Korean J Physiol Pharmacol
Vol 9: 165–172, June, 2005

Expression of Nitric Oxide Synthase and Endothelin-1 in Human Uterine Artery from Full-Term Pregnancies

Ook-Hwan Choi[1], Sun Hee Lee[2], Eun Jin Kim[2], Koan Hoi Kim[2], and Byung Yong Rhim[2]

Departments of [1]Obstetrics and Gynecology and [2]Pharmacology, College of Medicine, Pusan National University, Busan 602–739, Korea

The aim of this study was to determine the roles of ET-1 and NO on uterine blood flow in pregnancy. Uterine arteries were isolated from 17 nonpregnant and 12 pregnant women. Nonpregnant group included patients with median age of 48.6±2.3 years who underwent hysterectomy, because of myoma. Pregnant group included patients with median age of 31.3±1.4 years undergoing cesarean delivery. ET-1 and ET-2 induced concentration-dependent contraction in isolated nonpregnant and pregnant uterine arteries. The contractile response and maximal contraction were increased in pregnant uterine arteries. In nonpregnant uterine arteries, there was no contraction in response to ET-3, whereas pregnancy induced concentration-dependent contraction by ET-3. Tissue nitrite/nitrate level and immunohistochemical staining of eNOS and iNOS were increased in pregnant uterine arteries, compared with nonpregnant uterine arteries. In addition, the expressions of eNOS and iNOS mRNA were significantly increased in pregnancy. Moreover, contractions by ET isopeptides, including ET-1, were enhanced, and immunohistochemical staining of ET-1 and ET-1 mRNA expression was increased in pregnant uterine arteries. These results suggest that NO production by increased NOS activity, especially eNOS activity, is related to placental and uterine blood flow. Furthermore, ET-1 appears to play a pathophysiological role in pregnant complications such as hypertension.

Key Words: Nitric oxide, Endothelin-1, Nitric oxide synthase, Uterine artery, Full-term pregnancy

INTRODUCTION

It is well known that pregnancy induces changes in uterine vascular reactivity to various vasoconstrictors (Griendling et al, 1985; McLaughlin et al, 1989; Weiner et al, 1991; Steele et al, 1993). These changes in vascular reactivity play an important role in mediating elevation of uterine blood flow (Peeters et al, 1980). In addition, several studies have reported that pregnancy decreases vasoconstriction in response to noradrenaline or angiotensin II in uterine artery (Rosenfeld & Naden, 1989; Weiner et al, 1989a), however, increases production of prostacyclin and nitric oxide (Magness et al, 1985; Nelson & Suresh, 1989; Weiner et al, 1989b; Magness, 1991). There have been a great deal of disputes on placental vascular reactivity which affects placental blood flow, because it is different among animal species and varies during pregnancy.

Endothelin-1, a vasoconstrictor peptide isolated from the supernatant of cultured porcine endothelial cells (Yanagisawa et al, 1988), induces a potent and long-lasting contraction in all blood vessels and is known as one of the major factors which induce vasospasm. Endothelins (ET; ET-1, ET-2, ET-3) are a family of 21-amino acid peptides and have been identified in most of the mammalian species (Inoue et al, 1989; Saida et al, 1989), and induce potent contraction in many isolated arteries. These vasoconstrictor effects mediated by ET_A and ET_B receptors vary, depending on species and tissues. Many studies have shown that ET have a significant role in regulating vascular tension (Maggi et al, 1989; Warner et al, 1993; Battistini et al, 1994a; 1994b).

Because there is no distribution of autonomic nerves in the human placenta (Lees et al, 1967), the control of vascular tone via the placenta depends on locally released vascular activating factors (Reilly & Russell, 1977). And ET-1 has significantly been recognized as one of them (Poston et al, 1995): During pregnancy, ET-1 is produced mainly in the placenta and is thought to play an important role in the regulation of blood flow to the developing fetus via the placenta. (Myatt et al, 1992). Futhermore, the plasma ET-1 level increases gradually during normal pregnancy as the pregnancy advances, reaches a plateau in the 3rd trimester of pregnancy, and peaks during labor pain (Usuki et al, 1990). It has been suggested that the ET-1 levels are increased by hypoxia, the damage of vascular endothelial cells or the stimulation of other vascular activating factors. Therefore, ET-1 is expected to play a significant role in the increase of vascular resistance or pathophysiological states such as vasospasm and in regulation of normal blood flow in pregnancy. Furthermore, ET

Corresponding to: Byung Yong Rhim, Department of Pharmacology, College of Medicine, Pusan National University, 10 Ami-dong 1-ga, Seo-gu, Busan 602-739, Korea. (Tel) 82-51-240-7728, (Fax) 82-51-244-1036, (E-mail) byrhim@pusan.ac.kr

ABBREVIATIONS: ET-1, endothelin-1; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; EDRF, endothelium-derived relaxing factors; EDCF, endothelium-derived contracting factors.

is increased during pregnancy, especially in patients with preeclampsia (Iwata et al, 1991), indicating that ET-1 is important in regulation of blood flow in preeclampsia and late pregnancy. (Taylor et al, 1990; Nova et al, 1991). There are numerous studies about pharmacological action, role and characteristics of ET, however, the effects of ET on human vessels, especially in human uterine vessels, are poorly characterized.

Nitric oxide (NO) has been shown to mediate various biological reactions in many internal organs (Moncada, 1992), however, there are not many studies on whether about if NO or NO related substances mediate uterine blood flow.

Furthermore, studies on the relation between NO and gestosis such as hypertension during pregnancy report conflicting results. However, NO activity is elevated with increase of blood flow and decrease of pheripheral resistance in pregnancy, suggesting that NO plays a role in gestosis, but mechanisms involved in increasing NOS activity are largely not clarified (Anumba et al, 1999).

