



Characterization Of Vascular Endothelial Growth Factor (VEGF) “In The Uterine Cervix Over Pregnancy: Effects Of Denervation And Implications For Cervical Ripening

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Abstract

Bilateral neurectomy of the pelvic nerve (BLPN) that carries uterine cervix- related sensory nerves induces dystocia, and administration of its vasoactive neuropeptides induces changes in the cervical microvasculature, resembling those that occur in the ripening cervix. This study was designed to test the hypothesis that (a) the cervix of pregnant rats expresses vascular endothelial growth factor (VEGF) and components of the angiogenic signaling pathway [VEGF receptors (Flt-1, KDR), activity of protein kinase B, Akt (phosphorylated Akt), and endothelial nitric oxide synthase (eNOS)] and von Willebrand Factor (vWF) and that these molecules undergo changes with pregnancy, and (b) bilateral pelvic neurectomy (BLPN) alters levels of VEGF concentration in the cervix. Using RT-PCR and sequencing, two VEGF isoforms, 120 and 164, were identified in the rat cervix. VEGF, VEGF receptor-1 (Flt-1), eNOS, and vWF immunoreactivities (ir) were localized in the microvasculature of cervical stroma. Their protein levels increased during pregnancy but decreased to control levels by 2 days postpartum. VEGF receptor-2 (KDR)-ir was confined to the epithelium of the endocervix. BLPN downregulated levels of VEGF by a third. Therefore, the components of the angiogenic signaling pathway are expressed in the cervix and change over pregnancy. Furthermore, angiogenic and sensory neuronal factors may be important in regulating the dynamic microvasculature in the ripening cervix and may subsequently play a role in cervical ripening and the birth process

During pregnancy the quiescent uterus provides a favorable environment for the growing fetus and the nonpliable cervix ensures retention of the fetus in utero. However, at term the cervix undergoes tissue remodeling or cervical ripening with a progressive dissociation and disorganization of collagen fibers and bundles that leads to softening and dilatation of the cervix. At this time the uterus begins to contract and the cervix relaxes, and, collectively, these alterations facilitate a timely passage of the fetus at parturition (Kelly 2002). Failure or disruption of these processes causes complications

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at parturition, such as preterm or protracted labor, that account for 75% of all infant deaths (Challis 2000).

The complexity of cervical ripening is evidenced by the multiplicity of factors involved, many of which have been studied extensively, such as nitric oxide, cytokines, prostaglandins, relaxin, steroid hormones, and neuropeptides (Fuchs et al. 1983; Matsuzaki et al. 1993; Arntzen et al. 1998; Chaim and Mazor 1998; Collins et al. 2002; Mowa et al. 2003a,b). However, the exact mechanism(s) underlying the initiation and progression of cervical ripening remains elusive. Moreover, deletion of individual genes for key regulatory factors hitherto implicated in cervical ripening fail to completely block reproductive processes in mice (Kimura et al. 1999).

Uterine cervix-related sensory neurons (but not mo-

tor neurons) play an important role in parturition in that their denervation induces dystocia (Higuchi et al. 1987; Burden et al. 1990). Exactly how these neuropeptide-rich neurons influence parturition and cervical ripening remains to be elucidated. However, when the sensory neuropeptide substance P (SP) is administered to rats, it mimics aspects of neurogenic inflammation seen in the parturient cervix (Collins et al. 2002). Furthermore, production of SP and another sensory neuropeptide, calcitonin gene-related peptide (CGRP), in uterine cervix-related dorsal root ganglia (L6-S1 DRG) and the SP receptor NK1 in the cervix increase before parturition, coinciding with the initiation of cervical ripening (Collins et al. 2002; Mowa et al. 2003a,b). Because elimination of uterine cervix-related sensory nerves and their transmitters interferes with parturition (Higuchi et al. 1987, Burden et al. 1990) and because these nerves contain the vasoactive neuropeptides SP and CGRP (Papka 1990; Papka and Traurig 1993; Mowa et al. 2003a,b), which induce vascular changes resembling those induced by vascular endothelial growth factor (VEGF), we hypothesized the existence of a functional link between the sensory nerves and angiogenic factors in cervical ripening.

VEGF (VEGF-A) is a member of a family of closely related growth factors that includes VEGF-A, -B, -C, -D, -E, and placenta growth factor (PlGF) (Tischer et al. 1991; Conn et al. 1990; Park et al. 1994; Shima et al. 1996; Ferrara and Davis-Smyth 1997). VEGF-A has several splice variants, i.e., 120, 164, 188, and 205 representing the number of amino acids (+1 in human), and its biological effects have been well established (Ferrara and Davis-Smyth 1997). VEGF 164/165 is the most potent, abundant, and best-characterized variant (although transcripts of VEGF 120 may be more abundant in some tissues Zachary and Gliki 2001). The biological effects of VEGF are largely mediated by two receptors, kinase domain region (KDR) and fms-like tyrosine kinase-1 (Flt-1) (de Vries et al. 1992; Ferrara and Davis-Smyth 1997; Shibuya 1995; Mustonen and Alitalo 1995; Shibuya et al. 1999) [a third VEGF receptor 3 mediates the effects of VEGF in the lymph vessels (Shibuya et al. 1999)]. VEGF induces angiogenesis primarily via protein kinase B (Akt), phosphorylated at serine 473 (Six et al. 2002). Akt activates endothelial nitric oxide synthase (eNOS)-derived nitric oxide (NO) through phosphorylation (Fulton et al. 2002). Consequently, it is essential to characterize the VEGF isoforms present in the cervix and to profile the basic signaling machinery likely to mediate the effects of VEGF.

The present study was designed to address the hypothesis that the microvasculature of the uterine cervix undergoes remodeling during cervical ripening which is likely to be regulated, in part, by VEGF. Three aims were undertaken for this goal: (a) characteriza-

tion of VEGF isoforms present in the cervix; (b) localization and profiling the expression of VEGF and the basic VEGF angiogenic signaling factors [VEGF receptors Flt-1 and KDR, eNOS, and phosphorylated Akt (pAkt)] over pregnancy; and (c) determination of the effects of transecting the uterine cervix-related sensory nerves on VEGF levels in the cervix.

Materials and Methods

Animals

Timed-pregnant Wistar rats at pregnancy days 8 ($n=10$), 15, 20, 22, and 2 days postpartum were used in these studies ($n=8$ per time-point). Uterine cervical tissues were analyzed by immunohistochemistry (IHC), Western blot analysis, ELISA, and RT-PCR. To examine links between sensory nerves and VEGF, bilateral pelvic neurectomies (BLPNs; $n=4$) were performed on day 9 of pregnancy (described below) and rats were killed on day 22. BLPN-shams ($n=4$) were used as controls. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the local institution and the NIH guidelines (NIH publication number 86-23), and efforts were made to minimize both animal suffering and numbers of animals used.

