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Pregnancy reduces noradrenaline but not neuropeptide levels in the uterine artery of the guinea-pig

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Summary. Using histochemical, immunohistochemical and biochemical techniques, noradrenaline-, neuropeptide Y-, vasoactive intestinal polypeptide-, substance P- and calcitonin gene-related peptide-containing nerve fibres were studied in the uterine artery of virgin, progesterone-treated and pregnant guinea-pigs. Morphological changes following hormone treatment or in pregnancy were also evaluated in a quantitative study on semithin sections of the uterine artery. In late pregnancy, the number of noradrenaline-containing nerve fibres, which formed the densest plexus in virgin animals, was significantly decreased, a finding supported by a significant reduction in noradrenaline levels. This reduction was not mimicked by systemic progesterone treatment. In contrast, the innervation of the uterine artery by neuropeptide Y-containing nerve fibres was increased in pregnancy, while the other peptidergic nerves and peptide levels were unchanged after progesterone treatment and in pregnancy. These changes led to a predominance of innervation by neuropeptide Y- rather than noradrenaline-containing nerve fibres in late pregnancy. No morphological changes were detected following progesterone treament, but pregnancy led to a marked increase in the cross-sectional area of the vessel accompanied by an increase in the thickness of the media.

Key words: Pregnancy – Progesterone – Noradrenaline levels – Neuropeptides – Uterine artery – Guinea-pig

It has been reported that noradrenergic nerves disappear from the uterus of several animal species during late pregnancy (Sjöberg 1968; Owman et al. 1975; Alm et al. 1979; Thorbert et al. 1979). By use of electron microscopy (Sporrong et al. 1981), a pregnancy-induced degeneration of uterine noradrenergic nerves has been shown to occur in the guinea-pig. A concomittant decrease of neuropeptide Y (NPY)- and vasoactive intestinal polypeptide (VIP)-immunoreactive nerves and levels has been demonstrated in the uterus of guinea-pigs (Fried et al. 1985) and rats (Stjernquist et al. 1985), respectively. In addition, pharmacological responses of the uterine artery to noradrenaline (NA) are unchanged, while cholinergic responses are increased in late pregnancy (Bell 1968, 1969). It has been reported that transmural nerve stimulation of the uterine artery fails to evoke vasomotor responses in pregnancy (Tare et al. 1988). Progesterone treatment has also been shown to cause a reduction of NA levels in the uterus and uterine artery (Bell and Malcolm 1978).

Since perivascular nerves in the uterine artery contain various neuropeptides, including VIP, NPY, dynorphin and calcitonin gene-related peptide (CGRP), as well as the catecholamine-synthesising enzyme, dopamine β-hydroxylase (Morris et al. 1985, 1987; Uddman et al. 1986), we have investigated the expression of neuropeptides as well as NA in the uterine artery of the guinea-pig in late pregnancy. In addition, the ability of progesterone treatment to mimic the effects of pregnancy was examined. A combination of quantitative histochemical, immunohistochemical and neurochemical techniques was used to investigate nerve distribution and neurotransmitter levels in the uterine artery from control, pregnant and progesterone-treated guinea-pigs.

A quantitative morphological study was also carried out on semithin sections, and all the results were evaluated in the light of the morphological changes that were shown to occur in the uterine artery during late pregnancy.

Materials and methods

The study was carried out on Dunkin-Hartley guinea-pigs. Three groups of animals were used:
(1) virgin guinea-pigs (3–5 months of age);
(2) late pregnant guinea-pigs (about 50 days gestation);
(3) guinea-pigs injected with 5 mg progesterone (Paines & Byrne Ltd., Greenford, UK) i.m. every day, for 45 days.

The treatment with progesterone was started at the end of the oestrus phase (as checked by the presence of leucocytes in vaginal smears); the gestation time was estimated from the crown–rump lengths of the foetuses (Draper 1920). Only bilaterally pregnant animals were used in this study.

Animals were killed by cervical dislocation. The right uterine artery of each animal was taken for biochemical analysis (immediately frozen in liquid nitrogen). The left uterine artery was taken for morphological studies (see below).

Immunohistochemical study

Pieces of tissue, 1 cm in length, corresponding to the uterine horn, were removed, opened longitudinally and stretched

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to their original length and circumference onto sheets of silicone elastomer (Sylgard; BDH Ltd., Poole, UK) with micropins.

