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Important differences in nitric oxide synthase activity and predominant isoform in reproductive tissues from human and rat

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Abstract

For the extrapolation of data obtained from experimental animals to the human situation, it is important to know the similarities and differences between human and animal species. Some important characteristics of nitric oxide synthase (NOS) in myometrium and vagina from human and rat were compared. NOS-activity was measured by the formation of ^{14}C-citrulline from ^{14}C-arginine and the expression of NOS isoforms was examined by Western blotting. NOS activity in human uterus and vagina was significantly lower than in the tissues from rat. In contrast to the rat where NOS activity was predominantly found in the cytosolic fractions, NOS activity in particulate and cytosolic fractions from both human myometrium and vagina was similar. Data from Western blots confirmed that eNOS and nNOS isoforms were concentrated in the particulate and cytosolic fractions, respectively. Estrogen treatment of rats resulted in a down regulation of uterine cytosolic NOS activity. A down regulation of NOS in the cytosolic fraction was also seen in the human pregnant myometrium as compared with the nonpregnant myometrium. The vaginal NOS activity was considerably higher than the uterus in both species. In spite of some clear-cut qualitative and other differences between human and rat tissues, there are some interesting similarities. Downregulation in pregnancy of human uterine NOS is probably due to, at least in part, the influence of estrogen and progesterone.

Background

During the past decade there has been a tremendous interest in nitric oxide (NO), which has now come to be accepted as an important mediator of multiple cellular function including smooth muscle relaxation. In living cells, NO is synthesized from L-arginine via the catalytic action of the enzyme, NO synthase (NOS). Three types of enzymes have been identified and characterized. Two of the three are constitutive and expressed in specific cell types (NOS I or neuronal and NOS III or endothelial), whereas the expression of the third isoform (NOS II or inducible) can be induced by cytokines.

In the area of human reproduction, there is now considerable evidence albeit not conclusive suggesting that NO-cGMP system plays an important role in uterine and feto-placental blood flow, quiescence of myometrium and ripening of cervix [1,2]. Evidence that NO has an important role in myometrial function comes largely from studies showing that NO has a relaxing effect on the myometrium and this relaxation is specifically blocked by inhibitors of NOS [3,4]. NO exerts its smooth muscle relexant effect by stimulating soluble guanylate cyclase, thereby increasing cyclic GMP [1–4].

Several recent studies show the presence of NOS in the uterus from animal species [5–10]. However, there appear to be differences with respect to the predominant form, subcellular localisation, and Ca^{2+} dependence in different species. These may be due to real differences in species, hormonal status of the animal, or methodology employed or a combination of these.

We have reported that in the non-pregnant rabbit uterus, cytosolic NOS was the predominant form and that NOS activity in both cytosolic and particulate fractions was highly Ca^{2+}-dependent indicating the presence of constitutive NOS [9]. We also demonstrated for the first time that under well-controlled conditions, there is a clear down-regulation by estrogen of rabbit uterine and vaginal NOS [9,10]. Interestingly, the down-regulation was seen in cytosolic NOS, which was the predominant form, and not particulate NOS. Similar findings were recently reported in the female rat reproductive organs [11]. Interestingly, in both rabbits and rats NOS activity was considerably higher in the vagina than uterus.

There are several published studies in which NOS activity was characterised in uterine tissue from animal species, but there is relatively little information on human uterus and practically none in human vaginal tissue. Although considerable evidence suggests that NO plays an important role in the regulation of myometrial activity, particularly in maintaining uterine quiescence, the role of NO in vaginal physiology is not known.

In our preliminary observations in human reproductive tissues, we found NOS activity to be very low compared with the rabbit or the rat tissues. In the present study, we have partially characterized NOS activity in human myometrium and vagina and have made qualitative and quantitative comparison with the data obtained in the rat tissues. In addition, to judge the extent to which sex steroids and pregnancy can influence the NOS activity, uterine tissue from both pregnant and non-pregnant women and rat tissues from ovariectomized and estrogen treated animals were also examined.

Material and Methods

Materials

All chemicals were obtained commercially from the following suppliers. From Sigma-Aldrich (Stockholm, Sweden): Reduced β-nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), calmodulin, tetrahydro-L-biopterin (BH_4), N^G-methyl-L-arginine, aprotinin, dithiothreitol (DTT), leupeptin and trypsin inhibitor. From Bio Rad Laboratories AB (Sundbyberg, Sweden): running buffer, sample buffer, polyvinylidene difluoride membrane (PVDF), dry milk and ready gels (7.5%, 50 μl). Standard kaleidoscope was used as a size marker for Western blot. From Amersham Pharmacia Biotech AB (Uppsala, Sweden): ECL-kit and Hyperfilm ECL. From Merck Eurolab (Stockholm, Sweden): Whatman filters. From Scandinavian Diagnostic Services (Falkenberg, Sweden): Cruz Marker MW standard, primary polyclonal eNOS (Santa Cruz sc-654) and nNOS (Santa Cruz sc-648) antibodies and anti-rabbit IgG-HRP (Santa Cruz sc 2030).

Tissues from women

The study was approved by the local Ethics committee (University Hospital, Lund, Sweden) and informed consent was obtained from all patients.