The aim of this study was to determine the roles of ET-1 and NO in uterine blood flow during pregnancy. Thus, we examined 1) the effects of pregnancy on the expression of ET-1 and NOS mRNA in human uterine arteries, and 2) changes of ET-1 and NOS distribution using immunohitochemistry.

METHODS

Tissue preparations

Human uterine arteries were obtained from 17 nonpregnant and 12 pregnant women. Nonpregnant women were those undergoing hysterectomy, because of myoma, while pregnant women were patients undergoing cesarean delivery. No patients with vascular complications such as hypertension, diabetes and arteriosclerosis were included in the study. Patients with preeclampsia were excluded from the pregnant group.

All experimental protocols were approved by the Scientific Committee of Medical Research Institute, Pusan National University Hospital, and the patients' written consent and ethical approval were obtained.

The vessels were placed in cold physiological salt solution (PSS) immediately after hysterectomy and cesarean delivery. Each tissue was cleaned of adhering fat and connective tissue. Some of the isolated uterine arteries were used for the measurement of isometric tension in the very same day, and others were immediately frozen at $-70^{\circ}C$ for the measurement of nitrite/nitrate, immunohistochemistry and RT-PCR.

Measurement of isometric tension

Uterine arteries were placed in a wax block containing oxygenated physiological salt solution (PSS), and fat and connective tissue were removed. Uterine arteries were cut into ring (2~3 mm) and mounted on parallel wires in 5 ml muscle chamber which were thermoregulated at 37°C. The medium (PSS) consisted of 130 mM NaCl, 4.7 mM KCl, 1.18 mM NaH_2PO_4, 1.17 mM $MgSO_4$, 1.6 mM $CaCl_2$, 14.9 mM $NaHCO_3$ and 5.5 mM dextrose and was maintained at pH 7.4 with 95% O_2~5% CO_2. Rings were stretched to optimal resting tensions of 2.0 g. Isometric tension was measured using Polygraphy (Grass Instrument Co., 7E), force-displacement transducer (Grass Instrument Co., FT03) and was recorded on a computer by use of chart v 3.6/s software and a MacLab 8/e data acqusition System (ADInstruments). Following equilibration period, the rings were exposed to 60 mM KCl, and contractile response was obtained. The concentration of agonist required to produce a half maximal response, EC_{50}, was obtained from each agonist dose-response curves.

Measurement of nitrite/nitrate level

The uterine arteries were frozen in liquid nitrogen and homogenized in 5 volumes of 0.1 M phosphate buffer. The homogenates were centrifuged (1,500×g for 20 min), and protein concentration in the supernatant was measured by Bio-Rad protein assay (Bradford, 1976) method. Subsequently, an equal volume of Griess reagent (800 μl) was added to deproteinated tissue homogenate, and they were reacted for 10 min at 25°C. Nitrite concentrations were determined by measuring optical density at 554 nm and by comparing with standard solutions of sodium nitrite (Green et al, 1982).

Immunohistochemistry

The uterine arteries were placed in mixture of 0.01 M picric acid and 2% paraformaldehyde (in 0.1 M sodium phosphate buffer) for fixation. Then, the sections were sequentially immersed in 7.5%, 15% and 30% sucrose solution to dehydrate and then frozen after embedding in OCT compound (Tissue-Tek, Miles Scientific Inc.). The sections were cut in 5 μm thick sections and mounted on poly-L-lysine coated slide glass and air-dried overnight. After fixation in cold acetone for 20 min, the sections were exposed to 0.3% hydrogen peroxide solution for 20 min to inhibit endogenous peroxidase activity and washed three times in phosphoric buffer solution (PBS) at 10-min interval. The sections were sequentially exposed to 2% bovine serum albumin (BSA, blocking antibody) for 1 hour to block nonspecific binding of antibody, and then to ET-1 antibody (mouse monoclonal, anti-ET-1, Oncogene Research Products), eNOS antibody (mouse monoclonal, Oncogene Research Products) and iNOS antibody (mouse monoclonal, Oncogene Research Products) diluted in buffer (20 mM PBS contained 0.05% BSA) at 4°C overnight. They were then washed with PBS. Biotinylated goat anti-mouse IgG (Oncogene Research Products) was added to the sections and incubated for 2 hours. After washing with PBS, the sections were incubated with avidin and biotinylated horseradish peroxidase macromolecular complex (Vector Laboratories Inc., Burlingame, USA) for 1 hour. Peroxidase activity was visualized using diaminobenzidine (DAB) containing substrate solution (Vector Laboratories, Inc.). The sections were dehydrated in graded alcohols and xylen, and coverslipped with malinol (Muto Pure Chemicals Ltd.). Quantitative analysis of eNOS, iNOS and ET-1 was performed with image analysis system (Media Cybernetics, Silver Spring, USA).

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated by TRIzol reagent. Concentrations of RNA were determined by measuring absorbance

at 260 nm (A_{260}). The A_{260}/A_{280} ratio of the samples ranged from 1.5 to 2.0 RNA was then reverse transcribed in 50 ml of reaction mixture containing 100 U MMLV reverse transcriptase. The sense primer for ET-1 was 5'-TGCT CCTGCTCGTCCCTGATGGATAAAGAG-3', and the antisense primer was 5'-GGTCACATAACGCTCTCTGGAGGGCTT-3'. The sense primer for eNOS was 5'-CGGCTTGTCACCTCCT GG-3' and the antisense primer was 5'-GACATTGAGAGCA AAGGGCTGC-3'. The sense primer for iNOS was 5'-TCCAT GCAGACAACCTT-3', and the antisense primer was 5'-GCC TCGCTCTGGAAAGA-3'. The sense primer for GAPDH was 5'-TGAAGTCAGAGGAGACCACC-3', and the antisense primer was 5'-CTTCACCACCATGGAGGAG G-3'. Each 50 μl reaction mixture contained 1 μl of cDNA, 1 μl of each primer (100 pmol/L), 1 unit of Taq DNA polymerase, 5 μl of 10× Taq polymerase buffer, and optimal concentration of $MgCl_2$. Samples were placed onto a thermal cycler and preheated for 1 min at 94°C.

