Regulation of myometrial contractivity during pregnancy in the rat: potential role for DDAH

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ABSTRACT: There has been little information demonstrating the roles of dimethylarginine dimethylaminohydrolase (DDAH), which is the hydrolyzing enzyme of endogenous nitric oxide synthase (NOS) inhibitors and, in turn, modulates the intracellular concentrations of NOS inhibitors, in the myometrium during the course of pregnancy. Therefore, the present experiments were designed to investigate whether or not DDAH activity, protein and mRNA expression levels are altered during gestation of the rat and, if altered, those changes reflect on the levels of endogenous inhibitors and endothelin-1 (ET-1), and NO-dependent cyclic GMP generation in the myometrium. The up-regulated changes in DDAH activity, DDAH-2 protein and DDAH-2 mRNA expression at mid-gestation were accompanied by the reduced mono-methylarginine and asymmetric dimethylarginine as NOS inhibitors, and ET-1 levels, and by the enhanced NO-dependent cyclic GMP production. At term gestation, on the other hand, down-regulated changes in DDAH activity, DDAH-2 protein and DDAH-2 mRNA expression were accompanied by the increased NOS inhibitors and ET-1 levels, and decreased NO-dependent cyclic GMP generation. These results suggest that alterations in DDAH/NOS inhibitors/NO-dependent cyclic GMP/ET-1 pathway are possibly involved in maintaining myometrial quiescence during gestation and controlling delivery at term.

Key words: DDAH-1 and DDAH-2 / MMA and ADMA / NOS / NO-dependent cyclic GMP / endothelin-1

Introduction

Mechanisms of inducing uterine quiescence during gestation and initiating the delivery at term are not fully understood. Since it has been reported that nitric oxide (NO) has an ability to produce relaxation of the myometrium (Izumi et al., 1993; Kaya et al., 1998), attention has been paid to the role of NO for the uterine quiescence. However, according to Hennan and Diamond (1998), Word and Cornwell (1998) and Momohara et al. (2004), NO per se does not cause directly the myometrial relaxation although the NO-cyclic GMP generation system exists in rat myometrium. The failure of the uterus to relax in the face of cyclic GMP elevation and cyclic GMP-dependent protein kinase (PKG) activation is suggested to be due to a lack of phosphorylation of the PKG substrate identified in the rat aorta (Hennan and Diamond, 2001). In addition, Word and Cornwell (1998) clearly demonstrated that myometrial tissues from pregnant rats were not sensitive to relaxation by sodium nitroprusside (SNP) as an NO donor and that the insensitivity to SNP was accompanied by progesterone-mediated decreases in the level of PKG expression.

In addition to those findings, we have reported that exogenously applied endothelin-1 (ET-1) causes myometrial contractions under the non-pregnant state and greatly increased the myometrial contractions and tone at term gestation (Sakamoto et al., 1999), and that the decreased NO production due to accumulated endogenous NO synthase (NOS) inhibitors such as monomethylarginine (MMA) and asymmetric dimethylarginine (ADMA) results in the increased ET-1 level, thereby increasing myometrial contractions and tone at term gestation (Momohara et al., 2004). At term gestation, indeed, the determined concentrations of MMA and ADMA sufficiently inhibited NOS activity and impaired NO-dependent cyclic GMP production (Momohara et al., 2004).

As one of the mechanisms of accumulating MMA and ADMA, it is worthy to note the impaired activity of dimethylarginine dimethylaminohydrolase (DDAH), an enzyme that hydrolyzes MMA and ADMA to L-citrulline and methylamines (Ogawa et al., 1989). DDAH is
composed of two isoforms, DDAH-1 and DDAH-2 (Leiper et al., 1999). These isoforms have distinct tissue distributions, but similar enzymatic activities (MacAllister et al., 1996; Leiper et al., 1999). DDAH-1 is typically found in tissues expressing neuronal NOS, whereas DDAH-2 predominates in tissues containing endothelial NOS (Leiper et al., 1999). Changes in DDAH activity may play important roles in regulating the intracellular MMA and ADMA levels. The decreased DDAH activity possibly brings about an accumulation of the NOS inhibitors in myometrium, resulting in the impairment of NO production and, in turn, increases the ET-1 level (Momohara et al., 2004), which accelerates the myometrial contractions and tone. Therefore, the present experiments were designed to investigate whether or not DDAH activity, protein and mRNA expression levels are altered during gestation of the rat and, if altered, those changes reflect on the levels of endogenous NOS inhibitors and ET-1, and NO-dependent cyclic GMP generation in the myometrium.

