Nitric oxide increases matrix metalloproteinase-1 production in human uterine cervical fibroblast cells

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Since uterine cervical ripening is an active biochemical process similar in part to an inflammatory reaction, nitric oxide (NO) has been proposed as a key mediator of this event. However, the mechanism by which NO modulates human cervical ripening has not been fully elucidated. In the present study we investigated the presence of NO synthases in human uterine cervix by immunohistochemistry and reverse transcriptase–polymerase chain reaction analysis. Furthermore, we examined the presence of NO-mediated regulation of matrix metalloproteinase-1 (MMP-1) production in cultured human uterine cervical fibroblast cells using enzyme-linked immunosorbent assay and Northern blot analysis. Endothelial and inducible NO synthases were detected in the form of mRNA and protein expression in pregnant uterine cervix. Interleukin-1α (IL-1α) increased the expression of inducible NO synthase mRNA in cultured human uterine cervical fibroblast cells. IL-1α also increased MMP-1 secretion from the cultured cervical fibroblast cells. This IL-1α-augmented MMP-1 secretion was partially but significantly blocked by an NO synthase inhibitor, Nω-nitro-L-arginine methyl ester. On the other hand, NO donors increased MMP-1 production in the cultured cervical fibroblast cells. These findings together suggest that NO contributes to the process of cervical ripening via enhancement of MMP-1 production, and that IL-1α increases MMP-1 secretion from cervical fibroblasts at least in part via NO synthesis.

Key words: interleukin-1α/matrix metalloproteinases/nitric oxide/nitric oxide synthase/uterine cervical ripening

Introduction

Uterine cervical ripening and dilatation are essential events in a non-complicated vaginal delivery. Uterine cervical ripening is an active biochemical process similar in part to an inflammatory reaction, and involves a complex cascade of sequential reactions, including degradation of extracellular matrix proteins and glycoproteins, disruption of tightly aligned collagen fibrils, and increase in hydration caused by hyaluronan (Junqueira et al., 1980; Uldbjerg et al., 1983a; El Maradny et al., 1997).

The central event in the complicated process of cervical ripening is the degradation of collagens, since the human uterine cervix is composed mainly of fibroblast cells and fibrous connective tissues in which collagen and glycosaminoglycans predominate (Rorie and Newton, 1967; Uldbjerg et al., 1983b). Accordingly, collagenases, recently termed matrix metalloproteinases (MMPs), are believed to be the key enzymes in the degradation of collagens during cervical ripening (Ito et al., 1991, 1998; Imada et al., 1997). The MMPs are a family of at least 16 different types of zinc-dependent enzymes, capable of degrading collagen as well as other extracellular matrix components such as proteoglycans, fibronectin and laminin present in the interstitial matrix and basement membranes (Hulboy et al., 1997; Konttinen et al., 1999). Each MMP has substrate selectivity. The major extracellular matrix proteins in the human uterine cervix are collagen type I and type III (Kleissl et al., 1978; Minamoto et al., 1987) which are the preferred substrates of MMP-1 (Hulboy et al., 1997). Thus, it is plausible that MMP-1 is involved in the degradation of collagens during the process of cervical ripening. Indeed, MMP-1 mRNA expression has been reported in cultured human uterine cervical fibroblast cells (Sugano et al., 2000) and in cultured human uterine cervical smooth muscle cells (Watarai et al., 1999).

Nitric oxide (NO), originally identified as a potent vasodilative substance, has been reported to contribute to the maintenance of pregnancy, by allowing myometrial relaxation and an increase in uterine blood flow during pregnancy (Weiner et al., 1994; Kublickiene et al., 1997; Magness et al., 1997). We
have previously reported that maternal plasma NO metabolite concentrations increase during pregnancy, and that the maternal plasma concentration of cyclic guanosine 3′,5′-monophosphate (cGMP), a second messenger of NO, is increased in women with mild pre-eclampsia, supporting the possibility that NO plays physiological roles in pregnancy (Itoh et al., 1997; Nanno et al., 1998).

