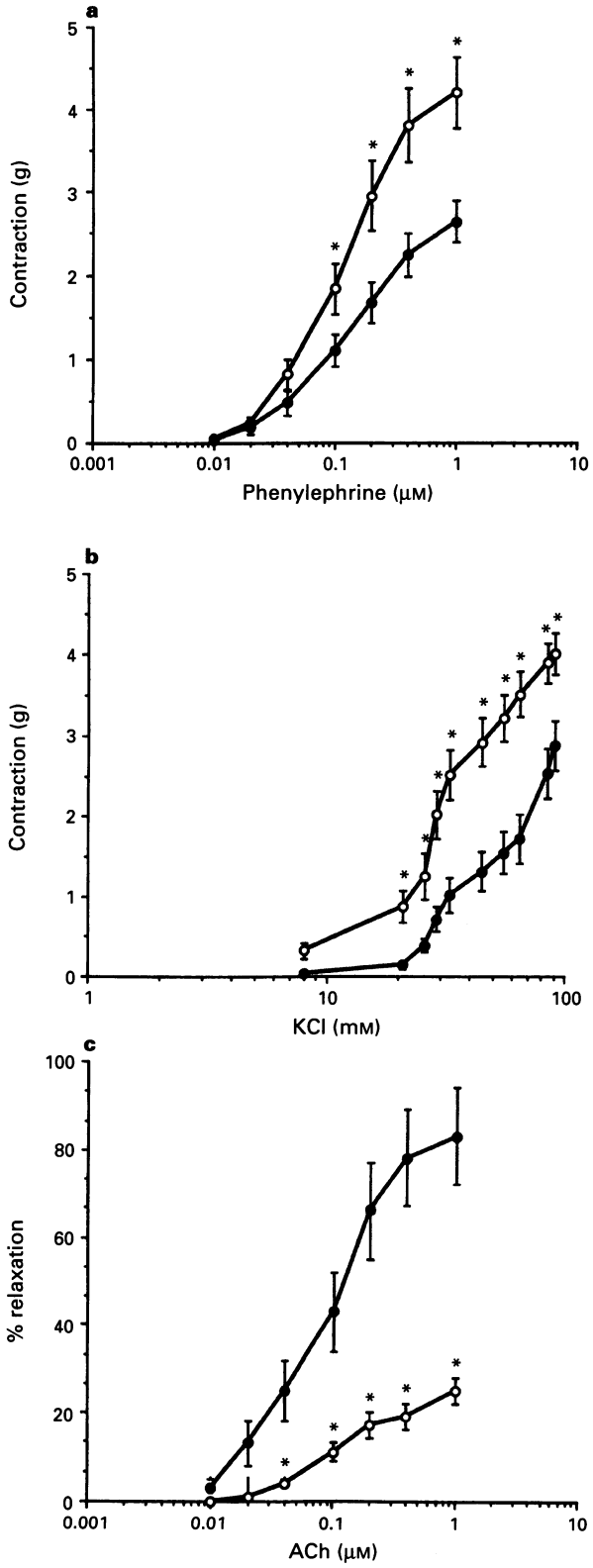

Figure 1 Concentration-response curves to vascular agonists in aortic rings from male and female rabbits. Aortic rings from male (○) or female (●) rabbits were stimulated with increasing concentrations of phenylephrine (a), KCl (b) and acetylcholine (ACh, c). Each curve represents the mean of 9 different experiments; vertical lines show s.e.mean. *$P<0.05$ when compared to rings from female rabbits.