To localise noradrenergic nerves, vessels were incubated in 2% w/v glyoxylic acid in 0.1 M phosphate buffer at pH 7.2 for 1.5 h at room temperature (Lindvall and Björklund 1974). During the last 10 min of incubation, the whole-mount stretch preparations were counterstained with 0.5% Pontamine Sky Blue and 0.1% dimethyl sulphoxide in the glyoxylic acid solution to reduce background autofluorescence (Cowen et al. 1985). After drying, vessels were stretched on glass slides to their original size, heated at 100° C for 4 min, and mounted in liquid paraffin.

For immunohistochemistry, whole-mount stretch preparations of the uterine arteries were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1.5 h, dehydrated in 80% ethanol and incubated with 0.1% Triton X-100 in PBS, before incubation with the specific antisera overnight at room temperature.

All the antisera were polyclonal and raised in rabbits: anti-NPY (Cambridge Research Biochemicals, CRB, Cambridge, UK) diluted 1:400; anti-VIP (Peninsula Laboratories Inc., St. Helens, UK) diluted 1:1000; anti-substance P (SP) (CRB, UK) diluted 1:400; anti-CGRP (CRB, UK) diluted 1:400. The vessels were then washed with PBS and incubated with anti-rabbit IgG conjugated with fluorescein isothiocyanate diluted 1:50 for 1 h at room temperature. Before mounting, vessels were stained with 0.05% Pontamine Sky Blue for 10 min (Cowen et al. 1985). The preparations were stretched to their original size on glass slides, mounted in a PBS/glycerol mixture (Citifluor; City University, London, UK) and observed under a Zeiss photomicroscope equipped with epi-illumination.

Measurements

Using an ocular grid and a ×16 Neofluor objective lens, the nerve fibres that intersected an ocular grid line along the opened circumference of each vessel in three different regions were counted. The mean number of nerve fibre intercepts per vessel circumference in the uterine artery of the three groups of animals was compared. Nerve bundles were also examined with a ×40 objective lens in order to distinguish single fibres within the bundles.

Other segments of the uterine arteries were also used: (i) as a control for the specificity of the immunostaining, by incubating them with the antisera preabsorbed with an excess (10^{-3} M) of the appropriate neurotransmitter (SP, VIP, NPY, CGRP; CRB, UK) for 24 h at 4° C prior to use, and (ii) to study regional differences in the density of neurotransmitter-containing nerves along the uterine artery. No differences in the density and pattern of innervation could be detected for any neurotransmitter-containing nerves in that part of the uterine artery corresponding to the uterine horn, in all 3 groups of animals.

Biochemical studies

Noradrenaline assay. NA levels were measured using high performance liquid chromatography with electrochemical detection. The length and weight of the arteries were measured before extraction. Samples were homogenized in 500 µl of 0.1 M perchloric acid containing 0.4 mM sodium bisulphite and 12.5 ng of an internal standard, dihydroxybenzylamine (DHBA). The extraction procedure, slightly modified by the addition of 0.1 mM ethylenediaminetetraacetate (EDTA) in the solution used for washing the alumina, was that of Keller et al. (1976). Chromatography was carried out using a mobile phase consisting of 0.1 M sodium dihydrogen phosphate, 0.1 mM EDTA, 5 mM heptane sulphonate (pH 5.0) containing 10% (v/v) methanol on a µ-Bondapak C-18 reverse phase column (Moyer and Jiang 1978). Detection and quantification was accomplished with a glassy carbon electrode set at a potential of +0.72 V. NA levels were corrected for loss during the extraction procedure using the DHBA internal standard. Results were expressed as nmol/cm length.

Peptide assays. After measuring the length and weight of the arteries, samples were placed in 0.5 M acetic acid (1 ml) in polypropylene tubes in a boiling water bath for 15 min. The samples were homogenized, centrifuged for 30 min at $3500 \times g$ and lyophylized. VIP, NPY, SP and CGRP levels were quantified using an inhibition enzyme-linked immunosorbent assay (Stjernschantz et al. 1982) as previously described (Belai et al. 1985). Results were expressed as fmol of VIP, NPY, SP or CGRP per cm of artery. Samples from control, progesterone-treated and pregnant animals were extracted and assayed together to counteract any interassay variabilities.