Bilateral Pelvic Neurectomy

Transecting the pelvic nerves bilaterally, to eliminate sensory nerves innervating the cervix at gestational day 9, is compatible with pregnancy but parturition is blocked (Burden et al. 1990). On day 9 of pregnancy, rats were anesthetized (50 mg/kg sodium pentobarbital IP) and a laparotomy was performed to identify the pelvic paracervical ganglion adjacent to the junction of the cervix and vagina. The ganglia were followed laterally to identify the pelvic nerve, a 5-mm segment was removed from the nerve on each side, and the wound was closed. The animals were allowed to recover and to progress towards term. If animals did not deliver normally and showed signs of protracted labor, they were killed by an overdose of anesthesia (100 mg/kg sodium pentobarbital IP) and perfused transcardially with saline. The cervixes were harvested, processed, and evaluated for changes in VEGF levels using ELISA. For sham control animals, rats on day 9 of pregnancy were handled as in bilateral pelvic neurectomy above except that the pelvic nerves were touched but not transected.

IHC Staining

Angiogenic Factors. IHC studies were undertaken to determine the cellular sites and expression patterns of VEGF, Flt-1, KDR, vWF, and eNOS in the cervixes of rats in early (day 10) and late (day 22) pregnancy. After deeply anesthetizing the animals (ketamine HCl 100 mg/kg IP), the cervixes were rapidly removed, frozen in liquid nitrogen, and 8- μ m-thick cryostat sections were cut in a transverse plane. The sections were mounted on glass slides, fixed in acetone for 10 min at 4°C, air-dried and processed for IHC. Commercially avail-

able and well-characterized (Jesmin et al. 2003) antibodies were used [VEGF, rabbit generated against human polyclonal antibodies (Immunological Laboratories, Fujioka, Japan); KDR and Flt-1, rabbit generated against human polyclonal antibodies (Santa Cruz Biotechnology; Santa Cruz, CA); eNOS, rabbit generated against human polyclonal antibodies (Affinity BioReagents; Golden, CT)]. To prevent non-specific staining, the sections were blocked by nonimmune serum (1% bovine serum albumin in Tris) for 30 min at room temperature. After overnight incubation at 4°C with primary antibodies, the sections were rinsed in phosphate buffer solution and then exposed to the secondary antibody, Cy3-conjugated AffiniPure goat anti-rabbit IgG or fluorescein isothiocyanate (FITC)-conjugated AffiniPure goat anti-rabbit IgG (Jackson Immunoresearch Laboratories; West Grove, PA) for 2 hr according to the manufacturer's instructions. Sections processed without primary antibodies served as negative controls. The coverslips were mounted with Immunon (Thermo Shandon; Pittsburgh, PA). Immunofluorescent images were viewed using a laser scanning confocal imaging system (model MRC-1024; Bio-Rad, Hertfordshire, UK). In some cases serial sections were stained with hematoxylin and eosin (H and E) and antibodies to identify the cell types expressing the proteins of interest.

Capillary Morphology. To identify and assess capillary morphology and expansion, some cervixes were fixed and either frozen and sectioned or embedded in paraffin and sectioned and immunostained with vWF, a marker for endothelial cells. After immunostaining with vWF primary antibody (goat generated against mouse antibody; DAKO, Hamburg, Germany), sections were incubated with Cy3-conjugated AffiniPure donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories).

Western Blot Analysis

Angiogenic Factors. Western blot analysis was used to complement immunohistochemical experiments by facilitating the quantification of the angiogenic factors in the cervix at different time points of pregnancy [days 8, 15, 20, and 22 of pregnancy and 2 days postpartum (2dp)]. After removing and rinsing the cervixes in ice-cold PBS, the tissues were minced with scissors, homogenized, and centrifuged at 500 × g for 15 min to pellet any insoluble material. The protein concentration of the supernatants was determined using the bicinchoninic acid protein assay (Pierce; Rockford, IL). Samples were run on SDS-PAGE using a 7.5–15% polyacrylamide gel and electrotransferred to polyvinylidene difluoride filter (PVDF) membrane. To reduce nonspecific binding, the PVDF was blocked for 2 hr at RT with 5% nonfat milk in PBS containing 0.1% Tween-20 (TPBS). The PVDF was incubated overnight at 4°C with specific antibodies for VEGF, KDR, Flt-1, and eNOS (as described above; 1:100–1,000 dilution) in TPBS, washed thrice in TPBS, and then incubated with horseradish peroxidase-conjugated anti-rabbit antibody (Amersham; Poole, UK) diluted at 1:2000–10,000 in TPBS at RT for 60 min. The blots were visualized with the enhanced chemiluminescence detection system (Amersham), exposed to X-ray film, and analyzed by free NIH image software.

ELISA

ELISA, a sensitive technique for determining tissue protein concentration, was used to determine VEGF and phosphorylated Akt (pAkt) protein concentration in the cervixes of pregnant rats.

VEGF. VEGF levels in the rat cervixes of pregnant, bilateral pelvic neurectomy (BLPN), and BLPN-sham rats ($n=4$) were estimated using a commercially available ELISA kit (VEGF Immunoassay; R and D Systems, Minneapolis, MN) according to the manufacturers' instructions.

Phosphorylated Akt. The level of protein kinase B (Akt), phosphorylated by VEGF at serine residue 473, in tissue extracts of cervixes of pregnant rats, was estimated by ELISA Akt kit [pS473] (BioSource International; Richmond, CA).

Semiquantitative RT-PCR

Primers for RT-PCR were designed to detect all five isoforms of VEGF (Burchardt et al. 1999) to screen VEGF variants expressed in the cervix around the onset of cervical ripening. The lung and penis, which express various VEGF variants, were used as positive control tissues (Burchardt et al. 1999).

mRNA: PCR. Total RNA Isolation. Rats in late (day 20) pregnancy were anesthetized and perfused only with saline. The cervixes were removed and stored at -80°C until processing. Total RNA was isolated from cervixes of individual animals using the RNeasy Mini Kit (Qiagen; Valencia, CA). The amount and purity of total RNA for each sample were estimated by spectrophotometric analysis at A260 and A280. The quality of RNA was determined by agarose gel electrophoresis after ethidium bromide staining. Aliquots of total RNA were diluted in diethylpyrocarbonated (DEPC)-treated water and stored at -80°C.