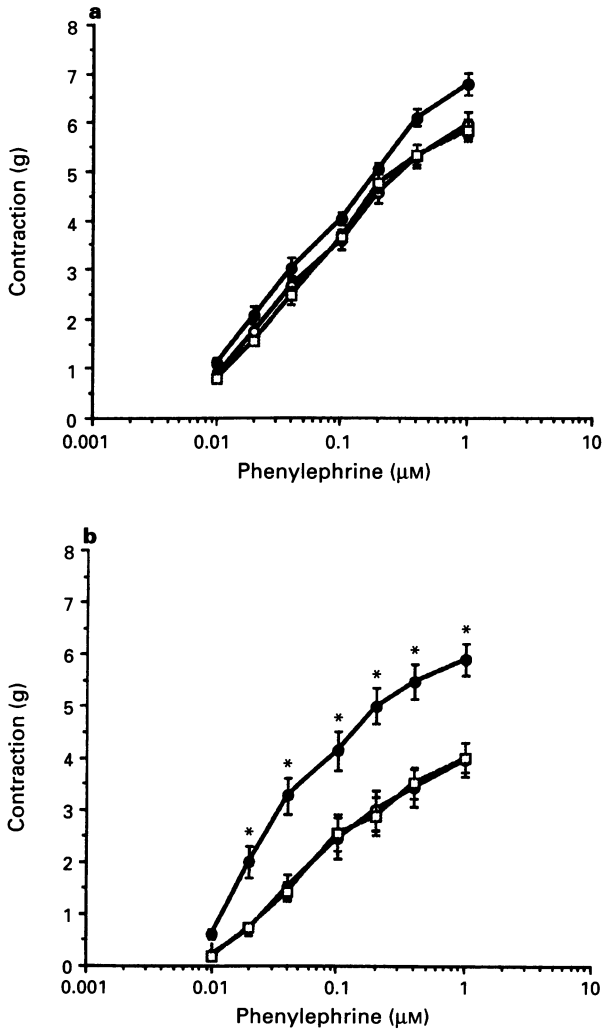

Figure 2 Effect of nitric oxide inhibition on phenylephrine-induced contraction. Aortic rings from male (a) or female (b) rabbits were stimulated with increasing concentrations of phenylephrine in the absence (○) or presence (●) of the nitric oxide synthase inhibitor L-NAME (100 μM). Time related control (□). Each curve represents the mean of 10 different experiments; vertical lines show s.e.mean. *$P<0.05$ when compared to L-NAME.

in the phenylephrine concentration-response curve were observed in the time related control for male or female rabbits (Figure 2a and b). When the area between concentration-response curves (with and without L-NAME) as an index of nitric oxide production, as previously described (Chu & Beilin, 1993), was calculated, it was observed that the area was higher in the case of female rabbits than the area of male rabbits (73 ± 16 g μM^{-1} and 199 ± 31 g μM^{-1} for male and female rabbits, respectively $P < 0.05$). Moreover, when the vasoconstrictor effect of phenylephrine in aortic rings from female vs. male rabbits was compared in the presence of L-NAME, the differences previously observed (Figure 1a) disappeared (Figure 2a and b).

Nitrite production by aortic rings

Thirty min incubation of the aortic rings in Krebs solution resulted in a basal release of nitrites from the aortic rings. The rings from female rabbits released higher amounts of nitrites than the rings from male or ovariectomised rabbits (Figure 3). As compared with the basal values, ACh increased the release of nitrites. Following addition of ACh to the aortic rings, the differential release of nitrites between the various groups tested became more evident (Figure 3). Furthermore, basal or ACh-stimulated release was inhibited $60 \pm 5\%$ by L-NAME.

Effect of ovariectomy on vascular reactivity of aortic rings

In ovariectomised rabbits, blood levels of oestrogen decreased from 47 ± 9 pg ml^{-1} to 27 ± 11 pg ml^{-1}, while testosterone levels remained unchanged (0.3 ± 0.3 pg ml^{-1} versus 0.2 ± 0.02 pg ml^{-1} for control and ovariectomised rabbits, respectively). When aortic rings were obtained from rabbits which had been ovariectomised 2–3 weeks previously, the response of the arteries to phenylephrine was higher than the control female rabbits. The maximal tension, developed upon stimulation with phenylephrine was 3.2 ± 0.3 g and 2.6 ± 0.2 g for ovariectomised and control female rabbits, respectively. Similarly, KCl-induced contractions increased significantly after ovariectomy. The maximal tension developed upon stimulation by KCl (90 mM) was 3.4 ± 0.3 g and 2.8 ± 0.3 g for ovariectomised and control female rabbits, respectively. ACh relaxation decreased significantly in the rabbits that had been ovariectomised. Relaxation produced by ACh in rings from ovariectomised rabbits was $29 \pm 5\%$, compared with $83 \pm 11\%$ relaxation in rings from female rabbits. Thus, these changes in vascular reactivity after ovariectomy eliminated the differences in response between aortic rings from male and female rabbits previously obtained (Figure 1). Therefore, if we compare the effect of phenylephrine, KCl and ACh on aortic rings from male rabbits versus aortic rings from ovariectomised rabbits, no significant differences are seen in the concentration-response curves (Figure 4a,b,c).