Each cycle consisted of three periods : denaturation for 1 min at 95°C, annealing for 1 min at 55°C for ET-1 and iNOS, and at 56°C for eNOS, and extension for 1 min at 72°C. The PCR products were separated by 2% agarose gel electrophoresis.

Statistics

Values are expressed as means±SEM. Results were statistically evaluated by Students' t-test for the differences between pregnant and nonpregnant groups. $P<0.05$ was accepted as statistically significant.

RESULTS

Selection of patients

In the present study, uterine arteries were isolated from 17 nonpregnant and 12 pregnant women. Nonpregnant group included patients undergoing hysterectomy because of myoma, and their median age was 48.6±2.3 years (range, 19~82 years), gravity 3.43±0.45, and parity 3.15± 0.32. Pregnant group included patients undergoing cesarean delivery with a median age of 31.3±1.4 years (range, 24~39 years), gravity of 2.64±0.43, parity of 1.27±0.28, and gestational age of 38.3±0.4 weeks (Table 1).

KCl-induced contraction

KCl-induced contraction was tested to observe the change of contractile response by pregnancy. Contraction in response to 60 mM KCl by hyperpolarization was suppressed in pregnant uterine arteries ($P<0.05$) (Fig. 1).

Table 1. Clinical profile of patients subjected to experiment

	Nonpregnant (n=17)	Pregnant (n=12)
Age (yrs)	48.6±2.3 (39~82)	31.3±1.4 (24~39)
Gravidity	3.43±0.45 (3~8)	2.64±0.43 (1~6)
Parity	3.15±0.32 (1~5)	1.27±0.28 (0~2)
Gestational age (wks)	–	38.3±0.4 (31~40)

Values are expressed as mean±SEM (range).

Contractions in nonpregnant and pregnant uterine arteries by ET derivatives

ET-1 and ET-2 induced concentration-dependent contraction in isolated nonpregnant and pregnant uterine arteries. The contractile response and maximal contraction were increased in pregnant uterine arteries. In addition, EC_{50} (the concentration producing 50% of the maximal response) of ET-1 and ET-2 were decreased from 6.3×10^{-8} M to 1.6×10^{-8} M and 126.0×10^{-8} M to 1.9×10^{-8} M, respectively. In nonpregnant uterine arteries, there was no contraction in response to ET-3, whereas pregnancy induced concentration-dependent contraction to ET-3, and the maximal contraction was 58.1±11.2% at the concentration of 10^{-7} M. ET contractions to peptides were expressed as a percentage of the contraction to 60 mM KCl (Fig. 2).

Nitric oxide contents

Nitrite/nitrate concentration in nonpregnant uterine arteries was 0.45±0.12 nmol/mg protein, and it was significantly increased to 0.94±0.27 nmol/mg protein in pregnant uterine arteries (Fig. 3).

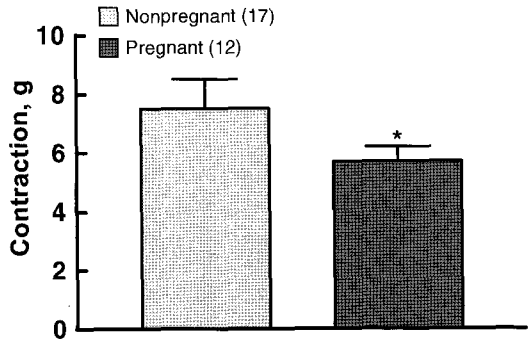

Fig. 1. Increase in active tension (contraction, g) to 60 mM KCl. Responses are expressed as mean±S.E.M. Numbers in parentheses represent the number of materials. *P<0.05 vs. Nonpregnant.

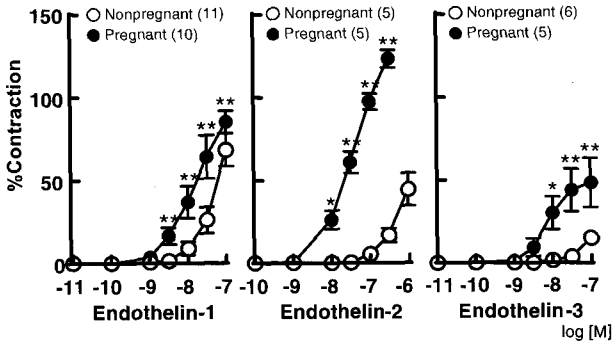

Fig. 2. Concentration-dependent contractile responses to endothelins in isolated uterine arteries of uterus from nonpregnant and pregnant women. Values are expressed as means±S.E.M. Contraction to peptides is expressed as a percentage of the contraction to 60 mM KCl. Numbers in parentheses represent the number of experiments. *P<0.05; **P<0.01 vs. Nonpregnant.

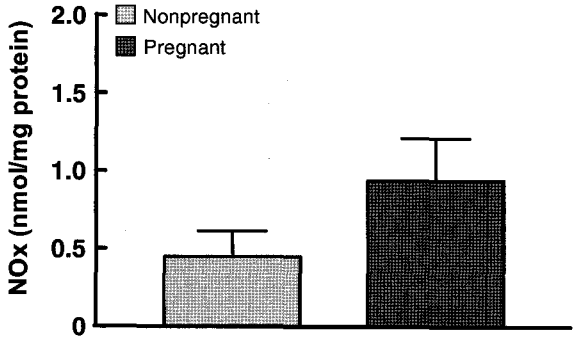

Fig. 3. Nitrite/nitrate (NOx) levels in the uterine artery from nonpregnant and pregnant women. Values are expressed as means ±S.E.M. from 4~8 experiments.