Semiquantitative RT-PCR. RT-PCR was utilized to identify VEGF isoforms present in the cervix using rats at day 20 of pregnancy. Total RNA was reverse-transcribed and amplified in an Eppendorf Master Cycler, using reagents from Gene AMP Gold RNA PCR Kit (PE BioSystems; Foster City, CA). Briefly, 0.5–1 µg total RNA was reverse transcribed and amplified in a 50 µl reaction mixture containing the following: 5 × RT-PCR buffer, 1.75 mM MgCl₂, 1.2 mM dNTPs, 10 U RNase inhibitor, 5.0 mM DTT, 1 µM random hexamer, 200 nM VEGF (forward, 5'-TGC-ACC-CAC-GAC-AGA-AGG-GGA-3'), 200 nM VEGF (reverse, 5'-TCA-CCG-CCT-TGG-CTT-GTC-ACA-3'), 5.0 U AmpliTaq Gold DNA polymerase, 30 U MultiScribe reverse transcriptase, and DEPC-treated water. The RT-PCR reaction was normalized across the runs using GAPDH gene (Primers: forward, 5'-GAG-GAC-CAG-GTT-GTC-TCC-TG-3'; reverse, 5'-GGA-TGG-AAT-TGT-GAG-GGA-GA-3') as a standard. The PCR products were separated using agarose gel electrophoresis, visualized after staining with SYBR Green 1, and scanned using the Kodak 1D Image Station (Rochester, NY). The cDNA bands for VEGF were isolated from the gel and sequenced.

Statistical Analysis

Values are shown as means + SD. Statistical analysis was performed by ANOVA with multiple comparisons by Fisher's protected least-significant difference *t*-test. Nonparametric

data were analyzed by the Mann-Whitney's *U*-test or Wilcoxon signed-rank test. A $p < 0.05$ was considered significant.

Results

The Rat Cervix Contains VEGF 120 and 164

RT-PCR primers designed to detect all VEGF isoforms revealed the presence of VEGF-A splice variants 120 and 164 (Figure 1), with VEGF 164 being the predominant type in the cervix. The bands corresponded to those demonstrated from adult male lung and penis that were used as positive control tissues (Figure 1). The bands of the VEGF-A splice variants from the cervix were isolated from their electrophoresis gel, sequenced, and blasted, to determine their specific base pairs. A faint band, with a molecular weight corresponding to that of VEGF 188, was observed in cervixes sampled from parturient rats (data not shown). However, this band could not be confirmed by sequencing.

vWF Expression in the Cervical Microvasculature Changes During Pregnancy

vWF was used as a marker for endothelial cells to visualize the patterns and magnitude of capillary expansion that occur in the cervix during pregnancy. vWF immunolabeling was localized in the microvasculature of the endocervix and the base of the luminal epithelia of the cervix (Figures 2A and 2B). The relative abundance of expression of vWF appeared to increase over pregnancy (compare Figures 2A and 2B). Blood vessels immunolabeled with vWF tended to "aggregate" by day 22 of pregnancy, forming spindle-shaped structures (Figure 2B). The distribution pattern of vWF overlapped with those of VEGF receptors (Flt-1 and KDR; Figures 2E, 2F and 2G, 2H, respectively) and eNOS (Figures 2I and 2J), based on serial sections stained with H and E and the respective angiogenic molecules (H and E histology not shown).

VEGF Localization and Increases over the Course of Pregnancy

VEGF was localized by IHC to the stromal cells, largely in the endocervix, at days 8 and 22 of pregnancy (Fig-

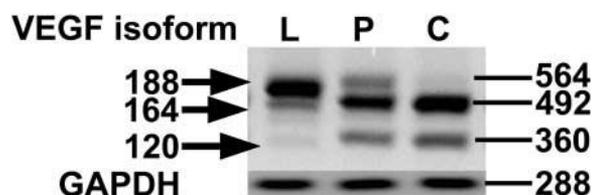


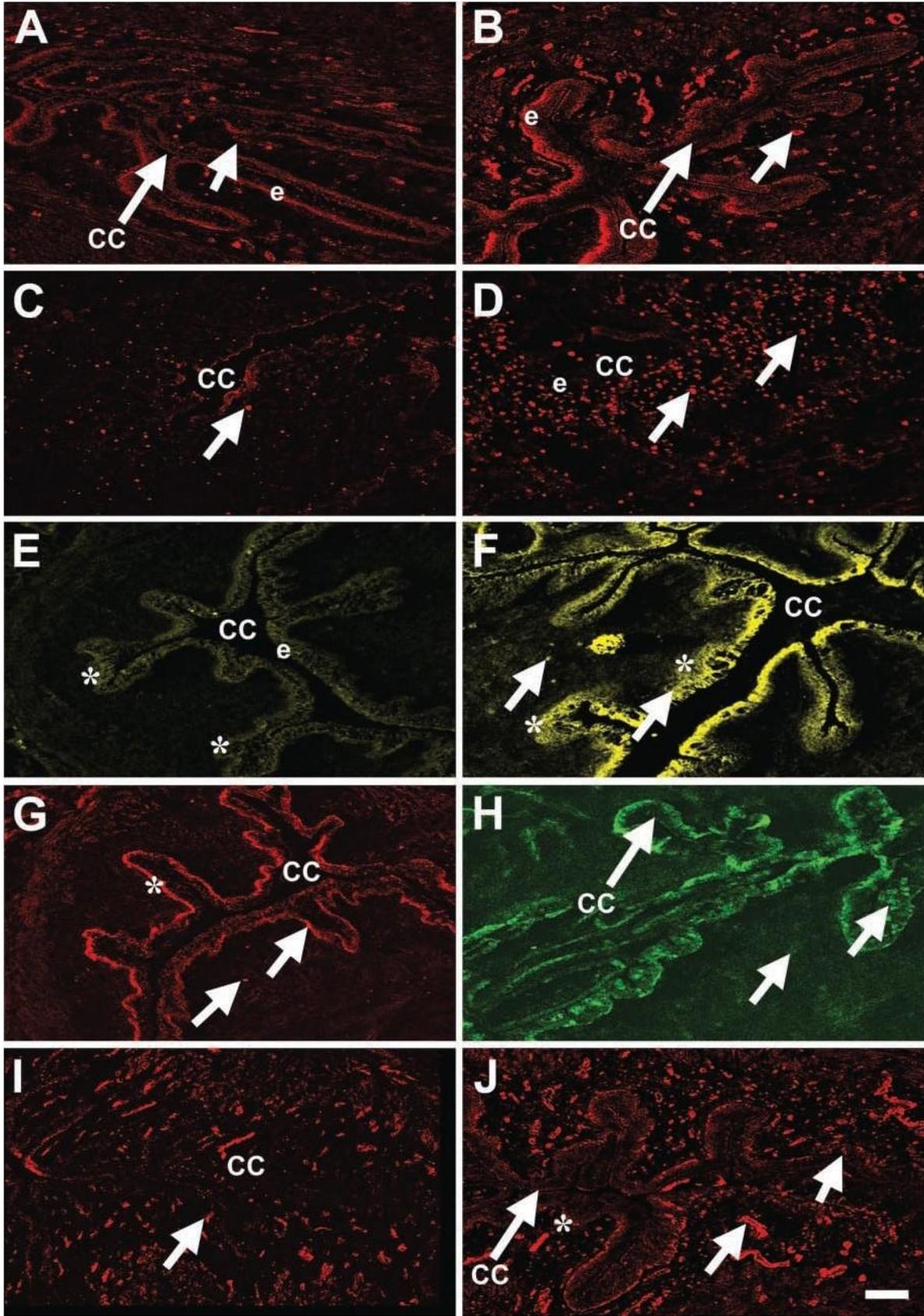
Figure 1 Characterization, using RT-PCR, of VEGF in the cervix (C). Two VEGF-A splice variants, VEGF-120 and -164, were identified in the cervix in late (day 20) pregnancy, with VEGF 164 being the predominant variant. The lung (L) and penis (P) of adult male rats, which predominantly express VEGF-188 and -164, respectively, were used as positive control tissues. The cDNA bands on the gel were identified on the basis of their molecular weight, after which they were isolated, sequenced, and blasted, to further confirm their identity. Bp, base pair. GAPDH gene was used as a normalizer.