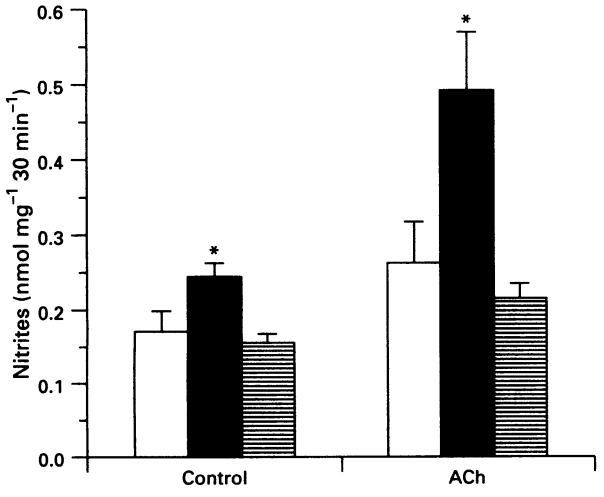

Figure 3 Basal and acetylcholine (ACh)-stimulated release of nitrites production. Aortic rings from male (open columns) female (solid columns) or ovariectomised (hatched columns) rabbits, were incubated with Krebs (control) or ACh (10 μM) for 30 min and nitrites in the solution were measured. Each column represents the mean of 5 different experiments; vertical lines show s.e.mean. *$P < 0.05$ when compared to rings from male or ovariectomised rabbits.

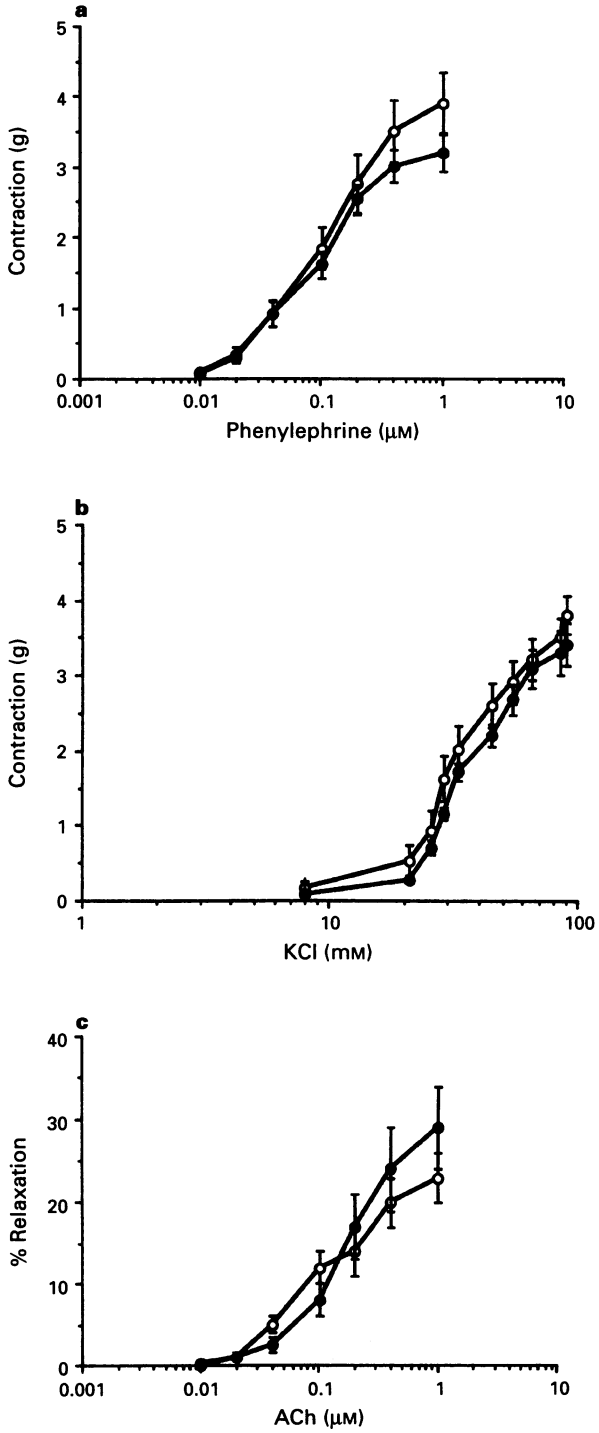

Figure 4 Concentration response curves to vascular agonists in aortic rings from ovariectomised rabbits. Aortic rings from male (○) or ovariectomised (●) rabbits were stimulated with increasing concentrations of phenylephrine (a), KCl (b) or acetylcholine (ACh, c). Each point represents the mean of 9 different experiments; vertical lines show s.e.mean.

Discussion

The present study demonstrates that vascular responses to phenylephrine and KCl are decreased and responses to ACh are increased in aortic rings from female rabbits as compared with responses in aortic rings from male rabbits. The differences in vascular response can be eliminated by either nitric oxide inhibition with L-NAME or ovariectomy of the rabbits. Additionally, we have demonstrated that aortic rings from female rabbits release nitrite in greater amounts than aortic rings from either male or ovariectomised female rabbits.

