Nitric oxide donors stimulate prostaglandin F$_{2\alpha}$ and inhibit thromboxane B$_2$ production in the human cervix during the first trimester of pregnancy

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Nitric oxide (NO) donors are capable of ripening the human cervix during pregnancy. The purpose of this study was to examine how NO donors may be involved in this process. Cervical biopsies were obtained from pregnant women randomized to receive isosorbide mononitrate (n = 10) or no treatment (n = 10) prior to suction termination. Enzyme-linked immunosorbent assays (ELISA) were performed on culture supernatant for interleukin (IL)-1, IL-6, IL-8, IL-10, IL-15, tumour necrosis factor-$\alpha$, monocyte chemotactant protein-1 and prostaglandin metabolites. Immunohistochemistry was performed to localize these cytokines, cyclooxygenase (COX)-1, COX-2 and prostaglandin dehydrogenase in cervical tissue and reverse transcription–polymerase chain reaction (RT–PCR) to identify COX-1 and COX-2 expression. Biopsies treated with the NO donor isosorbide mononitrate (IMN) produced significantly greater amounts of prostaglandin F$_{2\alpha}$ in culture and lower amounts of thromboxane B$_2$ than controls (572.8 versus 34.9 pg/ml, $P < 0.05$; 53.3 pg/ml versus 530.9 pg/ml, $P < 0.01$ respectively). The release of other prostaglandins and of cytokines was not affected by treatment with NO. Inflammatory mediators were localized to cervical tissue and COX-1 and COX-2 expression was confirmed by RT–PCR. In conclusion, the mechanism of NO donor-induced cervical ripening during pregnancy may be mediated in part via increased prostaglandin F$_{2\alpha}$ synthesis.

Key words: cervical ripening/cytokines/nitric oxide/pregnancy/prostaglandin

Introduction

Nitric oxide (NO), a gaseous free radical, is a potent inflammatory mediator and intercellular signalling molecule (Ånggård, 1994; Beck et al., 1999) which has recently been shown to be involved in various aspects of female reproductive physiology including the process of cervical ripening (Calder, 1998; Ekerhovd et al., 1998; Norman et al., 1998; Romero, 1998). In animal models, production of NO increases in the cervix in the later stages of pregnancy and at the onset of labour (Buhimschi et al., 1996; Ali et al., 1997; Chwalisz and Garfield, 1998) and cervical ripening can be mediated via the application of an NO donor (Qing et al., 1996; Chwalisz et al., 1997). We have also shown in humans that it is possible to effect cervical ripening in the first trimester of pregnancy using an NO donor, isosorbide mononitrate (Thomson et al., 1997) and that NO donors appear to have fewer side-effects than prostaglandins when used for this purpose (Thomson et al., 1998).

Spontaneous cervical ripening, which occurs prior to the onset of labour, is characterized by softening, effacement and dilatation of the cervix. However, the underlying mechanisms involved in the control of this crucial inflammatory process (Liggins, 1981) are not fully understood. Extensive tissue remodelling occurs associated with disorganization of collagen fibrils, alterations in glycosaminoglycan composition, stromal oedema, neutrophil influx (Junquiera et al., 1980) and possibly an increase in cell adhesion molecule expression (Winkler et al., 1998). Recent studies have also suggested that apoptosis may be involved (Leppert, 1998).

A wide variety of mediators has been implicated in the control of cervical ripening including prostaglandins and various inflammatory cytokines. Through observation of the effects of various antiprogestins in the cervix, it is clear that progestrone is also fundamentally involved in the hormonal regulation of these events (Chwalisz et al., 1994). There is evidence that various cytokines are also involved. Interleukin (IL)-8, a C-X-C chemokine, has been shown in vivo (Sennstrom et al., 1997) and in vitro (Barclay et al., 1993) to be produced in the cervix and to be capable of causing ripening when artificially applied to the cervix (Chwalisz et al., 1994). IL-1 can induce cervical ripening in animal models (El Maradny et al., 1995) and its mechanism of action may involve the co-induction of IL-8 (Uchiyama et al., 1992). Other cytokines, such as tumour necrosis factor-$\alpha$ (TNF$\alpha$) (Chwalisz et al., 1994) may act in concert with IL-6 to facilitate neutrophil chemotaxis, IL-1 gene expression and endothelial adhesion molecule upregulation (Rees, 1992) during this process.