ures 2C and 2D). VEGF was not visualized in the luminal epithelia of the cervix (Figures 2C and 2D). Profiles of the stromal cells expressing immunoreactivity for VEGF increased with advancing pregnancy, as revealed by IHC (Figures 2C and 2D). These IHC findings were corroborated by the Western blot and immunoassay data, both of which revealed an increase in VEGF levels between day 8 and days 15, 20, and 22 of pregnancy, with a decrease by 2 days postpartum (Figures 3A and 4A). The difference in relative levels of VEGF at days 15, 20, and 22 of pregnancy and at 2 days postpartum vs day 8 were statistically significant ($p < 0.05$).

VEGF Angiogenic Signaling Factors Increase in the Cervix During Pregnancy

VEGF Receptors. Flt-1 was localized in the microvasculature of the endocervix and the base of luminal epithelia on days 8 and 22 of pregnancy (Figures 2E and 2F). The immunostaining in the epithelium as well as the profiles of the microvasculature expressing Flt-1 appeared to increase by late pregnancy (compare Figures 2E and 2F), similar to the profiles immunoreactive for VEGF (Figures 2C and 2D). Western blots showed that Flt-1 increased between days 8 and 15, was unchanged between days 15 and 20, increased significantly between days 20 and 22, and then returned to day 8 levels by 2 days postpartum (Figure 3B).

Figure 2 Immunohistochemistry. Cryostat sections of the cervix from pregnant rats at days 8 (A,C,E,G,I) and 22 (B,D,F,H,J) of pregnancy immunostained for von Willebrand factor (vWF) (A,B), VEGF (C,D), Flt-1 (E,F), KDR (G,H), and eNOS (I,J). vWF immunolabeling (A,B) was localized to the microvasculature of the endocervix (arrows) and the base (*) of the luminal epithelia (e) of the cervix. The profiles of vWF immunoreactivity appeared to increase by late pregnancy (B) relative to early pregnancy (A). Vessels immunolabeled for vWF tended to "aggregate", forming spindle-shaped structures (arrows) (B). VEGF was localized (C,D) to the stromal cells of the endocervix (arrows). VEGF was not visualized in the luminal epithelia (e) of the cervix. The profiles of VEGF immunoreactivity (arrows) markedly increased in late pregnancy (compare C and D). Flt-1 immunoreactivity was localized (E,F) to the microvasculature of the endocervix and close to the base (*) of luminal epithelia (e) on days 8 and 22 of pregnancy. The intensity of Flt-1 immunoreactivity appeared to increase in late pregnancy (compare E and F). KDR immunoreactivity was concentrated largely at the base (*) of the luminal epithelia (e) of the cervix in early pregnancy, but appeared to extend to the microvasculature of the endocervix in late pregnancy (G,H, arrows). Endothelial NOS immunoreactivity (I,J) was expressed in the microvasculature of the endocervix (arrows) and appeared to moderately increase in relative abundance in late pregnancy (compare I and J). *, base of luminal epithelia (e); cc, cervical canal. Bar = 50 μ m.



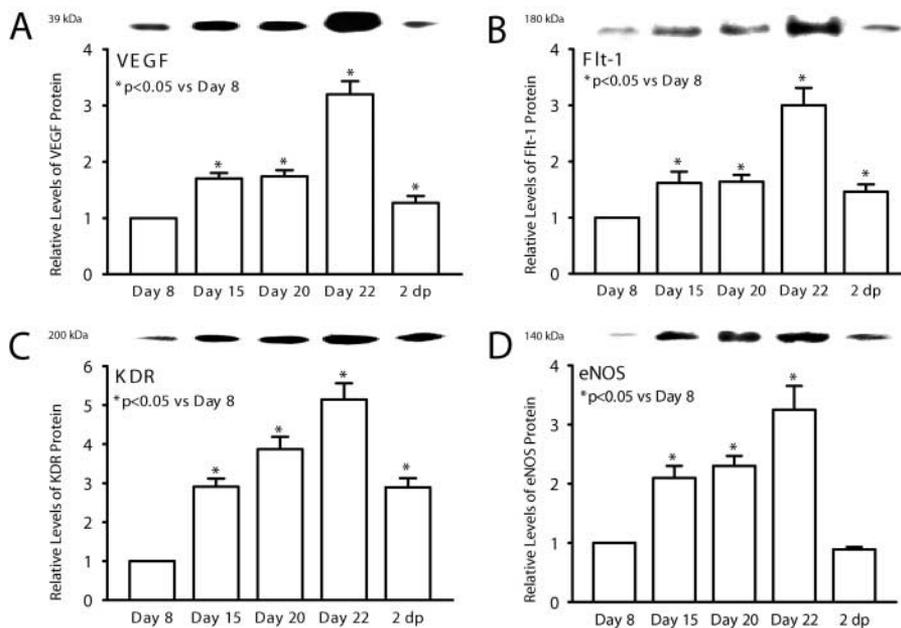


Figure 3 Western blot analysis showed that overall levels of all the molecules studied increased over the course of pregnancy. (A) When expressed as a percentage of day 8 levels, VEGF increased by 100% at days 15 and 20 and by 300% at day 22, but decreased to day 8 levels by 2dp ($p < 0.05$). (B) Flt-1 levels, expressed as a percentage of day 8 levels, increased 100% by days 15 and 20, and by 300% at day 22, but decreased to day 15 levels by 2dp. (C) When expressed as a percentage of day 8, KDR levels in the cervix increased 300% by day 15, 400% by day 20, 500% by day 22, and were falling by 2dp. (D) Endothelial NOS levels, expressed as a percentage of day 8 levels, increased 200% at days 15 and 20 and by more than 300% at day 22, but decreased to day 8 levels by 2dp ($n = 5$; $p < 0.05$).

KDR. KDR immunoreactivity was obvious at early and late pregnancy and was concentrated close to the base of the luminal epithelia of the cervix (Figures 2G and 2H). By late pregnancy, however, KDR immunoreactivity extended to the microvasculature of the endocervix (Figures 2G and 2H). As shown by Western blotting the relative abundance of KDR protein increased in late pregnancy (Figure 3C).