Non-pregnant myometrium and vaginal tissues were obtained from 11 women (55–65 years) in postmenopause. Tissue samples were taken from anterior wall of the uterus and anterior vaginal wall. Four patients had a second degree of prolaps and underwent vaginal hysterectomy and prolaps plasty. Five patients underwent only prolaps plasty. The remaining two patients were operated by total hysterectomy and salpingooophorectomy (ovarian diseases). Four patients had received a low dose of estriol (1 mg/day).

Pregnant myometrial tissues from the upper margin of the transverse section in the lower uterine segment were obtained from 14 women. All patients underwent elected Caesarean section delivery. Five women had labour with breech presentation, whereas another five due to personal apprehension preferred to deliver by Caesarean section. Among the remaining four patients two had fetal macrosomia, one had placenta praevia and one had vaginal cyst. The incised tissues were rinsed with ice-cold saline and frozen by immersion in liquid nitrogen and stored at -80°C until used (within 3 weeks) for the experiment.

Tissues from rats

Using Xylacin®, 10 mg/kg (im) and Ketamin® 90 mg/kg (im) as anaesthesia, mature female Sprague Dawley rats weighing 150 – 200 g were bilaterally ovariectomized at least 10 days before any further treatment. They were divided into two groups consisting of 10 animals in each group. While the control group (C) received no further treatment, the rats in estrogen (E_2)-treated group were given an injection (im) of 2 mg/kg polyestradiol phosphate (Estradurin, Pharmacia Upjohn) dissolved in saline solution. Data from a previous study in rabbits have shown that a single injection of polyestradiol phosphate maintains a steady and high level of plasma estradiol for at least 10 days [12]. The animals were killed after 7 days. The uterus and vagina were removed and placed in ice-cold saline solution. After excess fat and connective tissue had been trimmed, the tissues were washed with the saline solution, blotted dry and then weighed. Tissues were then

immediately frozen on dry ice and stored frozen at -80°C, until used (within 3 weeks) for the experiments.

Measurement of NOS activity

After thawing on ice, the tissues were homogenised in approximately 10 vol. of buffer containing 320 mM sucrose, 10 mM HEPES buffer (pH 7.5), 0.1 mM EGTA, 1 mM DTT, 10 μg/ml trypsin inhibitor, 10 μg/ml leupeptin and 2 μg/ml aprotinin with a Polytron homogenizer for four periods of 15 s (per gram tissue) with intermittent cooling pauses of 20 s. The homogenate was centrifuged at 1000 g for 10 min and the pellet was discarded. The supernatant was filtered through one layer of gauze and centrifuged at 40 000 g for 45 minutes and the pellet and supernatant were saved as particulate and cytosolic fractions, respectively.

NOS activity was determined immediately following the preparations of subcellular fractions by measuring the formation of [^{14}C]-L-citrulline from [^{14}C]-L-arginine essentially according to the procedure previously described (5,6). Incubation of cytosolic fraction (40 – 60 μg protein) with 100 μl of reaction mixture containing 50 mM K-phosphate (pH 7.5), 1.2 mM $MgCl_2$, 0.24 mM $CaCl_2$ (or 0.01 mM for human tissues), 50 mM valine, 0.1 mM NADPH, 1 mM citrulline, 2 μM FAD, 2 μM FMN, 10 μM BH_4 (or 50 μM for human tissues), 50 IU/ml calmodulin, 20 μM arginine and 0.6 – 0.8 μM [^{14}C] arginine (sp. Act 0.32 Ci/mmole). Samples were incubated for 20 min at 28°C. Reaction was terminated by removal of substrate and dilution by addition of 1.5 ml of 1:1 (v/v) pre-washed resin AG50W-X8, in ice-cold water, and 5 ml of distilled water. After 10 min, 2 ml of the supernatant was removed and radioactivity measured for quantification of [^{14}C] citrulline by liquid scintillation spectrometry. In preliminary experiments endogenous arginine was removed by passage of cytosol over a column (1 ml) of AG50W-X-8 resin. No significant difference between these or untreated cytosol was observed.

Calculation of NOS activity

Specific NOS activity in pmol/mg protein/min is reported as the difference between the activity (total) and activity measured in a medium lacking $CaCl_2$ and containing 1 mM N^G-methyl-L arginine and 1 mM EGTA (non-specific). Calcium independent activity was the activity measured in the incubation mixture lacking $CaCl_2$ and containing 1 mM EGTA. Protein concentration in the subcellular fractions was determined by the method of Peterson [13]. Data are expressed as mean ± SEM. Comparisons were done by using unpaired student's *t*-test.

Western blots

Aliquots of 50 μl of supernatant or particulate fraction were diluted 1:2 in electrophoresis sample buffer (62.5 nM Tris-HCL, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue and 5% β-mercaptoethanol) to yield a protein equalling to 10 μg/cell and 30 μg/cell for eNOS and nNOS analysis, respectively. The samples were reduced by boiling for 5 min. Human placenta and rat cerebral cortex were used as a positive controls for eNOS and nNOS, respectively. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a 7.5% polyacrylamide gel at 200 V for 30 min.

After electrophoretic transfer to a PVDF membrane at 10–15 V for 30 min, blocking with dry milk diluted in TBS-Tween was carried out over night at 4°C. The blots were then incubated with primary polyclonal antibodies diluted in TBS-Tween dry milk (eNOS 1:2000 and nNOS 1:1000) for 1 h at room temperature. The blots were rinsed in TBS-Tween for 1 × 15 min followed by 3 × 5 min and incubated with secondary anti-rabbit IgG-HRP diluted in TBS-Tween (1:10 000) for 1 h at room temperature. The blots were rinsed as above and the subjected to enhanced chemiluminescence (ECL) using the Western blot detection system. Autoradiographic film was applied to the blot until satisfactory exposure was obtained.