Morphological study

Lengths of right uterine arteries (2 cm) were pinned to Sylgard-lined dishes and fixed by immersion in 5% glutaraldehyde in 0.1 M sodium cacodylate buffer. Using a fine syringe, fixative was also passed through the lumen of each vessel. Excess surrounding fat deposits were removed and fixation continued for a total of 2 h. After washing in buffer, the samples were cut into convenient lengths, osmicated (1% osmium tetroxide in cacodylate buffer, 1 h), washed, stained en bloc (1% aqueous uranyl acetate, 1 h), dehydrated in ethanol and embedded in Spurr resin.

Transverse sections (1 µm thick) of the arteries were cut on an LKB ultramicrotome, dried, and mounted directly in DPX or after staining with a methylene-blue/azur-II/basic fuchsin recipe (Humphrey and Pittman 1974).

Counts of the total number of smooth muscle cell nuclei per cross-section were made directly from the stained material, and the mean thickness of the muscle and connective tissue layers of the artery wall was determined from several measurements on each section.

From tracings of photomicrographs at ×560 magnification, areas of the adventitial and medial layers were determined using a digitizing system (Houston Instruments tablet connected to an Apple IIe computer). As the cross-sectional area of the lumen could not be accurately measured, due to contraction of the artery wall to an unknown extent, the length of the convoluted internal elastic lamina was measured. Cross-sectional area of the lumen at maximal dilation could then be considered as that of a circle with a perimeter of this length (Todd et al. 1983).

Although the cross-sectional areas of the adventitia and media will remain constant with dilation of the vessel (Lee et al. 1983) the thickness of these layers will change. To overcome the inconsistencies due to contraction of the samples prior to fixation, thickness measurements were converted to the fully dilated condition.

Statistical analysis

Results were expressed as the mean ± SEM and the three groups of data were compared using analysis of variance followed by Dunnett's test for multiple comparison with a control (Dunnett 1964). A level of probability of 0.05 or less was considered to be significant.

Results

Quantitative analysis of the distribution of NA- and peptide-containing nerves in the uterine artery of virgin guinea-pigs, expressed as the number of nerve fibre intercepts per vessel circumference, showed that NA is present in the highest number of nerve fibres, followed by NPY-, VIP-, CGRP- and SP-like immunoreactive (LI) nerve fibres in descending order (Table 1).

Table 1. Innervation of the uterine artery in virgin, progesterone-treated and pregnant animals expressed as number of nerve fibre intercepts per vessel circumference ± SEM. The number of animals is given in brackets; $*p<0.05$ virgin versus pregnant; $**p<0.01$ virgin versus pregnant

Neuro-transmitter	Virgin	Progesterone-treated	Pregnant
NA	78.27 ± 5.16 (8)	82.60 ± 5.51 (6)	54.58 ± 4.35** (6)
NPY-LI	73.10 ± 2.42 (11)	74.35 ± 7.45 (4)	90.08 ± 6.68* (6)
VIP-LI	68.83 ± 2.60 (11)	70.17 ± 2.17 (5)	65.17 ± 6.47 (6)
CGRP-LI	58.52 ± 2.57 (6)	51.44 ± 3.18 (5)	50.39 ± 2.57 (6)
SP-LI	31.73 ± 1.50 (13)	32.82 ± 1.75 (5)	33.98 ± 2.39 (7)

Treatment with progesterone had no effect on any of the parameters studied of the perivascular nerves; however, the pregnant guinea-pigs had a significant reduction in the number of catecholamine-fluorescent nerve fibre intercepts per vessel circumference ($p<0.01$, Table 1) and a significant increase in the numbers of NPY-LI nerve fibre intercepts per vessel circumference ($p<0.05$, Table 1) (Fig. 1).

No differences were observed in the pattern of innervation by VIP-, SP- and CGRP-containing nerve fibres in virgin and pregnant guinea-pigs (Table 1, Fig. 2); this resulted in a relative predominance of NPY- and VIP-containing nerve fibres in the uterine artery in late pregnancy (see Table 1). Data from the biochemical assay for NA showed a marked reduction of NA content in the pregnant uterine artery ($p<0.01$, Fig. 3). The changes in NPY content during progesterone treatment and pregnancy were not significant (Fig. 3).