VEGF Signaling Molecules pAkt and eNOS Increase over the Course of Pregnancy. Endothelial NOS immunoreactivity was expressed in the microvasculature of the endocervix and appeared to increase moderately in relative abundance in late pregnancy, (day 8 vs day 22 of pregnancy (Figures 2I and 2J)). Endothelial NOS levels, when analyzed by Western blots, were markedly increased over pregnancy (Figure 3D). For example, when expressed as a percentage of day 8 levels, eNOS increased 200% at day 15 and 20 and by 300% at day 22, and then returned to day 8 levels by 2 days postpartum (Figure 3D).

Akt activity (as revealed by pAkt levels), expressed as a percentage of day 8 levels, increased by greater than 700% on day 20 but remained at levels close to those of day 8 on days 15, 22, and 2 days postpartum (Figure 4C).

Bilateral Pelvic Neurectomy Downregulates Levels of VEGF at Parturition

After BLPN at day 9, levels of VEGF protein in the uterine cervix at day 22 of pregnancy decreased by about a third compared with the BLPN-sham control animals (Figure 4B).

Discussion

The important findings of this study are as follows: (a) The rat cervix expresses two VEGF-A splice variants, VEGF 164 (predominant type) and VEGF 120, (b) VEGF and its signaling molecules Flt-1, KDR, eNOS, and activity of Akt (pAkt levels) increase with advancing pregnancy, peaking at the time of cervical ripening and parturition and then returning to base levels by 2 days postpartum, (c) in BLPN pregnant rats, levels of VEGF protein are lower than in intact pregnant rats. Collectively, these findings have led us to suggest that VEGF and its downstream signaling molecules could play a role in cervical ripening and that sensory nerves could influence parturition through actions on VEGF.

Angiogenesis, the development of new blood vessels from preexisting ones (Carmeliet and Jain 2000; Ferrara 2001), is a complex and tightly controlled process that occurs under both physiological and pathological conditions (Ferrara and Alitalo 1999). It involves endothelial cell survival, proliferation, and migration, which in the VEGF system are regulated by independent signaling pathways (Ferrara 2001). A key effector molecule of angiogenesis is VEGF. To visualize the microvasculature of the cervix we used immunostaining for vWF. There was a marked increase in the abundance of vWF-ir profiles over pregnancy, a finding we interpret as an indicator of expansion of the microvasculature in the endocervix over pregnancy. This indirectly implies that the changes occurring in the cervix during the ripening process may involve angiogenesis. Because cervical ripening entails a controlled inflammation-like process (Liggins 1981) and angiogenesis is intimately associated with inflamma-

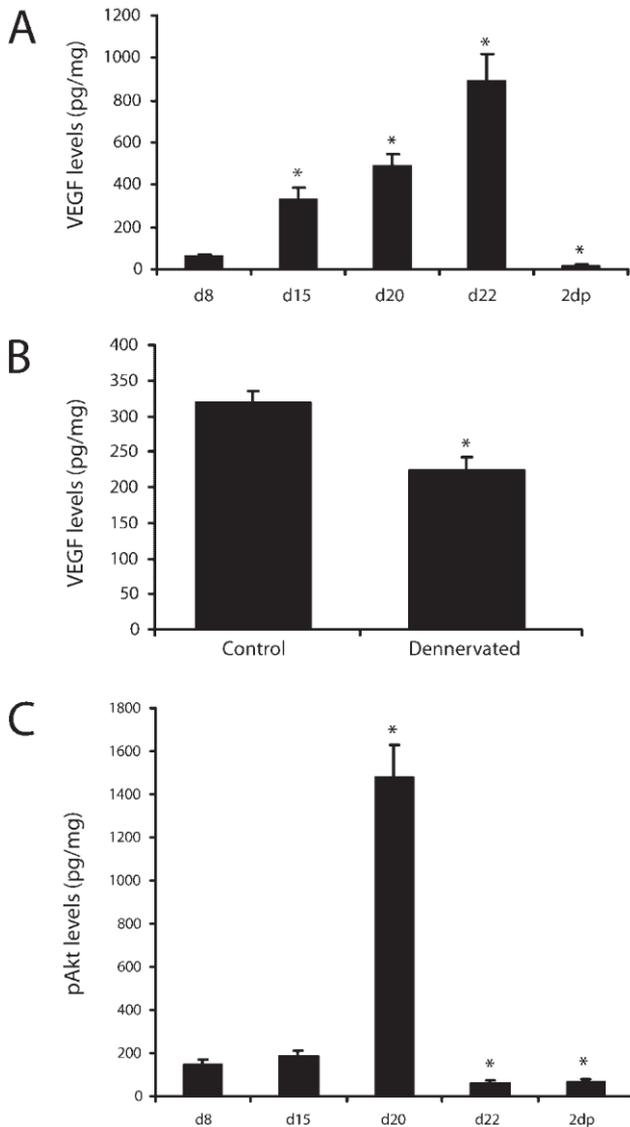


Figure 4 ELISA, illustrating protein concentration of VEGF over pregnancy (**A**) and after bilateral pelvic neurectomy (BLPN; **B**) and concentration of phosphorylated (activated) protein kinase B (pAkt; **C**) in the cervix. When expressed as a percentage of day 8 levels, VEGF (**A**) increased by 600% at day 15, 700% by day 20, and by 1300% at day 22, but decreased to levels less than day 8 by 2dp ($p < 0.05$). VEGF protein levels in the cervix were downregulated at day 22 of pregnancy after BLPN (**B**) performed at day 9 of pregnancy compared with sham controls. Levels of phosphorylated Akt (or activity) (**C**), expressed as a percentage of day 8 levels, increased by greater than 700% on day 20 but remained lower on days 15 and 22 of pregnancy and 2dp ($n=5$; $p < 0.05$).

tion (McDonald 2001), angiogenesis could be an important component of cervical ripening. These data provide the groundwork for future studies, which will examine specific patterns of endothelial cell activity, proliferation, and migration in the cervix during pregnancy and their impact on cervical ripening.

VEGF plays a critical role in angiogenesis and other vascular events, in part, by regulating proliferation, migration, and remodeling of endothelial cells (Ferrara et al. 1996; Ferrara and Davis-Smyth 1997; Ferrara, 2001). In female reproductive organs, VEGF mediates cyclical growth of blood vessels (Ferrara et al. 1998), e.g., treatment of rodents with a VEGF inhibitor [mFlt-(1-3)-IgG] virtually blocks ovarian corpus luteum angiogenesis and maturation of the uterine endometrium (Ferrara et al. 1998). With regard to the cervix, recruitment or mobilization of leukocytes into the cervical connective tissue at cervical ripening requires structural changes to the vasculature. These changes resemble those vascular alterations induced by VEGF, such as angiogenesis, vascular leakage, and vasodilation (Bates and Curry 1997; Murohara et al. 1998). Consequently, VEGF could play a role in the remodeling of cervical microvasculature during cervical ripening. The present study identified two VEGF-A splice variants, 120 and 164, in the cervix and demonstrated that the levels of total VEGF protein increased during late pregnancy, coincident with the expansion of cervical microvasculature during pregnancy. VEGF-120 and VEGF-164 have similar receptor binding characteristics, although they possess distinct properties that could influence their capacity to function in the ever-changing biochemical environment of the ripening cervix. For example, whereas VEGF-120 is acidic, fails to bind to heparin, and is freely diffusible (Robinson and Stringer 2001), VEGF-164 is basic, binds to heparin and, although secreted, has significant portions bound to the cell surface and extracellular matrix (Robinson and Stringer 2001). Because VEGF-164 is the most abundant in the cervix and has optimal characteristics for bioavailability and biological potency, it is likely to be the most important splice variant in the cervix. However, it is not yet clear whether VEGF-120 or VEGF-164, or both, are functionally critical in the cervix in late pregnancy.