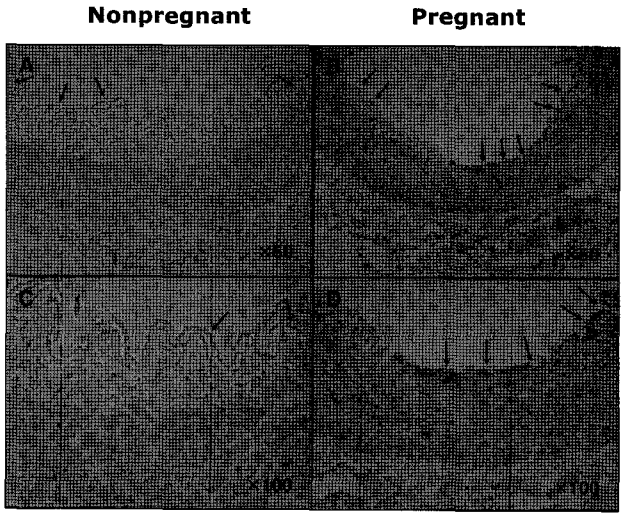

Fig. 4. Representative visualization by immunohistochemistry of the distribution of ET-1 in the section of uterine artery from nonpregnant (A and C) and pregnant (B and D) women. The expression of ET-1 was enhanced in luminal endothelium (arrows) of the artery from pregnant. Magnification: ×50 (A, B), ×100 (C, D).

The distribution of ET-1 by immunohistochemistry

We examined the distribution of ET-1 in uterine arteries. The immunohistochemical staining of ET-1 was enhanced in pregnancy. Quantitative analysis showed that ET-1 level was increased two-fold in pregnant uterine arteries, compared with nonpregnant uterine arteries (Figs. 4 and 7).

Distribution of NO synthase by immunohistochemistry

In nonpregnant uterine arteries, there was moderate staining of eNOS in the endothelium, but the staining of eNOS was greatly increased in pregnant uterine arteries (Fig. 5). Densitometric analysis of the immunohistochemical staining revealed that eNOS was increased approximately two-fold in pregnant uterine arteries, compared with nonpregnant uterine arteries (Fig. 7).

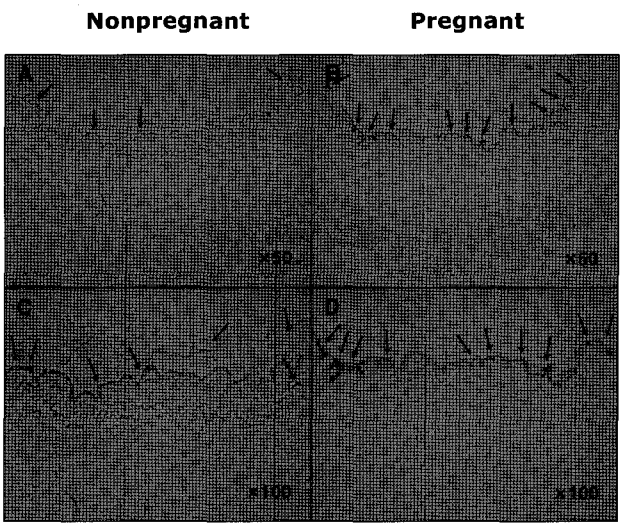

Fig. 5. Representative visualization by immunohistochemistry of the distribution of eNOS in the section of uterine arteries from nonpregnant (A and C) and pregnant (B and D) women. The expression of eNOS was enhanced in luminal endothelium (arrows) of the artery from pregnant. Magnification: ×50 (A, B), ×100 (C, D).

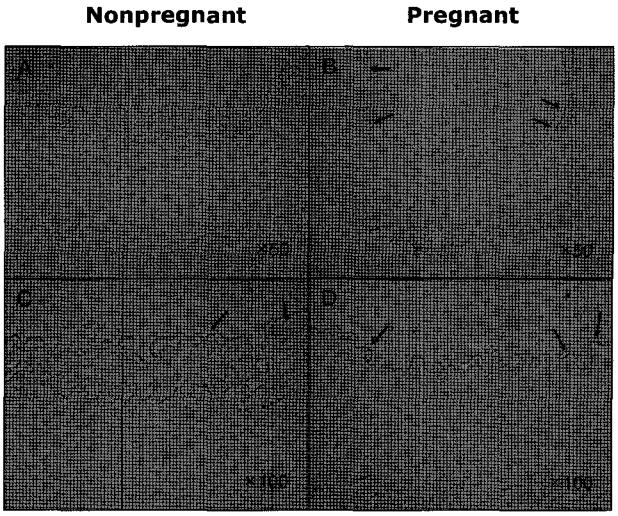

Fig. 6. Representative visualization by immunohistochemistry of the distribution of iNOS in the section of uterine artery from nonpregnant (A and C) and pregnant (B and D) women. Magnification: 50 (A, B),×100 (C, D).

Fig. 6 shows the distribution of iNOS, revealed by immunohistochemistry. Little iNOS was detected in nonpregnant uterine arteries, however, increased in pregnant uterine arteries. Quantitative analysis showed increased iNOS in pregnancy (Fig. 7). In nonpregnant uterine arteries, the quantity of iNOS was 10% of that of eNOS, however, it was increased to 30% in pregnant uterine arteries.

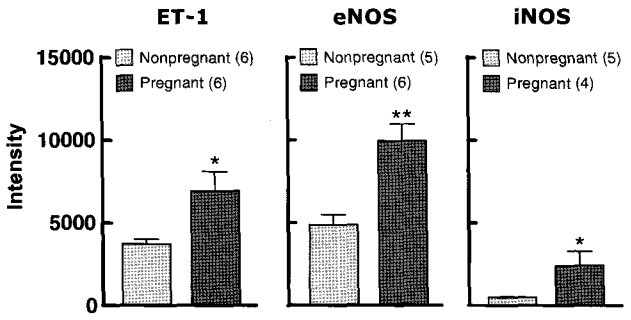

Fig. 7. Densitometric analysis of the immunohistochemical staining of ET-1, eNOS and iNOS. Results are represented as mean±S.E.M. of uterine arterial segments obtained from nonpregnant and pregnant women. Numbers in parentheses represent the number of experiments. *P<0.05; **P<0.01 vs. Nonpregnant.