Measurements of the weight per cm of the uterine artery of pregnant guinea-pigs showed a significant increase ($p<0.001$) when compared with control and progesterone-treated animals (Fig. 4). Results from the morphological studies (Table 2, Fig. 5) showed that there were no statisti-

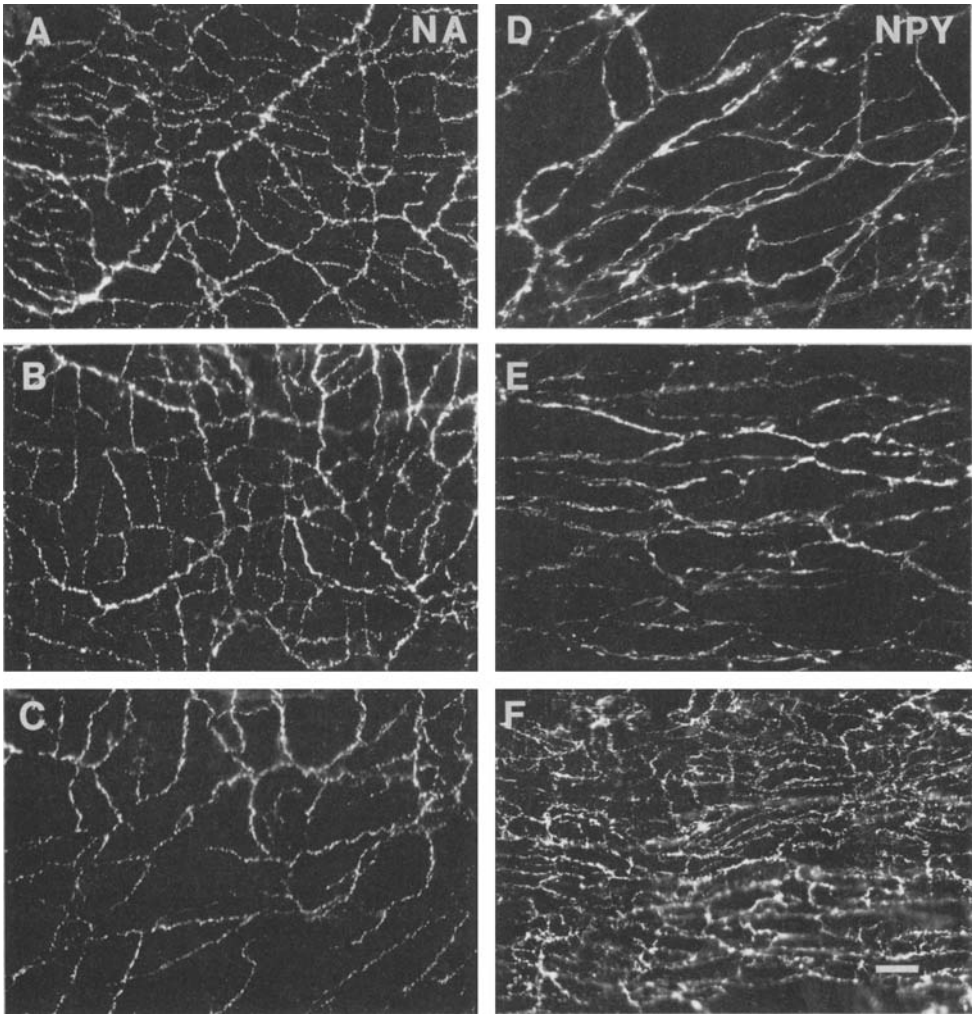

Fig. 1 A–F. Fluorescent micrographs of NA- (**A–C**) and NPY- (**D–F**) containing nerves in the uterine artery from virgin (**A, D**), progesterone-treated (**B, E**) and pregnant (**C, F**) guinea-pigs. Note the marked decrease of catecholamine fluorescence in pregnancy. *Calibration bar*: 50 μm