Results

Figure 1 shows NOS activity in both particulate and cytosolic fractions prepared from human non-pregnant and pregnant myometrium. Both total and Ca-independent activities are shown. NOS activity in particulate fractions was higher than in the cytosol for both types of tissues, but the difference only in case of pregnant myometrium was statistically significant ($p < 0.05$). Ca-independent activity in cytosolic fraction was very low in both types of tissues (< 10% of total), but it was substantial in the particulate fractions particularly in the pregnant myometrium as it was close to 50% of the total activity. The corresponding figure for the non-pregnant myometrium was < 15%. The activities in both cytosolic and particulate fractions from the non-pregnant myometrium were significantly higher than the pregnant myometrium indicating a downregulation during pregnancy. The statistical significance of the differences being $p < 0.005$ and $p < 0.05$ for cytosolic and particulate fractions respectively.

A comparison of the myometrial activity between human and rat is shown in figure 2. Although there were no noteworthy difference in NOS activities in particulate fractions of all tissues examined, the cytosolic NOS in the rat, which in contrast to the human is the predominant form, was 4–5-fold higher than the human tissues. This was true for comparisons of both non-pregnant human myometrium with ovariectomized rat myometrium ($p < 0.01$) and pregnant human myometrium and estrogenised rat myometrium ($p < 0.005$). More interestingly, however, was the downregulation in the cytosolic fractions in the pregnant

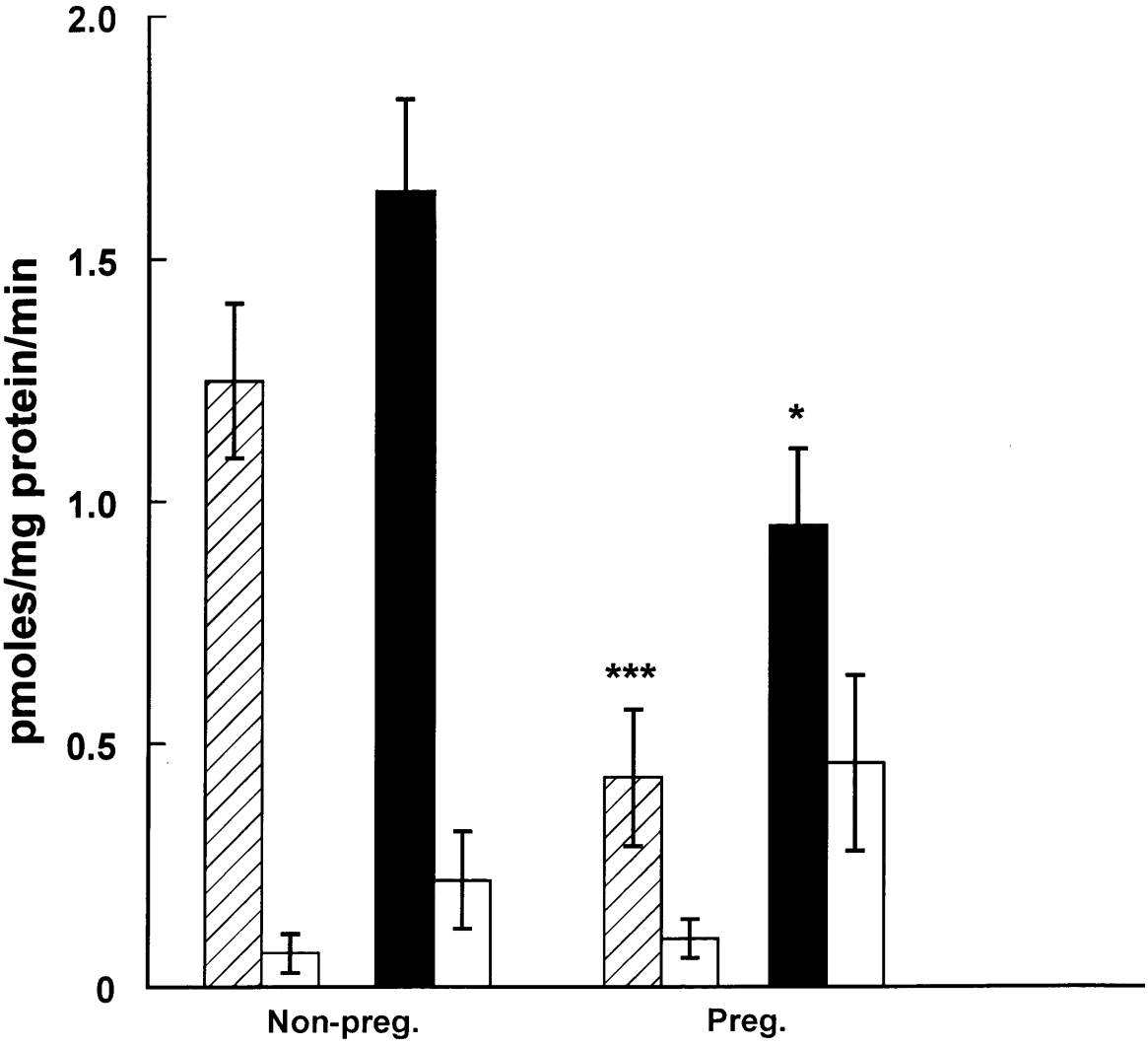

Figure 1
NOS activity in cytosolic (hatched bars), particulate (solid bars) and Ca-independent (empty bars), fractions isolated from non-pregnant and pregnant human myometrium. Values are means ± SEM. Significance of differences between non-pregnant and pregnant myometrium is shown by *($p < 0.05$) and ***($p < 0.005$).