Materials and Methods

Animals and tissues
Female Wistar rats, 12–18 week of age, were mated in the evening at pro-estrus cycle. If sperm was observed in the vaginal smear the next morning, that day was defined as Day 0 of gestation. Rats in estrus (non-pregnant) and at 7th, 14th and 22nd day of gestation were sacrificed by exsanguination under anesthesia with ether and hysterectomy. Immediately after the hysterectomy, both uterine horns were opened longitudinally, and fetuses and placentas were separated. The uteri were immersed in oxygenated and ice-cold modified Krebs solution (mmol/l) (115 NaCl, 4.7 KCl, 1.2 MgSO4·7H2O, 2.5 CaCl2·2H2O, 1.2 KH2PO4, 10 NaHCO3 and 10 glucose, pH 7.4) and stored at −80°C except for the fresh specimens for measuring cyclic GMP. For determinations of DDAH activity, DDAH-1 and DDAH-2 mRNA, protein expression levels, ET-1 concentrations and endogenous NOS inhibitors concentrations, endometrium and deciduous were removed with surgical knife. Histological examination was performed to confirm the successful removal of the endometrium.

The present experimental protocol complied with and was approved by the Animal Welfare Regulation of Tokyo Medical and Dental University and the Guiding Principles for the Care and Use of Laboratory animals approved by the Japanese Pharmacological Society.

Measurement of DDAH activity

The DDAH activity was determined by measuring the conversion of [H3]-MMA (N0-monomethyl-L-arginine (2,3,4-3H)) to l-[H3]-citrulline as described previously (Sasaki et al., 2007). Briefly, myometrial specimens were homogenized at 4°C in a buffer (0.1 mol/l sodium phosphate, 1 mmol/l phenylmethylsulphonylfluoride (PMSF), 2 mol/l 2-mercaptoethanol, 1 µmol/l pepstatin A and 2 µmol/l leupeptin) and sonicated at 50 W and 28 kHz for 15 s. The homogenates were centrifuged at 10,000 g for 20 min. Trichloroacetic acid (TCA) was added to 100 g/ml to precipitate proteins and followed by the centrifugation at 10,000 g for 20 min. The supernatants were filtered through a membrane filter (0.45 µm) and incubated with 500 µl of the supernatants (10 µg protein/µl) in a Polytron at a maximum speed for 20 s in 5 mol/l HEPES buffer. The homogenate was centrifuged at 10,000 g for 4°C for 20 min. The incubation was terminated by keeping the tubes on ice for 5 min. Samples were applied to a 1-ml column of AG 50W-X8 (Na+ form) to remove unmetabolized [3H]-MMA. The columns were then washed with 1.5 ml distilled water, and l-[H3]-citrulline was quantified in the flow-through fraction using a liquid scintillation counter (TRI-CARB 2750TR/LL; Packard Instrument, Meriden, CT, USA). DDAH activity was expressed as nmol l-citrulline/mg protein/2 h.

Determination of DDAH-1 and DDAH-2 mRNA

The rat DDAH-1 and DDAH-2 mRNA were quantified by RT–PCR. Total RNA was extracted using ISOGEN (Nippon gene, Tokyo, Japan) according to the manufacturer’s protocol. First-strand cDNA was synthesized by the reverse transcription using Superscript Firststrand System (Invitrogen, Carlsbad, CA, USA) equipped with program temperature control system (PC-818, ASTEC, Fukuoka, Japan). PCR condition was set at 94°C for 30 s, 55°C for 1 min and 72°C for 1 min, 27 cycles each. Primer design was as follows: DDAH-1-forward: 5'-CAACAGGAGTGC TGAAGATCTTGCC-3', DDAH-1-reverse: 5'-GGTAGCAGTAGGTGTC CTTAGGC-3', DDAH-2-forward: 5'-GCCACGACTAGTGCTCGACGT TC-3', DDAH-2-reverse: 5'-GGTACCGTGAAGACGGCAAGTC-3', GAPDH-forward: 5'-TCCCTCAAGATTGTCGCAA-3' and GAPDH-reverse: 5'-AGATCCCAACCGGATACATT-3'. The fluorescence data were analyzed using WELTEC Dolphin-DOC (Kurabo, Tokyo).