Consistent with the profile of their ligand VEGF, levels of VEGF receptors, KDR and Flt-1, increased in the cervix over pregnancy. These findings correlate with the expansion of cervical microvasculature demonstrated by increased vWF immunoreactivity. Collectively, these findings suggest that VEGF may underlie the expansion of the microvasculature during cervical ripening. Both KDR and Flt-1 have high affinities for VEGF, even though that of Flt-1 is higher than KDR (Terman et al. 1992; Seetharam et al. 1995; Shibuya 1995; Shibuya et al. 1999). Paradoxically, Flt-1 is more weakly phosphorylated by VEGF than is KDR and is, for this reason, considered to be a “decoy” receptor (Waltertnerberger et al. 1994). Moreover, data from knockout mice demonstrate that both receptors (KDR and Flt-1) are essential for embryonic vascular development (Shalaby et al. 1995; Fong et al. 1999).

At this point, the specific roles of KDR and Flt-1 in the cervix are unknown.

The parallel increase in VEGF and its receptors is indicative of an activated VEGF signaling. The activated form of the serine/threonine protein kinase B, Akt, is an attractive marker of VEGF signaling in that it is a critical downstream intermediate effector molecule that mediates multiple VEGF-induced cellular responses, including angiogenic processes and vascular permeability (Six et al. 2002). Consistent with our assumption, a remarkable correlation was found between the pattern of Akt activity (pAkt levels) and levels of VEGF/KDR/Flt-1 in the cervix over pregnancy. Together, these data support the existence of active VEGF/Akt signaling in the cervix during late pregnancy.

The role of NO in cervical ripening is well established (Dong et al. 1996; Yallampalli et al. 1996,1998; Ali et al. 1997; Kelly 2002; Maul et al. 2003; Tenore 2003). However, much of what we know about the role of NO in cervical events is based on its nonvascular effects, likely mediated by NO produced via inducible NOS (Yallampalli et al. 1996; Ali et al. 1997). NO in the ripening cervix may also arise from the endothelial cells, as demonstrated in the present study. Several recent reports have implicated eNOS-derived NO as a terminal effector molecule mediating various VEGF-induced vascular responses (Ziche et al., 1997; van der Zee et al., 1997; Tsurumi et al., 1997; Goligorsky et al. 1999; Servos et al. 1999) and that Akt phosphorylates eNOS at serine 1176 (Fulton et al. 2002). Indeed, the importance of eNOS in VEGF signaling is underscored by findings that mice lacking eNOS are resistant to VEGF-induced effects (Yang et al. 2001). Although in the present study the activated form of eNOS was not determined, levels of total eNOS protein correlated with those of VEGF, VEGF receptors, and pAkt. Consistent with our conclusion above, the correlation between VEGF/VEGF receptors, Akt activity, and levels of eNOS strongly suggests the presence of an active VEGF/Akt/eNOS signaling cascade in the ripening cervix; this could mediate vascular changes leading to increased leukocyte influx and enhanced cervical ripening. The precise mechanisms for the marked increase in VEGF and its signaling molecules reported here are not yet completely clear.

Many factors stimulate VEGF production, including interleukin-1, endothelin-1, calcium ions, phorbol esters, cytokines (PDGF, TGF, bFGF), heavy metals, hypoxia, and sex steroid hormones, e.g., estrogen (Toi 1995; Gu and Adair 1997; Detmar et al. 1997; Sandler et al. 1997; Christou et al. 1998; Okuda et al. 1998; Mueller et al. 2000). An important finding derived from the present study is that VEGF, in the cervix of pregnant rats, is influenced by sensory nerves. While recent observations indicate that VEGF directly stimulate growth, survival and axonal outgrowth of

neurons and glial cells (Carmeliet and Storkebaum 2002), to our knowledge, this study is the first to show that neuronal factors may have reciprocal effects on VEGF expression in peripheral tissues. Although, admittedly, it is too premature to speculate mechanisms that may underlie this effect at this point, it is possible that the vasoactive neuropeptides derived from cervix-related dorsal root ganglia, e.g., SP and CGRP, may account for this phenomenon for the following reasons: (a) SP has been shown to induce angiogenesis in the lungs (McDonald et al. 1996). However, it is important to state that it is not yet clear whether SP influences angiogenesis by acting via VEGF, (b) we previously have shown that when SP is administered to ovariectomized rats, it induces some of the "classical" vascular changes induced by VEGF, such as vascular leakage and vasodilation (Papka et al. 2001; Collins et al. 2002), (c) the close anatomic relationship between nerve terminals immunoreactive to SP and CGRP, their receptors, and the cervical microvasculature (Papka and Traurig 1993; Collins et al. 2000,2002; Pokabla et al. 2002; Mowa and Papka 2004) and stromal cells, which have been shown to express VEGF, (d) the temporal and spatial relationship between vasoactive neuropeptides (SP, CGRP) in uterine cervix-related DRG (Mowa et al. 2003a,b) and VEGF (present study). Whether the effect of BLPN on parturition (dystocia) is due to disruption of VEGF signaling, is yet to be confirmed. It is likely that VEGF signaling may be one of the mechanisms underlying this phenomenon since BLPN does not completely attenuate levels of VEGF in the cervix. VEGF levels are also likely to be influenced by high levels of circulating estrogen in late pregnancy. Studies on the potential mechanisms underlying the link between sensory nerves and angiogenic factors during cervical ripening are currently ongoing in our lab.

In conclusion, this study has shown that angiogenic molecules are increased over the course of pregnancy and peak during the time of cervical ripening. Moreover, the important factor VEGF is downregulated when the pelvic nerves are eliminated. Consequently, the dystocia seen with BLPN may be related to a reduction in angiogenic factors and events. Moreover, the data derived from this study provide the rationale to determine the specific roles of VEGF-120 and VEGF-164 signaling mechanisms in cervical ripening.