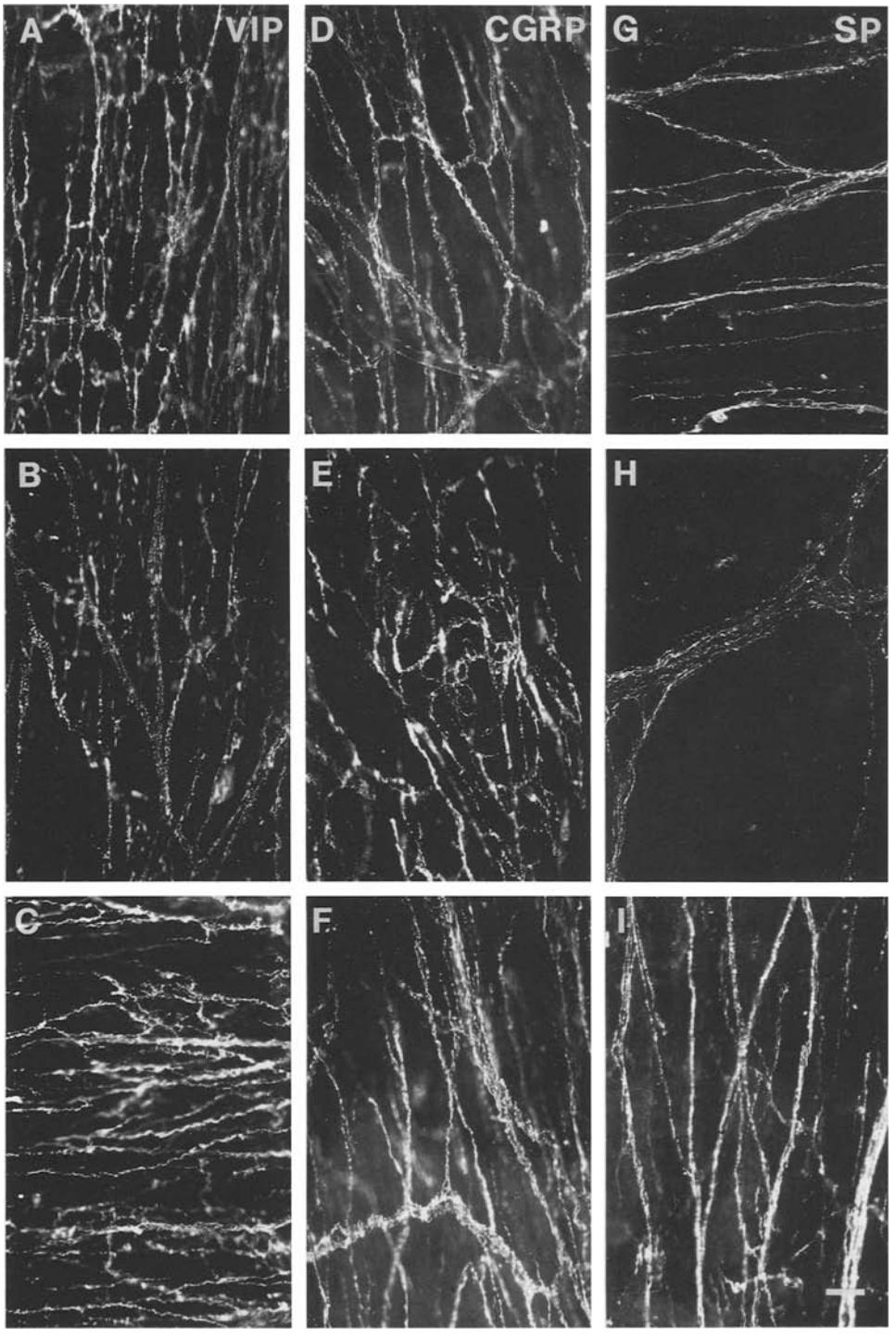

Fig. 2 A–I. Fluorescent micrographs of VIP- (**A–C**), CGRP- (**D–F**) and SP- (**G–I**) containing nerves in the uterine arteries from virgin (**A, D, G**), progesterone-treated (**B, E, H**) and pregnant (**C, F, I**) guinea-pigs. *Calibration bar*: 50 μm

cally significant changes in the uterine arteries of progesterone-treated animals compared with controls, for any of the parameters measured. Pregnancy, however, led to marked changes. The cross-sectional area of the lumen of arteries during pregnancy was increased by nearly three-fold, with the media increasing in size to a greater extent than that required simply to maintain the same thickness of muscle around the lumen. Although there was also a significant increase in the number of smooth muscle cells in the media during pregnancy, cell density (expressed as number of cells/1000 μm^2), in fact, decreased considerably (Table 2).

The surrounding adventitial layer increased substantially in cross-sectional area during pregnancy, but always remained the same thickness (Fig. 5). Comparison of the luminal areas, recalculated to simulate the condition of an internal elastic lamina forming a perfect circle, showed large differences in late pregnancy. The luminal area of uterine arteries during pregnancy increased by 157%, i.e. 2.5 times greater than controls (Table 2, Fig. 6).

Discussion

The quantitative analysis carried out in this study on guinea-pig uterine arteries demonstrated that NA-containing nerve fibres formed the densest perivascular plexus, compared with NPY-, VIP-, SP- and CGRP-LI nerve fibres.