Prostaglandins were previously thought to be the final common mediators of cervical ripening. Prostaglandin synthesis is controlled by the enzyme cyclooxygenase (COX) which converts arachidonic acid to the prostaglandins, prostacyclin (PGI$_2$) and thromboxane A$_2$ (TXA$_2$). COX-1 is the
constitutive form of the enzyme while COX-2 can be induced by a number of other mediators including proinflammatory cytokines and growth factors (DeWitt, 1991). Prostaglandin E₂ (PGE₂) and prostaglandin F₂₀ (PGF₂₀) have both been used to artificially mediate cervical ripening in the first trimester of pregnancy and at term (Neilson et al., 1983; Calder, 1990). However, other agents must also be fundamental to this process since the ripening action of antiprogestins in the cervix cannot be blocked by the use of indomethacin (Radestad and Bygdeman, 1992) or the specific COX-II inhibitor, flosulide (Shi et al., 1996). Candidate agents for cervical ripening include inflammatory cytokines and NO.

The mechanism of action of NO in the inflammatory cervical ripening process remains unknown. NO has been shown to stimulate prostaglandin production via induction of COX-2 (Salvemini et al., 1993; Sautebin et al., 1994) and also cytokine release (Brady et al., 1998; Cuthbertson et al., 1998) possibly through activation of the transcription factor nuclear factor kappa B (Umansky et al., 1988; Nathan, 1992).

The purpose of this study therefore was to test the hypothesis that NO mediates cervical ripening as part of an inflammatory reaction and that it does so via induction of a variety of inflammatory cytokines and prostaglandins. We also attempted to compare the effects of NO on the production of cytokines and prostaglandins with that of other known mediators of cervical ripening.

**Materials and methods**

All studies were approved by the local research ethics committees and written informed consent obtained from each woman prior to surgery.

**Subjects**

**Pregnant women**

Healthy women in the first trimester of pregnancy (7–12 weeks gestation, aged 17–41 years, mean age 28, n = 20) undergoing suction termination of pregnancy were recruited to the study. Women were randomized into two groups and treated with either: (i) 40 mg isosorbide mononitrate (IMN) tablet (Schwarz Pharma Ltd, East Street, Chesham, Bucks, England), an NO donor, per vaginam 2–3 h prior to surgery (n = 10); or (ii) no treatment (controls, n = 10).

Biopsies were taken from the anterior lip of the cervix using a scalpel following removal of the uterus. Tissue was dissected into 14–15 small pieces (1–2 mm³) and cultured in a 24-well plate in 1.5 ml Dulbecco’s medium supplemented with streptomycin 100 µg/ml, penicillin 100 U/ml and fungizone 100 U/ml in 5% CO₂ and 95% air for 24 h at 37°C. Biopsies were weighed after treatment and tissue was either snap-frozen in liquid nitrogen and stored at −80°C, or formalin-fixed and paraffin-embedded. Culture media were divided into two portions and either frozen in 250 µl aliquots at −20°C or treated with methyloximating solution (0.1 mol/l methoxylamine hydrochloride in 10% alcohol diluted in 1 mol/l sodium acetate, pH 5.6) prior to freezing.

**Cervical biopsies from non-pregnant women**

Biopsies (20–35 mg weight, 2–3 mm diameter and 2–3 mm length) were dissected into small pieces (1–2 mm³) and cultured in 24-well plates (Costar, High Wycombe, UK) in Dulbecco’s medium as previously described. Explants were treated with one of the following: (i) medroxyprogesterone acetate (MPA) 10⁻⁶ mol/l, (ii) MPA 10⁻⁶ mol/l with mifepristone 175 ng/ml (Roussel Uclaf, Cedex, France) PGE₁ 1.0 µg/ml, (iii) lipopolysaccharide (LPS) 1.0 µg/ml with interferon-γ (IFNγ) 60 U/ml, (iv) the nitric oxide donor S-nitroso-N-acetyl-d,l-penicillamine (SNAP) at 100 µmol/l, or (v) SNAP at 200 µmol/l. Experiments were run in triplicate, cultured and stored as previously described.