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Literature Cited

- Ali M, Buhimschi I, Chwalisz K, Garfield RE (1997) Changes in expression of the nitric oxide synthase isoforms in rat uterus and cervix during pregnancy and parturition. *Mol Hum Reprod* 3: 995-1003
- Arntzen KJ, Kjollesdal AM, Halgunset J, Vatten L, Austgulen R (1998) TNF, IL-1, IL-6, IL-8 and soluble TNF receptors in relation to chorio-amnionitis and preterm labor. *J Perinat Med* 26: 17-26
- Bates DO, Curry FE (1997) Vascular endothelial growth factor increases microvascular permeability via a Ca²⁺-dependent pathway. *Am J Physiol* 273:H687-694
- Burchardt M, Burchardt T, Wei Chen M, Shabsigh A, de la Taille A, Buttyan R, Shabsigh R (1999) Expression of messenger ribonucleic acid splice variants for vascular endothelial growth factor in the penis of adult rat penis and humans. *Biol Reprod* 60:398-404
- Burden HW, Price GT, Renegar RH, Hodson CA (1990) Effects of peripheral nerve lesions during pregnancy on parturition in rats. *Anat Embryol (Berl)* 182:499-501
- Carmeliet P, Jain RK (2000) Angiogenesis in cancer and other diseases. *Nature* 407:249-257
- Carmeliet P, Storkebaum E (2002) Vascular and neuronal effects of VEGF in the nervous system: implications for neurological disorders. *Semin Cell Dev Biol* 13:39-53
- Chaim W, Mazor M (1998) The relationship between hormones and human parturition. *Arch Gynecol Obstet* 262:43-51
- Challis JRG (2000) Mechanism of parturition and preterm labor. *Obstet Gynecol Surv* 55:650-660
- Christou H, Yoshida A, Arthur V, Morita T, Kourembanas S (1998) Increase vascular endothelial growth factor production in the lungs of rats with hypoxia-induced pulmonary hypertension. *Am J Respir Cell Mol Biol* 18:768-776
- Collins JJ, Usip S, McCarter KE, Papka RE (2002) Sensory nerves and neuropeptides in uterine cervical ripening. *Peptides* 23:167-183
- Collins JJ, Wilson K, Fischer-Colbrie R, Papka RE (2000) Distribution and origin of secretoneurin-immunoreactive nerves in the female rat uterus. *Neuroscience* 95:255-264
- Conn G, Bayne ML, Soderman DD, Kwok PW, Sullivan KA, Palisi TM, Hope DA, et al. (1990) Amino acid sequences of a vascular endothelial cell mitogen that is homologous to platelet-derived growth factor. *Proc Natl Acad Sci USA* 87:2628-2632
- Detmar M, Brown LF, Berse B, Jackman RW, Elicker BM, Dvorak HF, Claffey KP (1997) Hypoxia regulated the expression of vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) and its receptors in human skin. *J Invest Dermatol* 108: 263-268
- de Vries C, Escobedo JA, Ueno H, Houck K, Ferrara N, Williams LT (1992) The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science* 255:989-991
- Dong YL, Gangula PR, Fang L, Yallampalli C (1996) Differential expression of cyclooxygenase-1 and -2 proteins in rat uterus and cervix during estrous cycle, pregnancy, labor and myometrial cells. *Prostaglandins* 52:13-34
- Ferrara N (2001) Role of vascular endothelial growth factor in regulation of physiological angiogenesis. *Am J Physiol* 280:C1358-1366
- Ferrara N, Alitalo K (1999) Clinical applications of angiogenic growth factors and the inhibitors. *Nat Med* 5:1359-1364
- Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, et al. (1996) Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 380:439-442
- Ferrara N, Chen H, Davis-Smyth T, Gerber HP, Nguyen TN, Peers D, Chisholm V, et al. (1998) Vascular endothelial growth factor is essential for corpus luteum angiogenesis. *Nat Med* 4:336-340
- Ferrara N, Davis-Smyth T (1997) The biology of vascular endothelial growth factor. *Endocr Rev* 18:4-25
- Fong GH, Zhang L, Bryce DM, Peng J (1999) Increased hemangioblast commitment, not vascular disorganization, is the primary defect in flt-1 knockout mice. *Development* 126:3015-3025
- Fuchs AR, Periyasamy S, Alexandrova M, Soloff MS (1983) Correlation between oxytocin receptor concentration and responsiveness to oxytocin in pregnant rat myometrium: effects of ovarian steroids. *Endocrinology* 113:742-749
- Fulton D, Fontana J, Sowa G, Gratton JP, Lin M, Li KX, Michell B, et al. (2002) Localization of endothelial nitric-oxide synthase phosphorylated on serine 1179 and nitric oxide in Golgi and plasma membrane defines the existence of two pools of active enzyme. *J Biol Chem* 277:4277-4284
- Goligorsky MS, Abedi H, Noiri E, Takhtajan A, Lense S, Romanov V, Zachary I (1999) Nitric oxide modulation of focal adhesions in endothelial cells. *Am J Physiol* 276:C1271-1281
- Gu JW, Adair TH (1997) Hypoxia-induced expression of VEGF is reversible in myocardial vascular smooth muscle cells. *Am J Physiol* 273:H628-633
- Higuchi T, Uchida K, Honda K, Negoro H (1987) Pelvic neurectomy abolishes the fetus-expulsion reflex and induces dystocia in the rat. *Exp Neurol* 96:443-455
- Jesmin S, Hattori Y, Sakuma I, Liu M-Y, Mowa CN, Kitabatake A (2003) Estrogen deprivation and replacement modulate cerebral capillary density with vascular expression of angiogenic molecules in middle-aged female rats. *J Cereb Blood Flow Metab* 23: 181-189
- Kelly RW (2002) Inflammatory mediators and cervical ripening. *J Reprod Immunol* 57:217-224
- Kimura T, Ogita K, Kusui C, Ohashi K, Azuma C, Murata Y (1999) What knockout mice tell us about parturition. *Rev Reprod* 4:73-80
- Liggins GC (1981) Cervical ripening as an inflammatory reaction. In Ellwood DA, Anderson ABM, eds. *The Cervix in Pregnancy and Labor*. Edinburgh, Churchill Livingstone, 1-9
- Maul H, Longo M, Saade GR, Garfield RE (2003) Nitric oxide and its role during pregnancy: from ovulation to delivery. *Curr Pharm Des* 9:359-380
- Matsuzaki N, Taniguchi T, Shimoya K, Neki R, Okada T, Saji F, Nakayama M, et al. (1993) Placenta interleukin-6 production is enhanced in intrauterine infection but not in labor. *Am Obstet Gynecol* 168:94-97
- McDonald DM, Bowden JJ, Baluk P, Bunnett NW (1996) Neurogenic inflammation. A model for studying efferent actions of sensory nerves. *Adv Exp Med Biol* 410:453-462
- McDonald M (2001) Angiogenesis and remodeling of airway vasculature in chronic inflammation. *Am J Respir Crit Care Med* 164: S39-45
- Mowa CN, Papka R (2004) The role of sensory neurons in cervical ripening: effects of estrogen and neuropeptides. *J Histochem Cytochem* 52:1249-1258
- Mowa CN, Usip S, Storey-Workley M, Amann R, Papka R (2003a) Substance P in the uterine cervix, dorsal root ganglia and spinal cord during pregnancy and the effect of estrogen on SP synthesis. *Peptides* 24:761-771
- Mowa CN, Usip S, Storey-Workley M, Hargreaves KM, Papka R (2003b) The effects of pregnancy and estrogen on the expression of calcitonin gene-related peptide (CGRP) in the uterine cervix, dorsal root ganglia and spinal cord. *Peptides* 24:1163-1174
- Mueller MD, Vigne JL, Mincheko A, Lebovic DI, Leitman DC, Taylor RN (2000) Regulation of vascular endothelial growth factor (VEGF) gene transcription by estrogen receptor alpha and beta. *Proc Natl Acad Sci USA* 97:10972-10977
- Murohara T, Horowitz JR, Silver M, Tsurumi Y, Chen D, Sullivan A, Isner JM (1998) Vascular endothelial growth factor/vascular permeability factor enhances vascular permeability via nitric oxide and prostacyclin. *Circulation* 97:99-107
- Mustonen T, Alitalo K (1995) Endothelial receptor tyrosine kinases involved in angiogenesis. *J Cell Biol* 129:895-898
- Okuda Y, Tsurumaru K, Suzuki S, Miyauchi T, Asano M, Hong Y, Sone H, et al. (1998) Hypoxia and endothelin-1 induce VEGF production in human vascular smooth muscle cells. *Life Sci* 63: 477-484
- Papka RE (1990) Some nerve endings in the female rat pelvic para-