Nitric oxide is also involved in inflammatory reactions (Cohen et al., 1998). The induction of inducible nitric oxide synthase (iNOS) can generate a sustained elevation of NO concentrations which may exert various pro-inflammatory effects, including vasodilation, oedema, cytotoxicity, tissue remodelling, and various other cytokine-dependent processes. Since uterine cervical ripening is an active biochemical process in some respects similar to an inflammatory reaction (Junqueira et al., 1980), the possible involvement of NO in cervical ripening has been proposed (Calder, 1998; Chwalisz and Garfield, 1998; Norman et al., 1998). Indeed, the augmentation of iNOS mRNA expression has been reported in the process of uterine cervical ripening in rats and humans (Ali et al., 1997; Tschugguel et al., 1999; Ledingham et al., 2000). Moreover, it has been reported that in-vivo treatment with NO donors causes uterine cervical ripening in rats, guinea-pigs and humans (Chwalisz et al., 1997; Thomson et al., 1998; Shi et al., 2000).

Thus, NO may be one of the more important modulators in the process of cervical ripening. However, Ledingham et al. have reported that NO donors do not alter MMP-2 or MMP-9 secretion from cultured human uterine cervical fibroblast cells (Ledingham et al., 1999). Thus, as far as we are aware, the mechanism by which NO induces uterine cervical ripening has not been fully elucidated. To clarify the possible effect of NO on the degradation of uterine cervical collagens, we investigated (i) whether iNOS and endothelial nitric oxide synthase (eNOS) are expressed in the uterine cervix; (ii) whether interleukin-1 (IL-1), one of the key modulators of cervical ripening (García-Velasco and Arici, 1999), augments iNOS mRNA expression in cultured uterine cervical fibroblast cells; and (iii) whether NO increases MMP-1 production in cultured cervical fibroblast.

Materials and methods

Reagents

All reagents were of analytical grade and purchased from Sigma Chemical Co. (St Louis, MO, USA), unless otherwise indicated.

Materials

Uterine cervical tissues were obtained with written informed consent from pre-menopausal non-pregnant (n = 5) and pregnant women (n = 7) who had undergone total hysterectomy for gynaecological reasons such as uterine cervical cancer (7, 9 and 11 weeks of gestation) ovarian cancer (12 weeks of gestation), and uterine leiomyoma (n = 8: five non-pregnant women in the early follicular phase and three women at 10, 10 and 11 weeks of gestation). Tissues were snap-frozen by liquid nitrogen and kept at −80°C until assayed. The experimental protocol was approved by our institutional ethical committee.

Figure 1. Immunofluorescence staining of vimentin (A), cytokeratin (B), α-smooth muscle actin (C), and normal mouse IgG negative control (D) in cultured human uterine cervical fibroblast cells at the sixth passage. Uterine cervical fibroblast cells were isolated from pregnant uterine cervical tissues as described in Materials and methods. Positive vimentin staining was detected in 99% of cells. By contrast, cytokeratin and α-smooth muscle actin were stained in <1% of cells. Original magnification ×400.

Preparation of cultured human uterine cervical fibroblast cells (CxF cells)

Cervical stromal tissues were obtained from non-pregnant women (n = 3) and first trimester pregnant women (n = 4: 9, 10, 10 and 11 weeks of gestation), rinsed several times in 10 mmol/l phosphate-buffered saline and minced into ~2 mm pieces, which were placed on 6 cm collagen-coated culture dishes and gently covered with a thin micro cover glass. Cells were cultured in Minimum Essential Medium (MEM) (Gibco BRL, Rockville, MD, USA) containing 10% fetal bovine serum, at 37°C in 5% air and 5% CO2 under humidified conditions. The cells from the tissues were grown to passage 6, and then used as CxF cells. At each passage, an aliquot of the cells was placed on several Neoprene-coated slide glasses (Nissin EM, Tokyo, Japan) and incubated for 24 h, and then fixed in acetone at −20°C for 5 min. The slides were incubated with anti-cytokeratin (1:100; Dako Co., Carpinteria, CA, USA), anti-vimentin (1:100; Dako), or anti-α-smooth muscle actin (1:100; Dako) monoclonal antibodies for 30 min at room temperature. After a 30 min incubation with fluorescein isothiocyanate-conjugated anti-mouse IgG rabbit serum as a second antibody (40 µg/ml; Dako), the slides were mounted with Perma Fluor Aqueous Mounting Medium (Immunocon, Pittsburgh, PA, USA), and examined under a fluorescence microscope by two individuals.