Western blotting or DDAH-1 and DDAH-2 proteins

Western blotting was performed as described previously (Momohara et al., 2004; Sakurada et al., 2008). Myometrial specimens were homogenized at 4°C in a lysis buffer containing 50 mmol/l Tris (pH 7.5), 300 mmol/l NaCl, 1% Triton X-100, 2 mol/l EDTA, 1 mmol/l PMSF and 2 µmol/l leupeptin and sonicated at 50 W and 28 kHz for 15 s. The homogenates were centrifuged at 10,000 g for 20 min at 4°C. After determining the protein concentration with bicinchoninic acid protein assay kit (Bio-Rad Laboratories), aliquots containing 20 µg of protein were separated by SDS–polyacrylamide gel electrophoresis using 12% gel and blotted onto polyvinylidene difluoride membranes (Amersham, Arlington Heights, IL, USA) in a wet transfer unit (Bio-Rad Laboratories). After blocking with 5% non-fat dry milk, the membranes were incubated overnight at 4°C with the anti-DDAH-1 antibody or DDAH-2 antibody. Primary antibodies used specifically recognized DDAH-1 (1:100), DDAH-2 (1:500, Abcam, Cambridge, MA, USA) and β-actin (1:500, Sigma, St Louis, MO, USA). Results were expressed as mean ratio of the DDAH-1 or DDAH-2 protein density to that of β-actin.

Measurement of endogenous NOS inhibitors

MMA and ADMA as endogenous NOS inhibitors within the myometrium were determined by means of automated HPLC according to the method described previously (Azuma et al., 1995, 1997; Hamasaki et al., 1997). In brief, myometrial specimens were minced with scissors and homogenized in a Polytron at a maximum speed for 20 s in 5 mol/l HEPES buffer. The homogenate was centrifuged at 10,000 g for 4°C for 20 min. Trichloroacetic acid (TCA) in a final concentration of 5% was added to the supernatant to precipitate proteins and followed by the centrifugation at 3000 g and 4°C for 15 min. The 100 µl of supernatant was used as a sample for HPLC.

Measurement of cyclic GMP level

Freshly isolated myometrial specimens (longitudinal specimens of ~10 mg wet weight) were pre-incubated in modified Krebs solution for 60 min at 37°C, transferred into fresh Krebs solution and followed by further 40 min.
incubation until the specimens were rapidly transferred into 10% TCA with liquid nitrogen in order to stop the reaction. All experiments were performed in the presence of 10 μmol/l 3-isobuty-1-methylxanthine as a non-selective inhibitor of phosphodiesterases. The cyclic GMP level was determined by radioimmunoassay kit according to the method described previously (Momohara et al., 2004; Hirata et al., 2006). The net production of cyclic GMP was expressed as the difference between the production with 300 μmol/l l-arginine and that with 300 μmol/l l-arginine plus 100 μmol/l nitroarginine as an inhibitor of NOS.

Measurement of ET-1 content within the myometrium

ET-1 within the myometrium was extracted according to the method described previously (Beppu et al., 2002). Briefly, myometrial specimens were minced with scissors and homogenized in a Polytron at a maximum speed for 20 s to a 25% homogenate in extracting buffer (1 mmol/l acetic acid containing 0.01% Triton X-100 and 10 μg/ml peptatin A). The homogenate was centrifuged at 25 000g for 30 min at 4°C after boiling in water for 10 min to inactivate neutral endopeptidase. Supernatant was collected and octadecylsilyl suspension was added to adsorb ET-1. This solution was centrifuged at 3000g, 4°C for 3 min. The solvent (4% acetic acid and 86% ethanol) was added to the precipitate and centrifuged at 3000g, 4°C for 3 min. Supernatant was evaporated and the residue was dissolved in 100% ethanol and evaporated again. The residue was dissolved finally in the buffer provided in the assay kit. ET-1 concentration was determined by ELISA kit for ET-1 (Wako Pure Chemical Industries, Japan) according to the method described previously (Momohara et al., 2004).

Statistical analyses

All values are expressed as mean ± SEM. Multiple comparisons between two groups were made by one-way analysis of variance. A value of P < 0.05 was considered statistically significant.

Results

DDAH activity

The DDAH activity was determined to be 598.0 ± 90.0 fmol l-citrulline/mg protein/2 h in the non-pregnant myometrium (n = 4) and significantly enhanced at early (7th day) and mid-gestation (14th day) (P < 0.05 and P < 0.001), with the highest value of 1601.6 ± 152.1 fmol l-citrulline/mg protein/2 h at mid-gestation. At term gestation (22nd day), on the other hand, the value was significantly (P < 0.05) reduced when compared with the highest value at mid-gestation. These results are shown in Fig. 1.