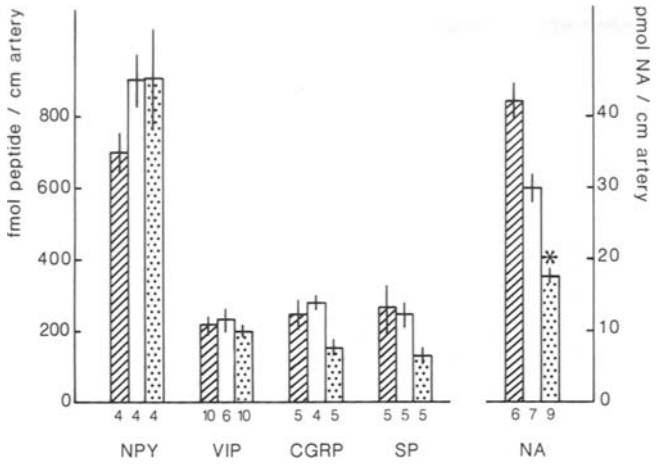

Fig. 3. Histogram of neurotransmitter levels in the guinea-pig uterine artery, expressed as fmol (peptides) or pmol (NA) per cm of artery. *Cross-hatched bars* controls, *clear bars* progesterone treated, *spotted bars* pregnant. The number of animals used in each group is given under each bar and the results given as the mean ±SEM; $*p<0.01$ virgin versus pregnant

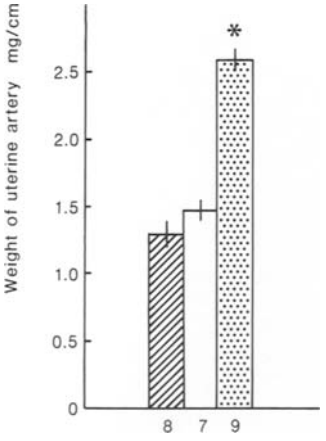

Fig. 4. Weight of the guinea-pig uterine artery per cm of vessel. *Cross-hatched bar* controls, *clear bar* progesterone treated, *spotted bar* pregnant. The number of animals for each group is given under each bar and the results given as the mean ±SEM; $*p<0.01$ virgin versus pregnant

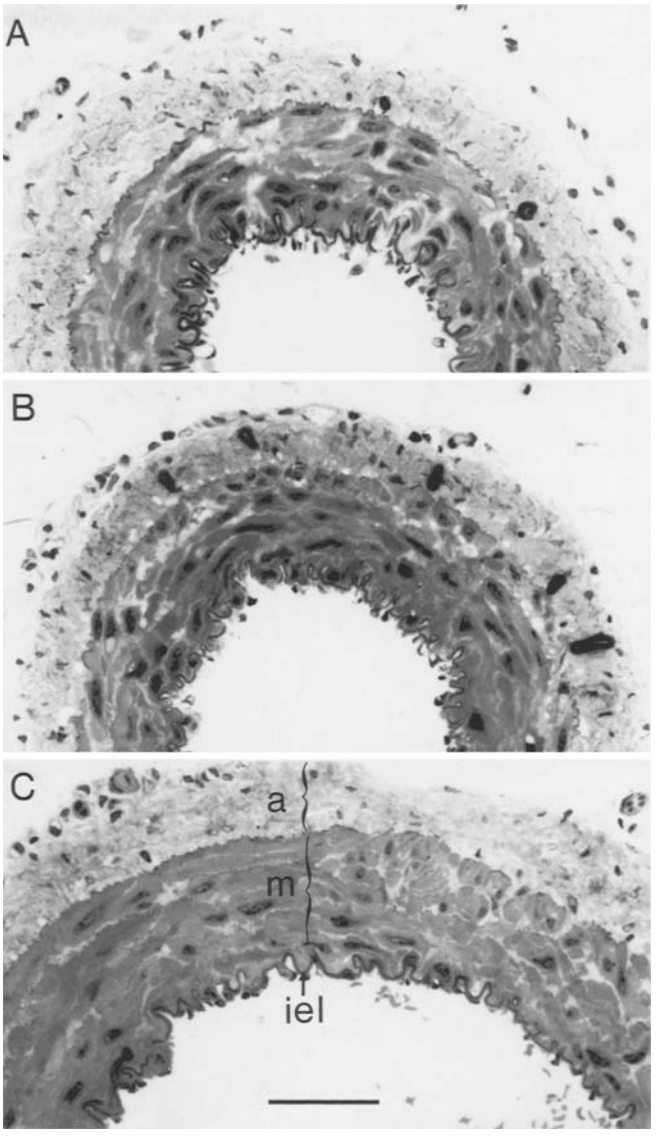

Fig. 5A–C. Transverse sections (1 μM) of the uterine artery from virgin (**A**), progesterone-treated (**B**) and pregnant (**C**) guinea-pigs; *a* adventitia; *m* media; *iel* internal elastic lamina. *Calibration bar*: 50 μm