The immunofluorescence study showed 99% positive staining for vimentin in cervical fibroblast cells at the sixth passage (Figure 1A), and <1% positive staining for both cytokeratin (Figure 1B) and α-smooth muscle actin (Figure 1C), indicating a high purity of cervical fibroblast cells. A negative control using normal mouse IgG showed minimal staining (Figure 1D). Thus, the cervical fibroblast cells at the sixth passage were confirmed to be of high purity. These cells were therefore used in the further experiments in this study.
Experimental protocol

When CxF cells at the sixth passage reached confluence, the medium was replaced with fresh MEM without serum. Subsequently, the cells were further incubated for 24 h in 12-well or 10 cm collagen-coated plates in the presence or absence of several substances. The culture medium was then collected and used for the MPM-1 enzyme-linked immunosorbent assay (ELISA) and the cells were used for reverse transcriptase–polymerase chain reaction (RT–PCR) and/or Northern blot analysis. The substances tested were sodium nitroprusside (SNP; a NO donor, 10−7–10−4 mol/l), diethylnitramine nitric oxide (DETA-NO; another NO donor, 10−6–10−4 mol/l), 8-bromo-1,2-guanosine cyclic monophosphate (8-bromo-cGMP; a membrane-permeable derivative of the NO second messenger, cGMP, 5×10−5–5×10−3 mol/l), Nω-nitro-l-arginine methyl ester (l-NAMe; an inhibitor of NO, 10–20 mmol/l), and interleukin-1α (IL-1α, 1.0 ng/ml; a kind donation from Daiichi Pharmaceutical Co. Ltd, Tokyo, Japan). All media samples were kept at −20°C until assayed.

Immunohistochemical analysis of iNOS and eNOS expression

Uterine cervical specimens were included in OCT compound (Sakura Finetek Inc., Torrance, CA, USA), and stored at −80°C. The sections were incubated for 1 h at room temperature with anti-iNOS monoclonal antibody (1.25 µg/ml) or anti-eNOS monoclonal antibody (2.5 µg/ml) (Transduction Co, Lexington, KY, USA). Staining was performed using a biotinylated horse anti-mouse IgG as the secondary antibody and the avidin–biotin–peroxidase (ABC) method (Elite ABC; Vector Laboratories, Burlingame, CA, USA) with 3,3′-diaminobenzidine (DAB).

RT–PCR analysis of iNOS and eNOS mRNA expression

To examine the expression of iNOS and eNOS, RT–PCR analysis was carried out. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene expression was also measured by RT–PCR as a control. Total RNA was extracted as previously described (Masuzaki et al., 1997). After reverse transcription of 2 µg of total RNA from human pregnant or non-pregnant uterine cervix or CxF cells using oligo(dT) primer (Promega, Madison, WI, USA) and Superscript™ II (Gibco BRL, Rockville, MD, USA), the resulting single-stranded cDNA was subjected to PCR. Forward and reverse primers used for amplification of the human iNOS (Chartrain et al., 1994) and eNOS coding regions (Nadaud et al., 1994) were: iNOS forward: 5′-GAGCTTCTACCTC-AAGCTATTC-3′; iNOS reverse: 5′-CTCTGATGGTGACTTTGGCTAG-3′; eNOS forward: 5′-GCACAGGAAGTTGTTCACTAC-3′; and eNOS reverse: 5′-CACAGTGTTGACTTTGGCTAG-3′. Forward and reverse primers for the human G3PDH coding region were purchased from Clontech Laboratories, Inc. (Palo Alto, CA, USA). The PCR was performed using TaKaRa Tag™ (Takara Shuzo Co. Ltd, Otso, Shiga, Japan), which recognizes both proMMP-1 and the active mature form of MMP-1, as total MMP-1. The ELISA was conducted in duplicated wells and inter- and intra-assay variations of this system were 10% each.

ELISA for MMP-1

The MMP-1 concentrations in the culture media from CxF cells were measured using a commercially available ELISA kit (Nippon Organon K.K., Osaka, Japan), which recognizes both proMMP-1 and the active mature form of MMP-1, as total MMP-1. The ELISA was conducted in duplicated wells and inter- and intra-assay variations of this system were 10% each.

Comparison between CxF cells established from pregnant women and those from non-pregnant women

In the current study, the responses of CxF cells from first trimester pregnant women (four cell lines) were similar to those from non-pregnant women (three cell lines), with respect to augmentation of iNOS expression by IL-1α, increase in MMP-1 secretion in response to NO donors, and the blocking effect of L-NAME on IL-1α-induced enhancement of MMP-1 secretion (data not shown). Based on these findings, we have only presented the data from experiments with the CxF cells established from pregnant women.

Statistical analysis

Values were expressed as mean ± SEM of quadruplicate dishes. Experiments were repeated three times and representative data are presented. The statistical significance was assessed by both the Mann–Whitney U-test and analysis of variance (ANOVA) followed by Fisher’s protected least significant difference test. P < 0.05 was regarded as significant.