DDAH-1 and DDAH-2 mRNA expression

Changes in myometrial DDAH-1 and DDAH-2 mRNA during gestation were determined. As shown in Fig. 2, RT–PCR revealed that DDAH-2 mRNA was significantly increased at mid-gestation (14th day) (P < 0.05), whereas DDAH-1 mRNA remained unchanged during gestation. Although the expression of DDAH-2 mRNA tended to be increased at 7th and 22nd day of gestation, those values were not significantly different from non-pregnant myometrium.

DDAH-1 and DDAH-2 protein expression

DDAH-1 protein was undetectable both in the non-pregnant and gestational myometrium. In contrast, DDAH-2 protein was detectable
in all myometrial specimens determined (non-pregnant, 7th, 14th and 22nd day of gestation). The DDAH-2 protein expression was significantly ($P < 0.05$) increased at mid-gestation (14th day) and decreased at term gestation (22nd day) (Fig. 3).

Changes in endogenous NOS inhibitors

In the non-pregnant myometrium, the contents of endogenous NOS inhibitors were determined to be $1.17 \pm 0.09$ nmol/g wet weight for MMA ($n = 3$) and $5.80 \pm 0.36$ nmol/g wet weight for ADMA ($n = 3$). Interestingly, the contents were significantly ($P < 0.005$) decreased ($0.53 \pm 0.03$ nmol/g wet weight for MMA, $n = 3$ and $1.83 \pm 0.34$ nmol/g wet weight for ADMA, $n = 3$) at mid-gestation (14th day), whereas significantly ($P < 0.005$) increased at term gestation (22nd day) ($3.83 \pm 0.26$ nmol/g wet weight for MMA, $n = 3$ and $8.70 \pm 0.38$ nmol/g wet weight for ADMA, $n = 3$). On the basis of the tissue water content at term gestation ($85.1 \pm 0.8\%$, $n = 5$), apparent concentrations of MMA and ADMA were estimated to be $3.26 \pm 0.22$ μmol/l ($n = 3$) and $10.22 \pm 0.44$ μmol/l ($n = 3$), respectively. MMA and ADMA effectively inhibited the NOS activity at the estimated concentrations ($51.3 \pm 1.1\%$ at $3.26$ μmol/l for MMA, $n = 3$ and $33.2 \pm 0.8\%$ at $10.22$ μmol/l for ADMA, $n = 3$). Results are shown in Fig. 4A as a value of MMA plus ADMA (nmol/g wet weight).

Changes in cyclic GMP production

In the non-pregnant myometrium, the basal cyclic GMP level was determined to be $0.30 \pm 0.06$ pmol/mg protein ($n = 3$). The nucleotide production was significantly ($P < 0.005$) increased in the presence of $300$ μmol/l L-arginine ($1.19 \pm 0.20$ pmol/mg protein, $n = 3$), which was significantly ($P < 0.005$) reduced by $100$ μmol/l nitroarginine as an NOS inhibitor ($0.35 \pm 0.14$ pmol/mg protein, $n = 3$). Net production expressed as the difference in the presence or absence of nitroarginine can be calculated as $0.84 \pm 0.16$ pmol/mg protein ($n = 3$). As shown in Fig. 4B, not only the basal level but also the net production of cyclic GMP was significantly increased at mid-gestation (14th day), whereas those were decreased at term gestation (22nd day).

ET-1 content within the myometrium

Results are shown in Fig. 4C. ET-1 was detectable at a level of $861 \pm 55$ pg/g wet weight in the non-pregnant myometrium ($n = 3$). Whereas, the content was significantly ($P < 0.005$ versus corresponding value in non-pregnant myometrium) decreased at mid-gestation (14th day) ($547 \pm 45$ pg/g wet weight, $n = 3$). Moreover, the ET-1 content was significantly ($P < 0.005$ versus corresponding values in non-pregnant and at mid-gestation) increased at term gestation ($2231 \pm 167$ pg/g wet weight, $n = 3$).

Discussion

To our knowledge, there has been little information demonstrating the roles of DDAH in the myometrium during the course of pregnancy. Therefore, the present experiments were designed to investigate the roles of DDAH/NOS inhibitors/NO-dependent cyclic GMP/ET-1 pathway during gestation in the rat.