The action of NA in virgin as well as pregnant uterine arteries has been studied in detail previously (Bell 1968; Tare et al. 1988).

NPY, which often coexists with dopamine β-hydroxylase and tyrosine hydroxylase in noradrenergic nerves in the uterine artery (Morris et al. 1985, 1987), causes a long-lasting vasoconstriction, similar to NA, when infused in the isolated uterine artery of virgin guinea-pigs (Morris et al. 1985; Morris and Murphy 1988). However, in the uterine artery, NPY-like immunoreactivity is also localized in non-noradrenergic axons containing the vasodilator neurotransmitter VIP, and/or with somatostatin- or dynorphin-like immunoreactivity (Morris et al. 1985, 1987). VIP and SP are potent vasodilators of the uterine vasculature (Clark et al. 1981; Ottesen and Fahrenkrug 1981; Gram and Ottesen 1982) and the same function can be attributed to CGRP, which is one of the most potent vasodilator neurotransmitters (Girgis et al. 1985). SP and CGRP are contained in sensory nerve fibres in the uterine artery, since both are depleted by capsaicin treatment (Gibbins et al. 1985).

In late pregnancy, the marked decrease of NA-containing nerve fibres is associated with changes in the pattern of the peptidergic innervation of the guinea-pig uterine artery. Our finding that in the uterine artery there is decreased noradrenergic innervation but increased number of NPY-LI nerve fibres during pregnancy, contrasts with the pregnancy-induced changes in the uterus itself, where substantial decreases in NA content and noradrenergic nerve fibres occurring at the end of pregnancy (Owman et al. 1975; Sporrong et al. 1981) are associated with a decrease of NPY-containing (Fried et al. 1985) and VIP-containing nerve fibres (Stjernquist et al. 1985). Moreover, the changes in the pregnant uterus are more extensive, in that there is a complete disappearance of noradrenaline (Sjöberg 1968) and degeneration of noradrenergic nerve terminals

Table 2. The effect of pregnancy and progesterone treatment on the morphology of the uterine artery. Results are the mean of five animals for each group ±SEM; $^{**}p<0.01$ virgin versus pregnant; $^{*}p<0.05$ virgin versus pregnant

Parameter	Virgin	Progesterone-treated	Pregnant
No. nuclei/ cross-section	88.0 ±4.2	94.6 ±4.4	141.7 ±6.0**
Cell density/ 1000 μm²	3.58±0.39	3.27±0.32	2.03±0.16**
Media area $\times 10^{-3}$ μm²	25.6 ±2.7	30.1 ±3.5	72.0 ±7.2**
Media thickness (μm)	19.2 ±1.59	22.4 ±1.74	30.8 ±3.48*
Adventitia area $\times 10^{-3}$ μm²	25.1 ±1.6	27.8 ±2.0	46.2 ±4.9**
Adventitia thickness (μm)	17.6 ±1.07	19.6 ±1.16	20.2 ±1.93
Lumen area (dilated) $\times 10^{-3}$ μm²	125.0 ±8.1	125.9 ±11.7	321.4 ±27.8**