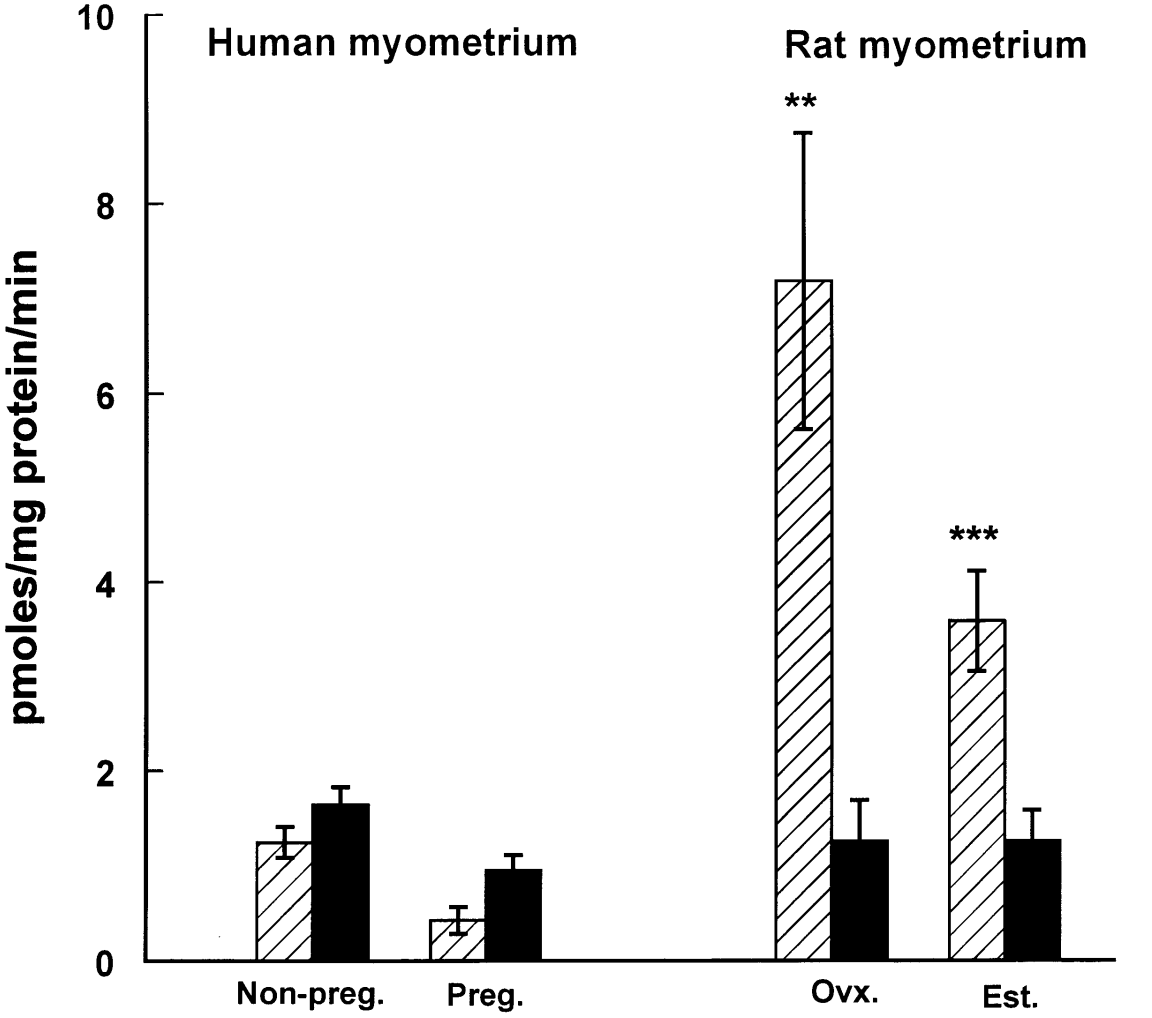

Figure 2
NOS activity in cytosolic (hatched bars) and particulate (solid bars) from non-pregnant and pregnant fractions isolated from human pregnant myometrium and from ovariectomised control (Ovx) and estrogen treated (Est) rat myometrium. Values are means ± SEM. Significance of differences between non-pregnant human and ovariectomized rats is shown by ($p < 0.01$), and between pregnant human and estrogenized rat is shown by ($p < 0.005$).

human myometrium as compared with the non-pregnant myometrium, which was also seen in cytosolic fraction of rat myometrium following estrogen treatment. The differences in both cases were statistically significant (human, $p < 0.005$ and rat, $p < 0.05$).