The up-regulated changes in DDAH activity, DDAH-2 protein and DDAH-2 mRNA expression at mid-gestation were accompanied by the reduced MMA and ADMA as endogenous NOS inhibitors, and ET-1 levels, and by the enhanced NO-dependent cyclic GMP production. It is well established that MMA and ADMA are hydrolyzed by DDAH and converted to L-citrulline and methylamines (Ogawa et al., 1989). Therefore, the hydrolyzing enzyme regulates the intracellular concentrations of NOS inhibitors (Ito et al., 1999), resulting in the significant changes observed in the present study.
in the changes in NO production (Momohara et al., 2004). If these findings are considered together, the enhanced DDAH activity due to up-regulated DDAH-2 mRNA and DDAH-2 protein at mid-gestation brings about the reduced concentrations of NOS inhibitors, and thus, accelerates NO/cyclic GMP generation pathway. The enhanced cyclic GMP generation was associated with significantly reduced myometrial ET-1 content, possibly reducing the myometrial contractility for uterine quiescence during gestation. This assumption appears to be supported in part by the findings that the enhanced NO-dependent cyclic GMP generation decreases ET-1 content in the myometrium (Momohara et al., 2004) and that ET-1 content becomes lower as endogenous NOS inhibitors were decreased (Momohara et al., 2004). In addition to these findings, exogenously applied ET-1 causes myometrial contractions (Sakamoto et al., 1999).

DDAH is expressed as type-I and type-2 (Leiper et al., 1999). DDAH-1 is widely expressed, especially in the liver (Nijveldt et al., 2003) and kidney (Tojo et al., 1997). DDAH-2 is expressed in the blood vessels, endothelium, heart, kidney and placenta (Aylng et al., 2006). In the present experiments, we demonstrated that functionally predominant DDAH isoform was DDAH-2 in the rat myometrium, since DDAH-1 mRNA was detectable but remained unchanged during gestation and DDAH-1 protein was undetectable in this tissue in spite of observable signals in liver in the preliminary experiments (data not shown). This dissociation between mRNA expression and protein level might be due to less sensitivity of the antibody for protein determination or modulation at the post-transcriptional step. However, further study should be performed to improve our understanding.

At term gestation, on the other hand, down-regulated changes in DDAH activity, DDAH-2 protein and DDAH-2 mRNA expression were accompanied by the increased NOS inhibitors and ET-1 levels, and decreased NO-dependent cyclic GMP production. The reduced DDAH activity due to down-regulated DDAH-2 mRNA and DDAH-2 protein brings about the accumulation of NOS inhibitors, and thus impairs NO/cyclic GMP generation pathway at term gestation. In addition to the effect of reduced DDAH activity on accumulation of MMA and ADMA, it seems reasonable to assume that the transmembrane transport of these NOS inhibitors would be enhanced at term, since the content of symmetric dimethylarginine, which is not a substrate for DDAH (Ogawa et al., 1989), was increased at term, although the content remained unchanged until 20th day of gestation (Momohara et al., 2004). The impaired NO-dependent cyclic GMP generation was associated with significantly increased myometrial ET-1 content, which possibly increases the myometrial contractions and tone for the labor onset. Sakamoto et al. (1999) demonstrated that the myometrial contractions and tone caused by exogenously applied ET-1 were greatly enhanced at term gestation. Moreover, ET-1 content became higher as endogenous NOS inhibitors (MMA and ADMA) were accumulated and as NO-dependent cyclic GMP generation was decreased (Momohara et al., 2004).

DDAH is subject to extensive post-transcriptional modulation by factors including sexual steroids, oxidative stress and intracellular signaling. DDAH activity is positively regulated by an antioxidant (Jiang et al., 2006), estradiol (Holden et al., 2003; Monsalve et al., 2007) and an antagonist of the transcription factor NFκB (Yang et al., 2005), whereas the enzyme activity was negatively regulated by lipopolysaccharide (Xin et al., 2007), high concentrations of glucose (Sorrenti et al., 2006), TNF-α (Ito et al., 1999) and cholesterol (Monsalve et al., 2007) in vitro. DDAH activity in the myometrium did not coincide with the plasma levels of estradiol, which peaks in early pregnancy (Watson et al., 1975). On the contrary, the changes in the time course of DDAH activity and DDAH-2 expression during pregnancy coincided well with peripheral plasma progesterone levels with highest levels at 13th day of gestation and decline near labor in the rats (Farina et al., 2004). These results indicate that the progesterone is possibly involved...
in the regulation of uterine DDAH-2 expression. However, further studies should be performed to improve our understanding of the regulation mechanisms of gestational changes in DDAH-2 expression.

In conclusion, we suggest that alterations in DDAH/NOS inhibitors/NO-dependent cyclic GMP/ET-1 pathway are possibly involved in maintaining myometrial quiescence during gestation and controlling delivery at term.

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