Results

Expressions of iNOS and eNOS in pregnant uterine cervical tissues and CxF cells

Strongly positive staining (brown) for iNOS was detected in cervical glandular cells and vascular endothelial cells of the
uterine cervix from first trimester pregnant women (Figure 2A). Staining in the stromal cells was diffuse but clearly positive (Figure 2A). These results indicate the production of NO in uterine cervical tissue. Positive staining for eNOS was mainly detected in cervical glandular cells and vascular endothelial cells (Figure 2B). Negative controls using normal mouse IgG showed minimal staining (Figure 2C).

RT–PCR analysis demonstrated iNOS mRNA expression in the uterine cervix from the first trimester of pregnancy (Figure 3A; lanes 1,2) as well as in the CxF cells (Figure 3A; lanes 3,4). On the other hand, eNOS mRNA expression was detected in the uterine cervix in the first trimester of pregnancy (Figure 3B; lanes 1,2), but not in the CxF cells (Figure 3B; lanes 3,4). G3PDH mRNA expression is shown in Figure 3C as a control.

**Figure 2.** Immunohistochemistry of inducible nitric oxide synthase (iNOS; A) and endothelial nitric oxide synthase (eNOS; B) in uterine cervical tissues at 9 weeks of gestation. Positive staining is brown. The negative controls, using normal mouse IgG, show minimal staining (C). The panels are representative stainings of four first trimester specimens (7, 9, 10 and 12 weeks of gestation). Original magnification ×200.

**Figure 3.** Reverse transcription–polymerase chain reaction analysis of inducible nitric oxide synthase (iNOS; A), endothelial nitric oxide synthase (eNOS; B), and glyceraldehyde-3-phosphate dehydrogenase (G3PDH; C). The expected final products from human iNOS, eNOS, and G3PDH cDNA were 312, 645 and 452 bp respectively. Lanes 1 and 2: uterine cervix at 10 weeks of gestation; lanes 3 and 4: cultured human uterine cervical fibroblast cells prepared from uterine cervix at 10 weeks of gestation.

**Figure 4.** The effect of interleukin-1α (IL-1α) on the inducible nitric oxide synthase (iNOS) mRNA expression in cultured human uterine cervical fibroblast (CxF) cells. Quantitative reverse transcriptase–polymerase chain reaction analysis of iNOS mRNA expression in CxF cells was performed using real time Taqman™ technology. The columns and error bars indicate the mean values [iNOS mRNA/glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA] from quadruplicate wells ± SEM respectively. Data are representative of three independent experiments with CxF cells prepared from uterine cervical tissue at 10 weeks of gestation. *P < 0.05 versus vehicle control.

**Effect of IL-1α treatment on iNOS mRNA expression in CxF cells**

Quantitative RT–PCR using Taqman™ technology revealed that iNOS mRNA expression (iNOS mRNA/G3PDH mRNA) in CxF cells after 3 and 6 h of treatment with 1.0 ng/ml IL-1α was 0.16 ± 0.02 and 0.35 ± 0.09 respectively. Both of these values were significantly higher than those in vehicle controls (0.01 ± 0.01 each) (n = 3; P < 0.05 for both comparisons, Figure 4). However, iNOS mRNA expression
Nitric oxide increases MMP-1

The present study demonstrated for the first time that the NO donors, SNP and DETA-NO, could augment MMP-1 mRNA expression in, and MMP-1 secretion from, cultured human uterine cervical fibroblast cells. A possible contribution of NO to the regulation of MMP-1 production has been previously reported for chondrocytes (Murrell et al., 1995; Lo et al., 1998). Since MMP-1 has a substrate selectivity for collagen type I and type III, the major components of the extracellular matrix of human uterine cervix, it is plausible that NO may directly stimulate MMP-1 production in uterine cervical fibroblast cells, thus promoting the degradation of extracellular collagens in the process of cervical ripening. In vascular smooth muscle cells, NO causes biological effects, including relaxation, by activating soluble guanylate cyclase, which generates cGMP as an intracellular second messenger (Garbers et al., 1994). However, 8-bromo-cGMP, a membrane-permeable stable derivative of cGMP, did not alter MMP-1 secretion from CxF cells (Figure 7).

After 24 h of incubation with various reagents used in the present study, the number of CxF cells was 2.5 × 10⁵ to 2.8 × 10⁵ cells/well in a 12-well plate. This was similar to that for the vehicle control.