The data in Figure 3 show activity in myometrium and vagina from non-pregnant women and ovariectomized rats. The NOS activity in both the vaginal and myometrial cytosolic fractions from rat was 4–5-fold higher than the corresponding fractions from human tissues and in either

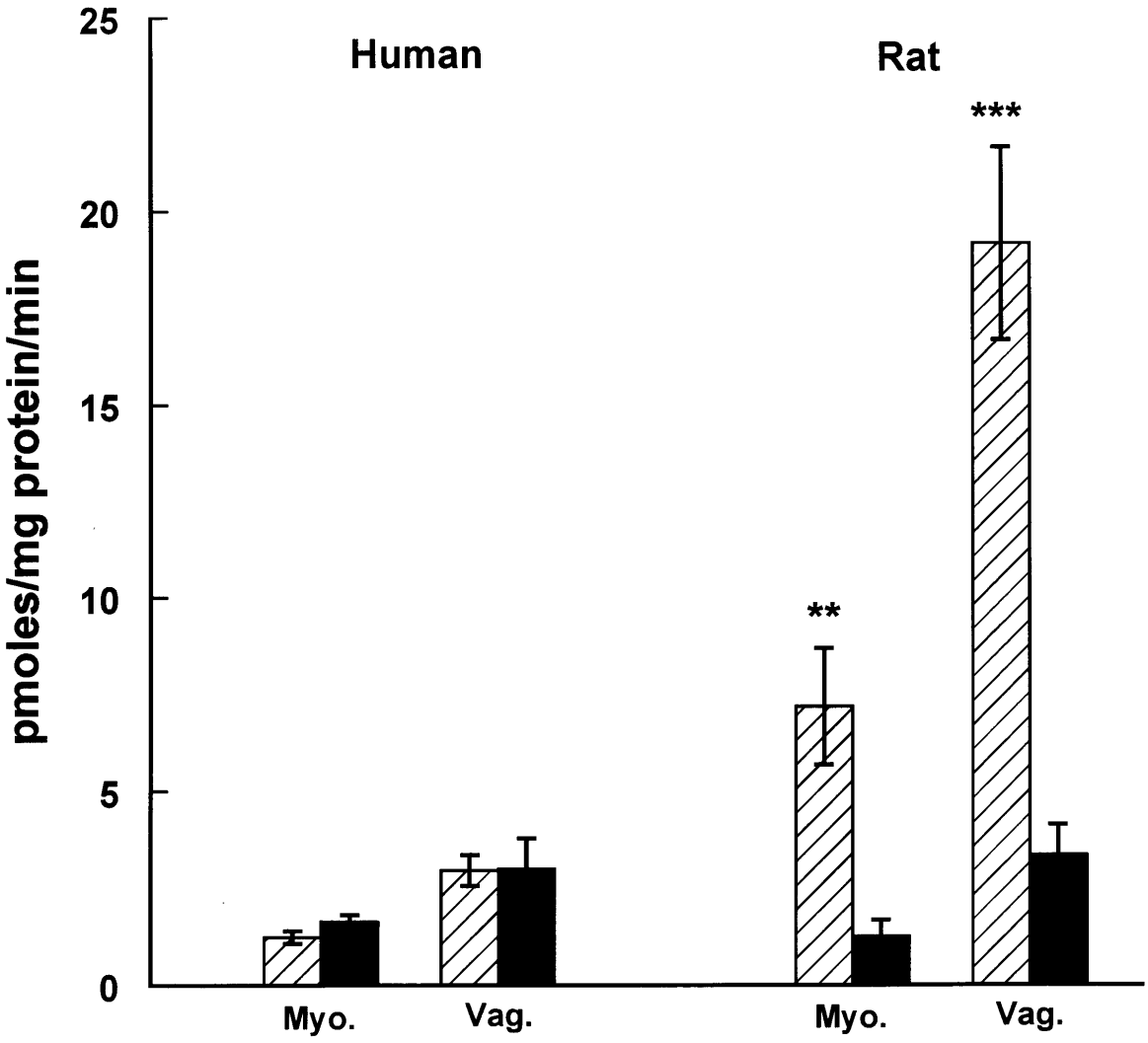

Figure 3
NOS activity in cytosolic (hatched bars) and particulate (solid bars) fractions isolated from non-pregnant human myometrium (Myo) and vagina (Vag), and rat ovariectomised rat myometrium and vagina. Values are means ± SEM. Significance of differences between human and rat tissues is shown by **($p < 0.01$) and ***($p < 0.005$).

species cytosolic NOS in vagina was at least 2-fold higher than myometrium ($p < 0.005$ in both species). NOS activity in vaginal particulate fraction from rat, but not human was also higher than the corresponding fraction from the myometrium ($p < 0.05$).