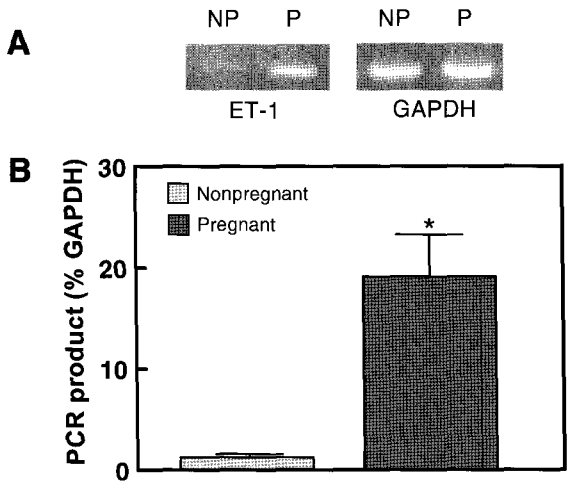

Fig. 8. Uterine arterial mRNA expression of the ET-1 genes. (A) Ethidium bromide stained gels of RT-PCR products that reflect ET-1 mRNA abundance in uterine artery mRNA from nonpregnant (NP) and pregnant (P) women. (B) Densitometeric analysis of the RT-PCR products. Results are expressed as means±S.E.M. from 4~5 experiments. *P<0.05 vs. Nonpregnant.

Expression of ET-1 mRNA by RT-PCR

RT-PCR was performed to determine the expression of ET-1 mRNA in human uterine arteries. ET-1 mRNA was scarcely detected in nonpregnant uterine arteries, but it was significantly increased in pregnant uterine arteries ($P < 0.05$) (Fig. 8).

Expression of NOS mRNA by RT-PCR

We then determined the expression of eNOS and iNOS mRNA by RT-PCR. eNOS mRNA was highly expressed in both groups, showing a tendency to increase in pregnant uterine arteries (Fig. 9). A small amount of iNOS mRNA was detected in nonpregnant uterine arteries, while it was markedly increased in pregnant uterine arteries (Fig. 10).

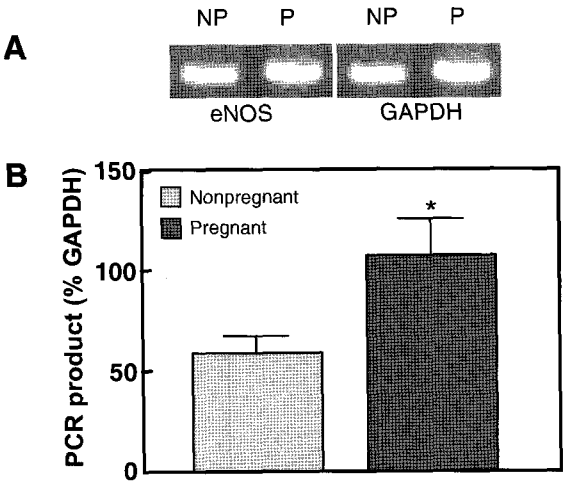

Fig. 9. Uterine arterial mRNA expression of the eNOS genes. (A) Ethidium bromide stained gels of RT-PCR products that reflect eNOS mRNA abundance in uterine artery mRNA from nonpregnant (NP) and pregnant (P) women. (B) Densitometeric analysis of the RT-PCR products. Results are expressed as means± S.E.M. from 4~5 experiments. *P<0.05 vs. Nonpregnant.

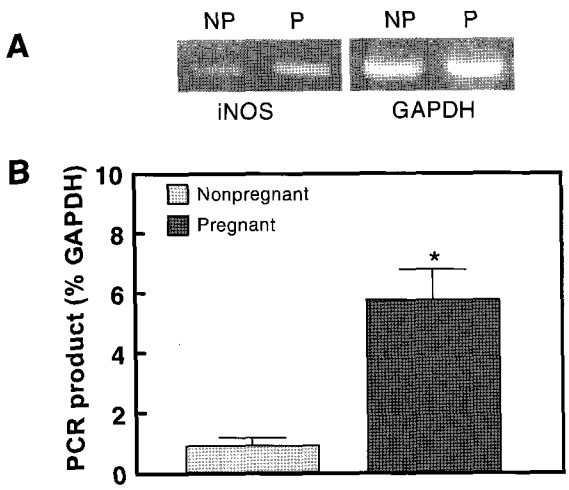

Fig. 10. Uterine arterial mRNA expression of the iNOS genes. (A) Ethidium bromide stained gels of RT-PCR products that reflect iNOS mRNA abundance in uterine artery mRNA from nonpregnant (NP) and pregnant (P) woman. (B) Densitometeric analysis of the RT-PCR products. Results are expressed as means±S.E.M. from 4~5 experiments. *P<0.05 vs. Nonpregnant.

DISCUSSION

The present study, contractions showed that contraction of human uterine arteries in response to ET-2 and ET-3 as well as ET-1 were enhanced. The immunohistochemical staining showed that the distribution of ET-1 centering around vascular endothelium was markedly increased in pregnant uterine arteries, suggesting that pregnancy increased the sensitivity of ET receptors, accompanied by increased expression of ET-1 mRNA (Napolitano et al, 2000; Dotsch et al, 2001). These findings are in good

agreement with other results. Thus, it seems quite likely that increased ET-1 level is associated with the regulation of placental and uterine blood flow in late pregnancy, although the mechanism is not yet fully understood.