Discussion

The present study demonstrated for the first time that the NO donors, SNP and DETA-NO, could augment MMP-1 mRNA expression in, and MMP-1 secretion from, cultured human uterine cervical fibroblast cells. A possible contribution of NO to the regulation of MMP-1 production has been previously reported for chondrocytes (Murrell et al., 1995; Lo et al., 1998). Since MMP-1 has a substrate selectivity for collagen type I and type III, the major components of the extracellular matrix of human uterine cervix, it is plausible that NO may directly stimulate MMP-1 production in uterine cervical fibroblast cells, thus promoting the degradation of extracellular collagens in the process of cervical ripening. In vascular smooth muscle cells, NO causes biological effects, including relaxation, by activating soluble guanylate cyclase, which generates cGMP as an intracellular second messenger (Garbers et al., 1994). However, 8-bromo-cGMP, a membrane-permeable stable derivative of cGMP, did not alter MMP-1 secretion from CxF cells (Figure 7).

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tissues of non-pregnant and pregnant women were different, the possibility is that the characteristics of the original cervical may not alter the regulatory mechanisms of the NO-associated women. These were similar to those observed in cells from non-pregnant established from uterine cervical tissue at 10 weeks of gestation. 

Figure 6. Northern blot analysis of pro-matrix metalloproteinase-1 (proMMP-1) mRNA expression in uterine cervical fibroblast (CxF) cells after treatment with or without nitric oxide donors. The 2.3 kb bands indicate proMMP-1 mRNA expression. Lane 1: placenta as a positive control; lane 2: vehicle control; lane 3: sodium nitroprusside (10⁻⁴ mol/l); and lane 4: diethylenetriamine nitric oxide (10⁻⁴ mol/l). The CxF cells used in this experiment were established from uterine cervical tissue at 10 weeks of gestation.

Figure 7. Matrix metalloproteinase-1 (MMP-1) secretion from cultured uterine cervical fibroblast (CxF) cells as measured by enzyme-linked immunosorbent assay. After 24 h of incubation with interleukin-1α (IL-1α) (1.0 ng/ml) in the absence or presence of N^2-nitro-l-arginine methyl ester (l-NAME, 10–20 mmol/l), MMP-1 concentrations were assayed. The columns and error bars indicate the mean MMP-1 concentrations from quadruplicate wells ± SEM respectively. The data are representative of three independent experiments with CxF cells prepared from uterine cervical tissue at 10 weeks of gestation. *P < 0.05 versus vehicle control. #P < 0.05 versus IL-1α 1.0 ng/ml.

MMP-1 production from the cervical fibroblast cells. Moreover, treatment with l-NAME, an inhibitor of NOS, significantly and dose-dependently attenuated the IL-1α-augmented MMP-1 secretion. However, treatment with l-NAME did not alter the basal secretion of MMP-1 from these cells. Since the concentration of IL-1α in cervical mucus is reported to increase during pregnancy and parturition (Cox et al., 1993), it is plausible that IL-1α may up-regulate MMP-1 secretion from uterine cervical fibroblast cells at least in part by stimulating local production of NO during parturition.

Moreover, all responses observed in cultured cervical fibroblast cells established from first trimester pregnant women were similar to those observed in cells from non-pregnant women. These findings suggest the possibility that pregnancy may not alter the regulatory mechanisms of the NO-associated increase of MMP-1 production in uterine cervix. Another possibility is that the characteristics of the original cervical tissues of non-pregnant and pregnant women were different, and that these differences were lost during culture in vitro. Another point to be considered is that the fibroblast cells used were obtained from pregnant women in the first trimester of pregnancy. Thus, the present study does not provide direct evidence of NO-mediated MMP-1 production in the uterine cervix at term.

To the best of our knowledge, this is the first report on the regulation of an MMP by NO as part of the complex cascade of cervical ripening. This provides a scientific basis for the recent report on the clinical application of NO donors as uterine cervical dilators (Thomson et al., 1998).

In summary, the present study demonstrated that iNOS is expressed in uterine cervix and cultured cervical fibroblast cells, and that treatment with IL-1α drastically increases the concentration of iNOS mRNA in the cervical fibroblast cells, suggesting a possible increase of NO production in the uterine cervix at parturition. Moreover, the study showed that treatment with NO donors enhances MMP-1 production in cervical fibroblast cells, and l-NAME partially, but significantly, blocks an augmentation of MMP-1 secretion by IL-1α from these cells. These data suggest a possible involvement of uterine cervical fibroblast NO production in the degradation of collagen during the process of cervical ripening.

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