Western blotting was performed on isolated fractions used for the measurement of NOS activity rather than the whole tissue lysates. Data on Western blot analysis using eNOS antibody are shown in Figure 4. The eNOS antibody reacted with a 133 kDa protein from human placenta villi (positive control), corresponding to the size of

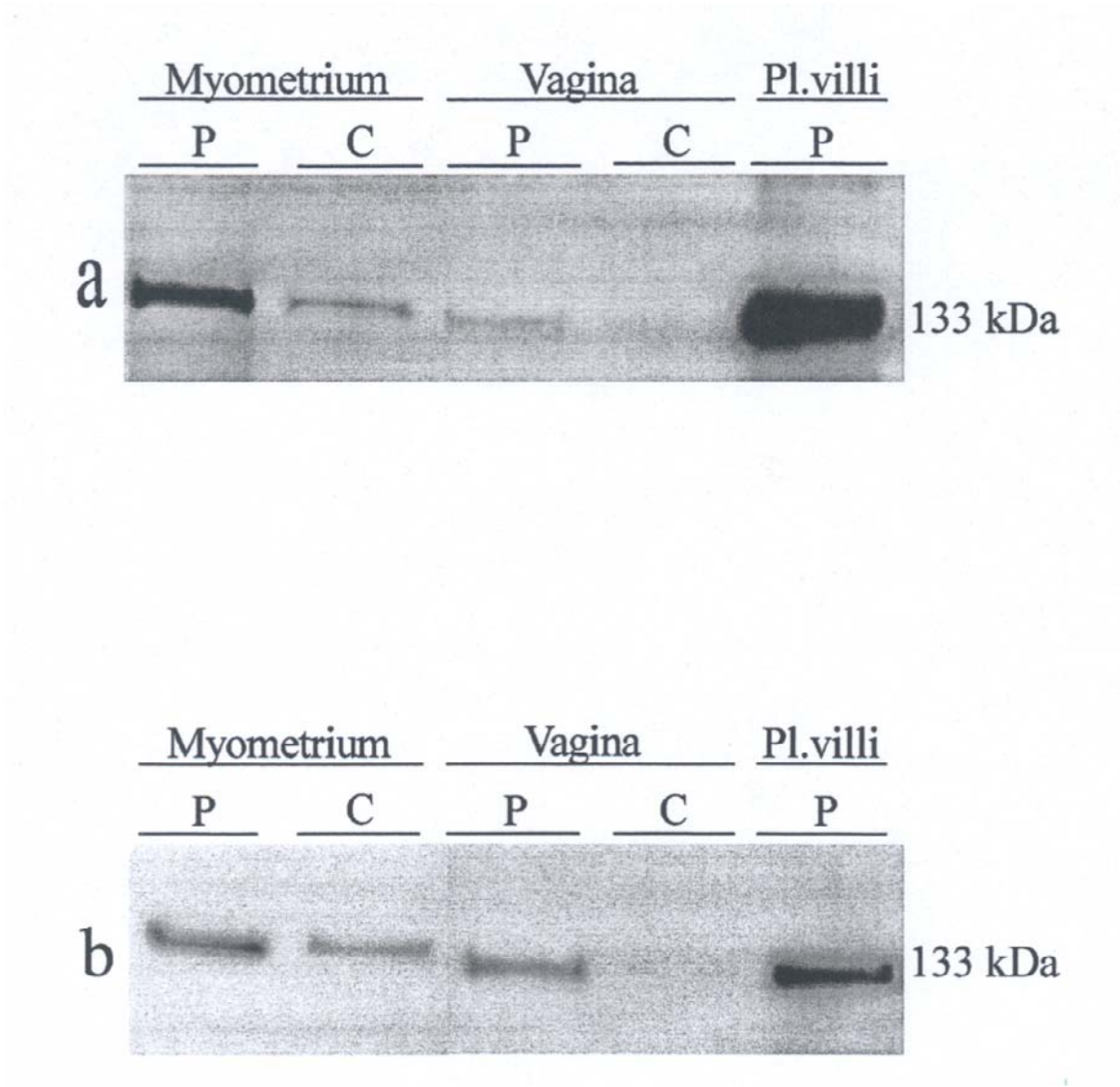

Figure 4
Western blots of particulate (P) and cytosolic (C) fractions isolated from non-pregnant human myometrium and vagina (a) and ovariectomised rat myometrium and vagina (b) using an antibody directed against eNOS. The putative bands are seen at 133 kDa corresponding to eNOS protein, for which human placental villi (Pl villi) were used as a positive control.

eNOS protein. The fractions from human myometrium (Fig. 4, a), and vagina showed a specific band of 133 kDa, corresponding to eNOS, which was detected in both particulate and cytosolic fractions of the myometrium although the density of the band in particulate fraction was much greater. Similar results were obtained in the vaginal fractions except the band in cytosolic fraction was hardly visible. The pattern of bands obtained in fractions from the rat tissues was very similar (Fig. 4, b) to the human as a clear expression of eNOS in the particulate fractions, a faint band in cytosolic fraction of the myometrium, but not vagina were visible.