Based on dynamical reports, pregnancy induces hypertension in 7~10% of late pregnant women, however, it seems clear that increased ET-1 level and enhanced sensitivity of uterine vessels by ET-1 are closely related to hypertension in late pregnancy, and they contribute to the development of preeclampsia. This hypothesis is supported by the facts that plasma ET-1 level is increased in the patients with preeclampsia (Nishikawa et al, 2000; Lowe, 2000), and that expression of ET-1 mRNA is increased in uterine vessels (Napolitano et al, 2000). In our preliminary study, ET was found to contract human uterine artery. ET induced much more potent contraction than other contractile agonists such as noradrenaline and 5-hydroxytryptamine (unpublished results).

Such strong vascular contraction was also shown in other vessels such as coronary artery, renal artery and arteries in skeletal muscle (Lippton et al, 1988; Franco-Cereceda, 1989; King et al, 1989; Pernow, 1989). ET-1 induced a slow and long-term contraction, and the contraction started at low concentration of 10^{-9} M and lasted for more than 1 hour, suggesting that ET-1 plays a role in vascular contractile disease related to uterine blood flow (Fried & Samuelson, 1991). This hypothesis is supported by the reports that plasma ET-1 level was elevated in disease states, including preeclampsia (Nova et al, 1991; Schiff et al, 1992), and also in normal pregnancy with no complications (Iwata et al, 1991). Therefore, we hypothesized that ET could play an important role in the regulation of uterine blood flow, expecially in late pregnancy, and investigated in the present study the responses to ET isopeptides in nonpregnant and pregnant uterine arteries.

As a preliminary experiment to test contraction of ET-1, we examined 60 mM KCl-induced contraction, resulting in suppression of contraction in pregnant uterine arteries. However, ETs-induced contraction was significantly increased in pregnant uterine arteries. ET-3 induced no contraction in nonpregnant uterine arteries, but induced a typical concentration-dependent contraction in pregnant uterine arteries. These results demonstrate that pregnancy leads to increase of sensitivity to ETs in human uterine arteries.

Furthermore, in this study, contractions in response to ET-1, ET-2 and ET-3 were found to be enhanced, manifesting either the increase of receptors or the augmented sensitivity of receptors. There are two main endothelin receptors, identified in human, ET_A and ET_B. It is known that ET_A receptor has the highest selectivity to ET-1 (Arai et al, 1990), and ET_B receptor has an equal selectivity to ET-1, ET-2 and ET-3. Sarafotoxin S6c is a highly selective ET_B agonist (Sakurai et al, 1990; Williams et al, 1991). Thus, our present results suggest that pregnancy stimulates the sensitivity of both ET_A and ET_B receptors. However, mechanisms involved in the increased activity of ET receptors remain uncertain at present.

NO is synthesized from the terminal guanidino nitrogen atom of L-arginine by NO synthase, also producing L-citrulline (Marletta, 1989; Lowenstein & Snyder, 1992). It is diffusible and rapidly destroyed by interaction with oxygen, making measurement difficult (Archer, 1993). Therefore, most of the studies on the role of NO have been performed using N-substituted L-arginine analogues which competitively inhibit the activity of NO synthase (NOS).

Three different isoforms of NOS have so far been identified; two constitutive isoforms, including neuronal NOS (nNOS) and endothelial NOS (eNOS), and inducible isoform (iNOS) first isolated from macrophage (Nathan, 1992). nNOS and eNOS are calmodulin-dependent and activated by intracellular Ca^{2+}, producing NO (Moncada, 1992). Small amounts of iNOS are found under normal condition, and it is induced by endotoxin, cytokine, gamma-interferon or lipopolysaccharide, and participates in DNA replication and protein synthesis. Being different from constitutive isoforms, iNOS has a Ca^{2+}-dependent activity and continuously produces NO.

In the current study, nitrite/nitrate level, which represents NO concentration, was increased by pregnancy, which means enhanced NOS activity. This result is in accord with other studies which reported that NO level was increased in pregnant uterine arteries (Conrad et al, 1989; 1993; Weiner et al, 1989b; Li et al, 1996; Magness et al, 1996; 1997). Therefore, it can be concluded that pregnancy induces NO production and that the NO contributes to the increase of uterine blood flow. This is further supposed by reports that the relaxation by acetylcholine, endothelium-dependent relaxing agent, was enhanced in late pregnancy, and it was suppressed by NOS inhibitors (Nelson et al, 1995; 1998; 2000).

Then, a question arises which mechanism is involved in increasing. NO production in pregnant uterine artery? Therefore, we examined whether it is by the enhanced activity of eNOS or iNOS, enhanced activity of NOS is related to increased expression of NOS mRNA and NOS is expressed in endothelium or in smooth muscle. Immunohistochemical result showed that both eNOS and iNOS were significantly increased in pregnant uterine endothelium, and that the increased NOS activity in pregnant smooth muscle was mainly due to increased eNOS activity (Nelson et al, 2000).

According to Nelson et al (2000), pregnancy increases Ca^{2+}-dependent NOS activity and eNOS protein expression, resulting in increase of NO production. In addition, bradykinin, known to increase cGMP levels by releasing NO from the endothelium, produced a much greater increase in cGMP levels in pregnant arteries than in nonpregnant arteries. Thus, it can reasonably be concluded that the pregnancy-enhanced NOS activity in uterine arteries reflects mainly an increased eNOS activity.

Our immunohistochemical results also revealed that eNOS is distributed more than 10 fold than iNOS, and this difference of distribution was maintained during pregnancy. In addition, our RT-PCR experiment showed that, in nonpregnant uterine arteries, eNOS mRNA was expressed as much as 60% of GAPDH mRNA expression, but iNOS was expressed less than 1%, indicating that iNOS has no physiological significance in normal nonpregnant states. However, two times more eNOS was expressed in pregnancy than in nonpregnancy. Pregnancy also increased iNOS mRNA expression, but it was just about 5% of GAPDH mRNA expression, implying that it is not pathophysiologically significant. Although iNOS mRNA expression was relatively small, compared with eNOS mRNA expression, pregnancy increased iNOS mRNA expression more than 10 fold, therfore, iNOS is expected to play some role in increasing uterine blood flow in late pregnancy. However, more future studies should be carried out.