- cervical autonomic ganglia and varicosities in the uterus contain calcium gene-related peptide and originate from the dorsal root ganglia. *Neuroscience* 39:459-470
- Papka RE, Collins JJ, McCarson KE (2001) Neuropeptides, neurogenic inflammation and ripening of the uterine cervix. *Neuropeptides 2001: Symposium and Meetings, Jerusalem, Israel. J Mol Neurosci* 16:80.
- Papka RE, Traurig HH (1993) Autonomic and visceral sensory innervation of the female reproductive system: special reference to neurochemical markers in nerves and ganglionic connections. In Maggi CA, ed. *The Autonomic Nervous System. Vol 6. Nervous Control of the Urogenital System*. London, Harwood Academic Publishers, 421-464
- Park JE, Chen HH, Winer J, Houck KA, Ferrara N (1994) Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. *J Biol Chem* 269:25646-25654
- Pokabla MJ, Dickerson IM, Papka RE (2002) Calcitonin gene-related peptide-receptor component protein expression in the uterine cervix, lumbosacral spinal cord, and dorsal root ganglia. *Peptides* 23:507-514
- Robinson CJ, Stringer SE (2001) The splice variants of vascular endothelial growth factor (VEGF) and their receptors. *J Cell Sci* 114:853-865
- Servos S, Zachary I, Martin JF (1999) VEGF modulates NO production: the basis of a cytoprotective effect? *Cardiovasc Res* 41: 509-510
- Sandner P, Wolf K, Bergmaier U, Gess B, Kurtz A (1997) Induction of VEGF and VEGF receptor gene expression by hypoxia: divergent regulation in vivo and in vitro. *Kidney Int* 51:448-453
- Seetharam L, Gotoh N, Maru Y, Neufeld G, Yamaguchi S, Shibuya M (1995) A unique signal transduction from Flt tyrosine kinase, a receptor for vascular endothelial growth factor. *Oncogene* 10: 135-147
- Shalaby F, Rossant J, Yamaguchi TP, Gertszenstein M, Wu XF, Breitman ML, Schuh AC (1995) Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 376:62-66
- Shibuya M (1995) Role of VEGF-Flt-1 receptor system in normal and tumor angiogenesis. *Adv Cancer Res* 67:281-316
- Shibuya M, Ito N, Claesson-Welsh L (1999) Structure and function of VEGF receptor-1 and -2. *Curr Top Microbiol Immunol* 237: 59-83
- Shima DT, Kuroki M, Deutsch U, Ng YS, Adamis AP, D'Amore PA (1996) The mouse gene for vascular endothelial growth factor. Genomic structure, definition of transcriptional unit, and characterization of transcriptional and post-translational regulatory sequences. *J Biol Chem* 271:3877-3883
- Six I, Kureishi Y, Luo Z, Walsh K (2002) Akt signaling mediates VEGF/VPF vascular permeability in vivo. *FEBS Lett* 532:67-69
- Tenore JL (2003) Methods for cervical ripening and induction of labor. *Am Fam Physician* 67:2123-2128
- Terman BI, Dougher-Vermazen M, Carrion ME, Dimitrov D, Armellino DC, Gospodarowicz D, Bohlen P (1992) Identification of the KDR tyrosine kinase, a receptor for vascular endothelial growth factor VEGF. *Biochem Biophys Res Commun* 187:1579-1586
- Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fides JC, Abraham JA (1991) The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *J Biol Chem* 266:11947-11954
- Toi M (1995) Endothelial growth factors: a target for angiogenesis. *Cancer J* 8:315-319
- Tsurumi Y, Murohara T, Krasinski K, Chen D, Witztenbichler B, Kearney M, Couffinhalt TT, et al. (1997) Reciprocal relation between VEGF and NO in the regulation of endothelial integrity. *Nature Med* 3:879-886
- van der Zee R, Murohara T, Luo Z, Zollmann F, Passeri J, Lekutat C, Isner JM (1997) Vascular endothelial growth factor/vascular permeability factor augments nitric oxide release from quiescent rabbit and human vascular endothelium. *Circulation* 95:1030-1037
- Waltertnerberger J, Claesson-Welsh L, Siegbahn A, Shibuya M, Heldrin CH (1994) Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *J Biol Chem* 269:26988-26995
- Yallampalli C, Buhimschi I, Chwalisz K, Garfield RE, Dong YL (1996) Preterm birth in rats produced by synergistic action of a nitric oxide inhibitor (NG-nitro-L-arginine methyl ester) and an antiprogesterin (onapristone). *Am J Obstet Gynecol* 175:207-212
- Yallampalli C, Dong YL, Gangula PR, Fang L (1998) Role and regulation of nitric oxide in the uterus during pregnancy and parturition. *J Soc Gynecol Investig* 5:58-67
- Yang HT, Yan Z, Abraham JA, Terjung RL (2001) VEGF₁₂₁- and bFGF-induced increase in collateral blood flow requires normal nitric oxide production. *Am J Physiol* 280:H1097-1104
- Zachary I, Gliki G (2001) Signaling transduction mechanisms mediating biological actions of the vascular endothelial growth factor family. *Cardiovasc Res* 49:568-581
- Ziche M, Morbidelli L, Choudhuri R, Zhang HT, Donnini S, Granger HJ, Bicknell R (1997) Nitric oxide synthase lies downstream from vascular endothelial growth factor-induced but not basic fibroblast growth factor-induced angiogenesis. *J Clin Invest* 99:2625-2634