The precise role of nitric oxide in sex-dependent vascular reactivity differences is not clear. However, considerable evidence suggests that NO synthesis may be regulated by sexual hormones. Recently, Weiner *et al.* (1994) showed that pregnancy caused a significant increase in the activity of calcium-dependent nitric oxide synthase (NOS) which could be inhibited by the oestrogen-receptor antagonist tamoxifen (Weiner *et al.*, 1994). Moreover, 17β-oestradiol treatment, induced eNOS expression and activity in human aortic endothelial cells (Hishikawa *et al.*, 1995). Our primary objective here, therefore, was to compare the vascular responses of aortic rings to different vascular agonists. We selected two vasoconstrictors; phenylephrine a receptor-mediated agonist and KCl a non-receptor-mediated agonist. The results clearly show that vasoconstriction in aortic rings from female rabbits is impaired. This hyporeactivity of the aortic rings from female rabbits seems to be associated with a generalised mechanism, since, it was evident with the two different vasoconstrictor stimuli, receptor and non-receptor mediated. Furthermore, a comparison of ACh-induced relaxation demonstrated that indeed, aortic rings from female rabbits are more sensitive to the vasodilator action of ACh. Thus, these results suggest that vascular tissues from female animals have higher capability to release vasodilator agents, than the vascular tissues from male animals, therefore, impairing the vasoconstrictor tone. Endothelium-derived NO has been shown to be a potent endogenous vasodilator which suppresses vasoconstrictor responses to various vasoactive substances (Palmer *et al.*, 1987; Myers *et al.*, 1989). Our observation, that phenylephrine-stimulated constriction of the aortic rings was enhanced by L-NAME suggests that NO release blunts the response of the aortic rings to phenylephrine from both female and male rabbits. However, the enhancement was higer in aortic rings from female rabbits than male rabbits. Most significantly, L-NAME abolished the differences in aorta reactivity to phenylephrine, previously observed. Since L-NAME treatment specifically inhibits the synthesis of NO (Rees *et al.*, 1990) an increase in NO synthesis and therefore release would appear to be responsible for the decreased sensitivity to phenylephrine and KCl in the aorta from female rabbits.

The NO-mediated decrease in the contractile response to vasoconstrictors, could result from either increased basal or stimulated release of NO. In the present investigation, we observed a significant increase in the ACh-induced relaxation in the aorta from female rabbits compared with that from male rabbits, suggesting that stimulated NO release is higher in the vascular tissue from female rabbits. We also showed that ACh-induced nitrites release was higher in the rings from female rabbits. Moreover, basal release of NO from endothelium intact aortic rings from female rabbits was significantly increased when compared with those from male and ovariectomised rabbits. NO synthesis can be inhibited by L-NAME and changes in vascular tone have been observed as a consequence of this elimination of the NO from the circulation (Rees *et al.*, 1990). Thus, differences in NO formation would, therefore, be reflected in the amount of contraction of the aortic rings in the presence of L-NAME. Addition of L-NAME to the incubation baths of the aortic rings produced a contraction which was significantly greater in magnitude in the aortic rings of female than those from male rabbits. The contractile effect of L-NAME was reversed by the additon of L-arginine, indicating that the effects seen were, indeed, due to inhibition of the synthesis of NO from L-arginine. Thus, these results further support our hypothesis that vascular tissue from female rabbits produces a higher release of NO and this could be responsible for the decreased vascular response to vasoconstrictor agonists.

Ovariectomy decreased circulating oestrogen levels, and NO release from aortic rings in this group of rabbits was significantly attenuated when compared with that seen in ovary-intact rabbits and did not differ from that seen in male rabbits. Furthermore, there were no differences in the responses of aortic rings from male rabbits and ovariectomised rabbits to the vascular agonists tested, confirming that oestrogen levels are important in regulating the release/synthesis of the potent vasodilator, NO. NO could counterbalance the vasoconstrictor effects of phenylephrine or KCl, thereby resulting in an important mechanism for the decreased vascular contraction in the tissue from female rabbits. Although, we did not address the precise mechanism(s) by which oestrogens increase NO release from aortic rings, several possibilities have been suggested, including an increase in NO synthase protein, an increase of cofactors, or decreased inactivation of NO. Recently, Rosseli *et al.* (1994) showed that oestrogen supplementation increased NO production *in vivo*, and that treatment of human aortic endothelial cells with increasing concentrations of 17β-estradiol resulted in an increase in NO release and induction of eNOS protein (Hishikawa *et al.*, 1995). However, further clarification of the exact mechanism by which oestrogen affects protein levels of eNOS is necessary.

In summary, the present study demonstrated differences in vascular reactivity in aortic rings from female and male rabbits. Studies with L-NAME suggested that endogenous NO influences vascular tone and may account for the increased vasodilator tone in the aortic rings from female rabbits, and that increased NO production may be regulated by the circulating oestrogens levels.

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