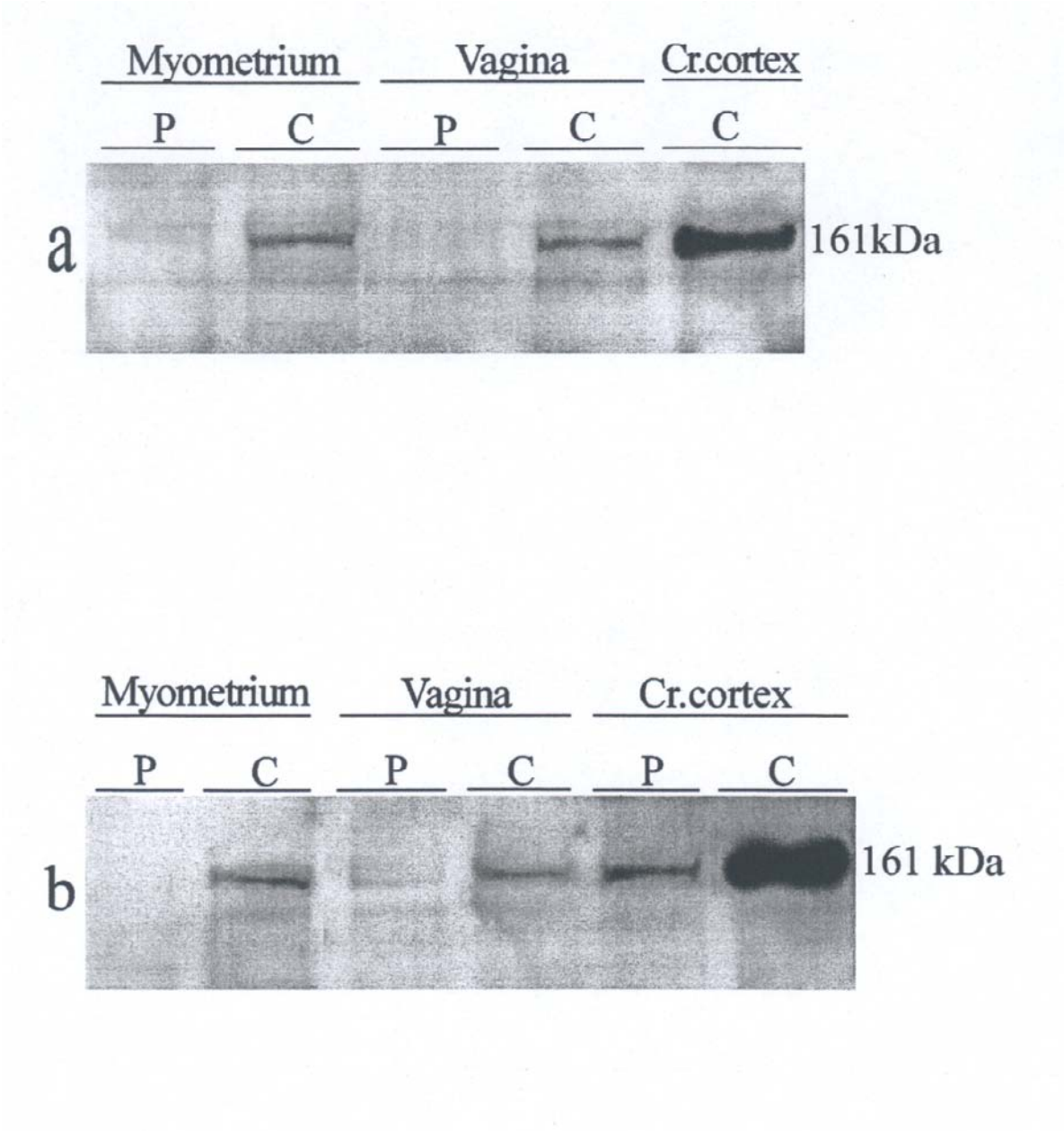

Figure 5
Western blots of particulate (P) and cytosolic (C) fractions isolated from human (a) and rat (b) tissues using an antibody directed against nNOS. The bands are seen at 161 kDa corresponding to eNOS protein, for which rat cerebral cortex was used as a positive control.

The nNOS antibody showed reactivity with a 161 kDa protein from rat cortex (positive control), corresponding to the nNOS size (Fig. 5). With this antibody the cytosolic fractions from both human myometrium and vagina showed a clear band with a density that was much greater than the band in the particulate fraction, which was nonetheless visible (Fig. 5, a).

Western blots analysis of rat tissues showed as expected a clear presence of nNOS in the cytosolic fraction and in eNOS in the particulate fraction (Fig. 5, b). With nNOS antibody, a clear and distinct band was seen in the cytosolic fractions and virtual absence of a band in the particulate fractions.

Discussion

Use of exactly the same method of preparation of subcellular fractions and assay conditions for human and rat tissues, allowed us to compare data on NOS activities and other parameters in the two species both qualitatively and quantitatively. Additionally, identification of NOS isoforms by Western blotting on isolated subcellular fractions provided information of subcellular distribution of eNOS and nNOS.

Our data show that in contrast to the rat, NOS activity in human uterus and vagina was nearly equally distributed between cytosolic and particulate fractions. In the rat NOS activity in the cytosolic fraction was 4–5 fold higher than the particulate fraction for both myometrium and vagina. In spite of the above difference in the subcellular distribution, the NOS activity in the vagina from both human and rat was approximately 2-fold higher than in myometrium. This was true for both particulate and cytosolic fractions. Whereas there was no significant difference in the NOS activity in particulate fractions from human and rat tissues, cytosolic NOS in the rat myometrium and vagina was 4–5 fold higher than corresponding human tissues. The very low NOS activity in the human myometrium compared with rat as shown here or that reported in the rabbit previously [9,10] would suggest that myometrial NOS does not play a pivotal role in myometrial activity in man.

In previous studies in the rabbit, we reported that NOS activity in the vagina was nearly 4-fold higher than the myometrium [9,10]. The present data in rat and human also show a significant higher activity of NOS in vagina than myometrium. Immunological studies have demonstrated the presence of NOS in the vagina from human and other species [14,15]. In our recent study we demonstrated NO-dependent relaxation of vaginal smooth muscle together with a rich supply of NOS containing nerves [10]. All these observations point to a physiological role of NO in vagina. NO may be important for the vasocongestion of the vagina and for the relaxation of the inner vaginal wall observed during sexual excitement [15]. Further studies are needed to define the role of NOS in vaginal physiology.