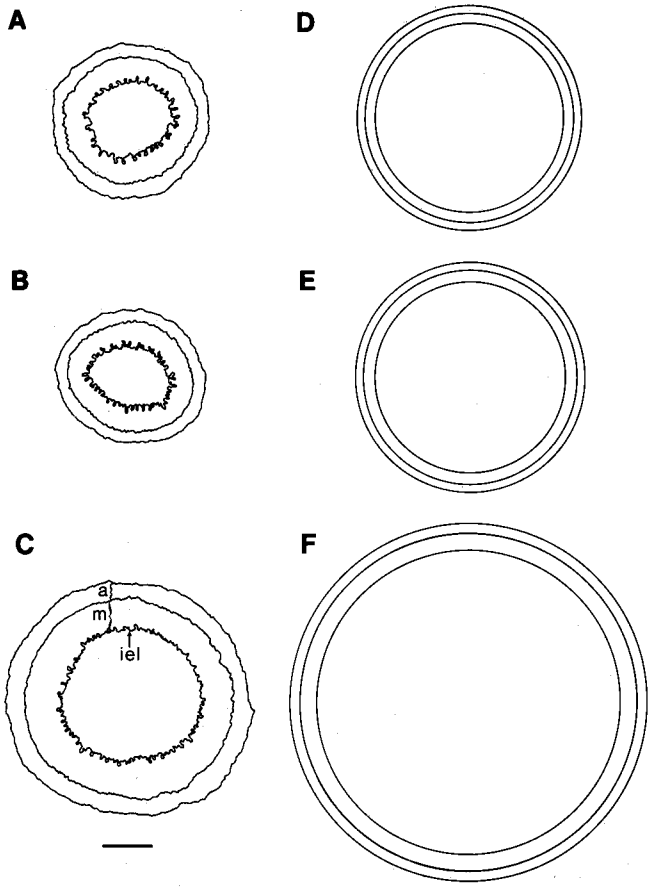

Fig. 6A–F. Tracings of typical transverse sections (**A, B, C**) of the uterine artery from virgin (**A, D**), progesterone-treated (**B, E**) and pregnant (**C, F**) guinea-pigs, and after expanding the internal elastic lamina (*iel*) to form a perfect circle (**D, E, F**) without changing the areas of the media (*m*) or adventitia (*a*). *Calibration bar*: 100 μm

is seen (Sporrong et al. 1981); this does not occur in the pregnant uterine artery (Cavanagh et al. 1988). Further studies are in progress in our laboratory to establish whether the decrease of noradrenaline in ultrastructurally intact sympathetic nerve fibres can be attributed to changes in synthesis, uptake or some other mechanisms.

Our data, showing unchanged noradrenergic innervation and NA levels in the uterine artery of progesterone-treated animals, indicate that systemic treatment with progesterone, unlike the direct application of the hormone (Bell and Malcolm 1978), was unable to affect noradrenergic nerves supplying the uterine artery.

Of major interest is the finding of an increase in the number of NPY-LI nerve fibres in perivascular uterine axons in late pregnancy, despite the decrease of NA levels and noradrenergic nerve density. The presence of NPY-like immunoreactivity in non-noradrenergic axons supplying some vascular beds (Morris et al. 1985; Gibbins and Morris 1988) would explain the lack of a disappearance of NPY-like immunoreactivity after chemical or surgical sympathectomy in the guinea-pig uterine artery and cerebral vessels, respectively. In the pregnant uterine artery, it is possible that the expression of the potent vasoconstrictor NPY increases in the perivascular nerve fibres as a consequence of the reduced availability of NA. Further studies are needed to establish whether the production of NPY increases in noradrenergic axons, which have decreased levels of NA, or whether it occurs in non-noradrenergic nerve fibres, where NPY coexists with the vasodilator neurotransmitter VIP (Morris et al. 1985).

Great care was taken in the analysis of nerve density and transmitter content to ensure that the changes detected reflect true changes in the nerve and do not represent a false impression as a consequence of muscle hypertrophy or distention. The influence of hypertrophy and distention on biochemical, histochemical and immunohistochemical results has been discussed previously (Lincoln et al. 1984). There was an increase in the number of smooth muscle cells observed in pregnant animals. In contrast, cell density, expressed as number of nucleate cells/1000 μm², showed a marked reduction during pregnancy. These values probably provide a more correct evaluation of the increase in the size of smooth muscle cells and lend support to the hypothesis that both hyperplasia and hypertrophy may contribute to the increase in the cross-sectional area and thickness of the muscular layer in uterine arteries in pregnancy. It is for this reason that the data for the innervation were expressed per vessel circumference rather than per unit surface area, and transmitter content was expressed per cm vessel length rather than per g wet weight tissue.

At this stage it is not possible to determine what effect the morphological changes in the muscle during pregnancy could have on neuromuscular transmission in the uterine artery. However, the findings of the present study provide evidence for a shift in the balance of the innervation of the uterine artery from a situation where nerves mediating vasoconstriction (NA-containing nerve fibres) predominate, to one where peptidergic nerves with multiple functions are dominant.

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