In summary, the above results suggest that NO produc-

tion by increased NOS activity, especially eNOS, is related to placental and uterine blood flow. ET-1 also appears to play a pathophysiological role in pregnant complications such as hypertension.

ACKNOWLEDGEMENT

This study was supported by Medical Research Institute Grant (2002-20), Pusan National University Hospital.

REFERENCES

Anumba DO, Robson SC, Boys RJ, Ford GA. Nitric oxide activity in the peripheral vasculature during normotensive and pre-eclamptic pregnancy. *Am J Physiol* 277: H848-H854, 1999

Arai H, Hori S, Aramori I, Ohkubo H, Nakanishi S. Cloning and expression of a cDNA encoding an endothelin receptor. *Nature* 348: 730-732, 1990

Archer S. Measurement of nitric oxide in biological models. *FASEB J* 7: 349-360, 1993

Battistini B, Warner TD, Fournier A, Vane JR. Characterization of ET_B receptors mediating contractions induced by endothelin-1 or IRL 1620 in guinea-pig isolated airways: Effects of BQ-123, FR139327 or PD145065. *Br J Pharmacol* 111: 1009-1016, 1994a

Battistini B, O'Donnell LJ, Warner TD, Fournier A, Farthing MJ, Vane JR. Characterization of endothelin (ET) receptors in the isolated gall bladder of the guinea-pig: evidence for an additional ET receptor subtype. *Br J Pharmacol* 112: 1244-1250, 1994b

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-254, 1976

Conrad K, Vill M, McGuire PG, Dail WG, Davis AK. Expression of nitric oxide synthase by syncytiotrophoblast in human placental villi. *FASEB J* 7: 1269-1276, 1993

Conrad K, Vernier KA. Plasma level, urinary excretion, and metabolic production of cGMP during gestation in rats. *Am J Physiol* 257: R847-R853, 1989

Dotsch J, Hogen N, Nyul Z, Hanze J, Knerr I, Kirschbaum M, Rascher W. Increase of endothelial nitric oxide synthase and endothelin-1 mRNA expression in human placenta during gestation. *Eur J Obstet Gynecol Reprod Biol* 97: 163-167, 2001

Franco-Cereceda A. Endothelin- and neuropeptide Y-induced vasoconstriction of human epicardial coronary arteries in vitro. *Br J Pharmacol* 97: 968-972, 1989

Fried G, Samuelson U. Endothelin and neuropeptide Y are vasoconstrictors in human uterine blood vessels. *Am J Obstet Gynecol* 164: 1330-1336, 1991

Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. *Anal Biochem* 126: 131-138, 1982

Griendling KK, Fuller EO, Cox RH. Pregnancy-induced changes in sheep uterine and carotid arteries. *Am J Physiol* 248: H658-H665, 1985

Inoue A, Yanagisawa M, Kimura S, Yoshitoshi K, Miyauchi T, Goto K, Masaki T. The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. *Proc Natl Acad Sci USA* 86: 2863-2867, 1989

Iwata I, Takagi T, Yamaji K, Tanizawa O. Increase in the concentration of immunoreactive endothelin in human pregnancy. *J Endocrinol* 129: 301-307, 1991

King AJ, Brenner BM, Anderson S. Endothelin: a potent renal and systemic vasoconstrictor peptide. *Am J Physiol* 256: F1051-F1058, 1989

Lees MM, Taylor SH, Scott DB, Kerr MG. A study of cardiac output at rest throughout pregnancy. *J Obstet Gynaecol Br Commonw* 74: 319-328, 1967

Li P, Tong C, Eisenach JC. Pregnancy and ephedrine increase the release of nitric oxide in ovine uterine arteries. *Anesth Analg* 82: 288-293, 1996

Lippton H, Goff J, Hyman A. Effects of endothelin in the systemic and vascular beds in vivo. *Eur J Pharmacol* 155: 440-444, 1988

Lowe DT. Nitric oxide dysfunction in the pathophysiology of preeclampsia. *Nitric Oxide* 4: 441-458, 2000

Lowenstein CJ, Snyder SH. Nitric oxide: a novel biologic messenger. *Cell* 70: 705-707, 1992

Maggi CA, Giuliani S, Patacchini R, Rovero P, Giachetti A, Meli A. The activity of peptides of the endothelin family in various mammalian smooth muscle preparations. *Eur J Pharmacol* 174: 23-31, 1989

Magness RR, Osei-Boaten K, Mitchell MS, Rosenfeld CR. In vitro prostacyclin production by ovine uterine and systemic arteries. *J Clin Invest* 76: 2206-2212, 1985

Magness RR. Endothelium-derived vasoactive substances and uterine blood vessels. *Semin Perinatol* 15: 68-78, 1991

Magness RR, Rosenfeld CR, Hassan A, Shaul PW. Endothelial vasodilator production by uterine and systemic arteries. I. Effects of ANG II on PGI_2 and NO in pregnancy. *Am J Physiol* 270: H1914-H1923, 1996

Magness RR, Shaw CE, Phernetton TM, Zheng J, Bird IM. Endothelial vasodilator production by uterine and systemic arteries. II. Pregnancy effects on NO synthase expression. *Am J Physiol* 272: H1730-H1740, 1997

Marletta MA. Nitric oxide biosynthesis and biological significance. *Trends Biol Sci* 14: 488-492, 1989

McLaughlin MK, Keve TM, Cooke R. Vascular catecholamine sensitivity during pregnancy in the ewe. *Am J Obstet Gynecol* 160: 47-53, 1989

Moncada S. The 1991 Ulf von Euler Lecture. The L-arginine: nitric oxide pathway. *Acta Physiol Scand* 145: 201-227, 1992