A comparison of NOS activity between non-pregnant and pregnant human myometrium showed a significantly lower NOS activity in both fractions from pregnant myometrium than from the non-pregnant myometrium. Previous studies on changes in NOS activity during human pregnancy have not provided any concrete evidence on the role of NOS in myometrial quiescence. In one study, no difference in either the expression or the activity of NOS was found in the myometrium before and during term labour [16]. In another study by the same group, increase in the expression of both eNOS and nNOS during human pregnancy was reported [17]. However, expression of NOS can not be equated with the enzymatic activity of NOS. Furthermore, quantification of expression can not be as accurate as activity measurements. Bansal *et al.* reported that the expression of iNOS in the myometrium was higher in preterm not-in-labour patients than in labour [18]. Yet in another study it was shown that the expression of myometrial NOS messenger-RNA did not change throughout gestation or with the onset of labour and that was true for both constitutive and inducible NOS isoforms [19].

Assuming that cytosolic and particulate fractions represent nNOS and eNOS, respectively, which is supported by the present data, our data show a significant downregulation of both eNOS and nNOS activities in samples of myometrium obtained from Caesarean sections at term compared with the non-pregnant myometrium. Keeping in mind the differences in the regional origin of the myometrial samples obtained from Caesarean sections and hysterectomies, it is suggested that sex steroids which increase approximately by 20-fold (progesterone) to 80-fold (estrogen) could contribute to the apparent downregulation of NOS activity in the myometrium. This is supported by the present data showing the effect of estrogen on rat myometrium and our previous data on rabbits showing a highly significant reduction of myometrial NOS activity by estrogen and estrogen and progesterone treatment [10]. Data from our previous studies in rabbit and rat led to the conclusion that there were no noteworthy differences in the NOS activities, subcellular distribution and even the regulation by sex steroids in the two species [10,11]. Since in a study by Weeks *et al.* a decline in estradiol resulting from gonadotrophin releasing hormone caused no change in myometrial NOS [20], it is suggested that rise in both estrogen and progesterone are required for the observed reduction of NOS at term pregnancy.

Table 1: NOS activity in isolated fractions of tissues from non-pregnant human and ovariectomised rat.

	Human		Rat	
Fraction	Myometrium	Vagina	Myometrium	Vagina
	NOS pmol/mg protein/min			
Cytosol	1.25 ± 0.16	2.97 ± 0.40	7.18 ± 1.56	19.17 ± 2.48
Particulate	1.64 ± 0.19	3.02 ± 0.78	1.26 ± 0.43	3.35 ± 0.80
(n)	8	11	10	10

Values are means ± SEM and the number of individual samples is indicated by (n).

Many years ago, it was shown that pregnancy as well as treatment with sex steroids led to a considerable loss of adrenergic nerves in guinea pig and rabbit [21,22]. Similar but less pronounced reduction of adrenergic nerves was seen in the human uterus [23]. Assuming that such is the case with also NO-generating nerves, the reduction of nNOS in the pregnant human myometrium and our observation of marked reduction of nNOS which was the predominant form in rat and rabbit myometrium following estrogen and progesterone treatment [10,11] could be explained by this phenomenon. However, other factors of fetal origin being responsible for the downregulation of NOS can not be ruled out [24,25]. A decrease in NOS activity in the myometrium toward the end of pregnancy probably represents a conditioning phase for the initiation of parturition. Up- or downregulation of other factors such as calcium channels, neurotransmitter receptors [26,27], calcium ion permeability of fetal membranes [24,25], and paracrine effects [28] or fetomaternal interaction [29] would also be important in preparing the uterus for the onset of labour. In a previous study in pregnant rabbit, convincing evidence was presented for the presence of myometrial inhibitory factors originating in the conceptus [30].

The present data from Western blot analysis support the generally accepted view that nNOS and eNOS are predominantly recovered in the cytosolic and particulate subcellular fractions. However, in contrast to nNOS, the presence of which was hardly detectable in the particulate fraction of either human or rat tissues, eNOS was clearly detectable in the cytosolic fractions in tissues from both rat and human. The simplest explanation for this unexpected observation is that cytosolic fraction is contaminated with small particles originally associated with the particulate fraction. This contamination, which would be expected to vary with the method of preparation, should be given serious consideration when comparing the subcellular distribution of NOS reported in various studies in addition to consideration of difference in organs and species.

In view of the very low NOS activity in the human myometrium as compared with rat shown here or rabbit reported previously [9,10], it is justified to importantly consider the possibility in human pregnancy of paracrine effects of fetal-placental NO in maintaining quiescence of the myometrium. Furthermore, caution should be exercised in explaining the role of NO in human pregnancy based on the data from studies in animals, a point previously emphasised in relation to hormonal control of myometrial function [20].

In summary our data show that in spite of some clear-cut quantitative differences in NOS activity and subcellular distribution, there are some interesting similarities between human and rat tissues. The vaginal NOS activity in both species was considerably higher than the myometrium. In pregnancy there appears to be a down-regulation of NOS activity in the human myometrium, which is probably due at least in part to hormonal influence, as substantiated with the data from rat and rabbit [9,10].

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