Myatt L, Brewer AS, Brockman DE. The comparative effects of big endothelin-1, endothelin-1, and endothelin-3 in the human fetal-placental circulation. *Am J Obstet Gynecol* 167: 1651-1656, 1992

Napolitano M, Miceli F, Calce A, Vacca A, Gulino A, Apa R, Lanzone A. Expression and relationship between endothelin-1 messenger ribonucleic acid (mRNA) and inducible/endothelial nitric oxide synthase mRNA isoforms from normal and pre-eclamptic placentas. *J Clin Endocrinol Metab* 85: 2318-2323, 2000

Nathan C. Nitric oxide as a secretory product of mammalian cells. *FASEB J* 6: 3051-3064, 1992

Nelson SH, Suresh MS. Pregnancy: endothelium-dependent cholinergic dilation of human uterine arteries [Abstract D43]. In: *Proceedings of the annual meeting of the Society of Obstetrical Anesthesiologists and Perinatologists.* San Francisco, Society of Obstetrical Anesthesiologists and Perinatologists, 1989

Nelson SH, Steinsland OS, Johnson RL, Suresh MS, Gifford A, Ehardt JS. Pregnancy-induced alterations of neurogenic constriction and dilation of human uterine artery. *Am J Physiol* 268: H1694-1701, 1995

Nelson SH, Steinsland OS, Suresh MS, Lee NM. Pregnancy augments nitric oxide-dependent dilator response to acetylcholine in the human uterine artery. *Hum Reprod* 13: 1361-1367, 1998

Nelson SH, Steinsland OS, Wang Y, Yallampalli C, Dong YL, Sanchez JM. Increased nitric oxide synthase activity and expression in the human uterine artery during pregnancy. *Circ Res* 87: 406-411, 2000

Nishikawa S, Miyamoto A, Yamamoto H, Ohshika H, Kudo R. The relationship between serum nitrate and endothelin-1 concentrations in preeclampsia. *Life Sci* 67: 1447-1454, 2000

Nova A, Sibai BM, Barton JR, Mercer BM, Mitchell MD. Maternal plasma level of endothelin is increased in preeclampsia. *Am J Obstet Gynecol* 165: 724-727, 1991

Peeters LL, Grutters G, Martin CB. Distribution of cardiac output in the unstressed pregnant guinea pig. *Am J Obstet Gynecol* 138: 1177-1184, 1980

Pernow J. Actions of constrictor (NPY and endothelin) and dilator (substance P, CGRP and VIP) peptides on pig splenic and human

skeletal muscle arteries: involvement of the endothelium. *Br J Pharmacol* 97: 983–989, 1989

Poston L, McCarthy AL, Ritter JM. Control of vascular resistance in the maternal and feto-placental arterial beds. *Pharmacol Ther* 65: 215–239, 1995

Reilly RD, Russell PT. Neurohistochemical evidence supporting an absence of adrenergic and cholinergic innervation in the human placenta and umbilical cord. *Anat Rec* 188: 277–286, 1977

Rosenfeld CR, Naden RP. Uterine and nonuterine vascular responses to angiotensin II in ovine pregnancy. *Am J Physiol* 257: H17–H24, 1989

Saida K, Mitsui Y, Ishida N. A novel peptide, vasoactive intestinal constrictor of a new (endothelin) peptide family. *J Biol Chem* 264: 14613–14616, 1989

Sakurai T, Yanagisawa M, Takuwa Y, Miyazaki H, Kimura S, Goto K, Masaki T. Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature* 348: 732–735, 1990

Schiff E, Ben-Baruch G, Peleg E, Goldenberg M, Rosenthal T, Alcalay M, Devir M, Mashiach S. Immunoreactive circulating endothelin-1 in normal and hypertensive pregnancies. *Am J Obstet Gynecol* 166: 624–628, 1992

Steele SC, Warren AY, Johnson IR. Effect of the vascular endothelium on norepinephrine-induced contractions in uterine radial arteries from the nonpregnant and pregnant human uterus. *Am J Obstet Gynecol* 168: 1623–1628, 1993

Taylor RN, Varma M, Teng NN, Roberts JM. Women with preeclampsia have higher plasma endothelin levels than women with normal pregnancies. *J Clin Endocrinol Metab* 71: 1675–1677, 1990

Usuki S, Saitoh T, Sawamura T, Suzuki N, Shigemitsu S, Yanagisawa M, Goto K, Onda H, Fujino M, Masaki T. Increased maternal plasma concentration of endothelin-1 during labor pain or on delivery and the existence of a large amount of endothelin-1 in amniotic fluid. *Gynecol Endocrinol* 4: 85–97, 1990

Warner TD, Allcock GH, Mickley EJ, Vane JR. Characterization of endothelin receptors mediating the effects of the endothelin/sarafotoxin peptides on autonomic neurotransmission in the rat vas deferens and guinea-pig ileum. *Br J Pharmacol* 110: 783–789, 1993

Weiner CP, Martinez E, Chestnut DH, Ghodsi A. Effect of pregnancy on uterine and carotid artery response to norepinephrine, epinephrine and phenylephrine in vessels with documented functional endothelium. *Am J Obstet Gynecol* 161: 1605–1610, 1989a

Weiner CP, Martinez E, Zhu LK, Ghodsi A, Chestnut D. In vitro release of endothelium-derived relaxing factor by acetylcholine is increased during the guinea pig pregnancy. *Am J Obstet Gynecol* 161: 1599–1605, 1989b

Weiner CP, Kang-Zhu L, Thompson LP, Herrig J, Chestnut DH. Effect of pregnancy on endothelium and smooth muscle: their role in reduced adrenergic sensitivity. *Am J Physiol* 261: H1275–H1283, 1991

Williams DL, Jones KL, Pettibone DJ, Lis EV, Clineschmidt BV. Sarafotoxin S6c: an agonist which distinguishes between endothelin receptor subtypes. *Biophys Biochem Res Commun* 175: 556–561, 1991

Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 332: 411–415, 1988