**Enzyme-linked immunosorbent assays (ELISA)**

**IL-1β assay**

Ninety-six-well plates (Costar, High Wycombe, UK) were passively coated overnight at 4°C with 4 µg/ml IL-1β capture antibody [R&D Systems, Abingdon, Oxon, UK; diluted in phosphate buffered saline (PBS), pH 7.2]. Plates were washed after incubation in cold water, coating solution added (polyvinylpyrolidone 2%, BSA 5 mg/ml, preservatives ([1 mol/l 2-methylisothiazolone and 1 mol/l bromonitrooxiane) Boehringer Mannheim UK Ltd, Lewes, East Sussex, UK; 0.1% (EDTA 5 mol/ml, Tris 50 mol/ml)] at 100 µl/well for 30 min, plates were then rewarshed, air-dried and stored at 4°C. Plates were washed once in cold water prior to adding standards [diluted in ELISA buffer (10 mol/ml) Tris pH 7.2, preservatives, BSA 2 mg/ml, 300 µl 0.5% Phenol Red solution/l, NaCl 9 g/l, EDTA 2 mmol/l, Tween-20 0.05% to final pH 7.2] and added at 100 µl/well with 250 pg/well as top standard. Samples were added (undiluted: 100 µl/well) and incubated overnight at 4°C. After washing ×4 in wash buffer (0.05% Tween-20, 9 g/l NaCl, 100 mmol/ml Tris, pH 7–7.5) detection antibody (25 ng/ml) was added (100 µl/well) and plates were incubated on an orbital shaker (1.5 h at 23°C) then washed ×4 as before. Strepavidin peroxidase (Boehringer Mannheim) was then added at 0.2 U/ml and plates were incubated at room temperature for 30 min. Plates were washed again and 100 µl tetramethyl benzidine (TMB) substrate added to each well. Plates were left for 20 min before quenching with 50 µl 2 N sulphuric acid and were read at 450 nm within 30 min of quenching. Detection limit of the assay was 1 pg/ml. The intra- and inter-assay coefficients were 4.4% and 8.4% respectively.

**IL-8 assay**

IL-8 ELISA was performed as previously described (Denison et al., 1999) using paired capture and biotinylated labelled detection antibodies. Capture antibody was used at 4 µg/ml with 100 µl /well and detection antibody at 30 ng/ml (both R&D Systems). Standards were donated from Toray Industries Inc., Tokyo, Japan with 500 pg/well as top standard. Strepavidin peroxidase was added to each well at 0.2 U/ml and antibody binding was detected using TMB as substrate. Detection limit of the assay was 15 pg/ml. The intra- and inter-assay coefficients were 9.1% and 11% respectively.

**IL-6 assay**

A similar protocol was followed for the detection of IL-6 with the use of capture and biotinylated secondary antibodies. Capture antibody
was used at 4 μg/ml and detection antibody at 50 ng/ml. Recombinant standards (R&D Systems) and samples were added to wells with 250 pg/ml as top standard. Plates were read and detected as before. Detection limit of the assay was 0.7 pg/ml. The intra- and inter-assay coefficients were 4.2% and 6.0% respectively.

**MCP-1 assay**
Monocyte chemotactant protein (MCP-1) ELISA was as previously described (Denison et al., 1997). Capture antibody (donated by Toray) was used at 4 μg/ml and peroxidase conjugated detection antibody added at 60 μl/well. Top standard was 500 pg/ml. Plates were read and detected as before. The intra- and inter-assay coefficients were 6.3% and 8.6% respectively. Detection limit of the assay was 7.5 pg/ml.

**IL-10 assay**
IL-10 assay was performed as previously described (Denison et al., 1999). Capture antibody (Pharmingen, Sandiego, USA) was used at 200 ng/ml and detection antibody at 125 ng/ml. Recombinant standards (Pharmingen, San Diego, CA, USA) were added with 500 pg/ml as top standard. Poly-peroxidase (CLB Laboratories, Amsterdam, Holland) was used at 1 ng/ml in ELISA buffer and plates read and detected as before. The intra- and inter-assay coefficients were 6.4% and 10.1% respectively. Detection limit was 15 pg/ml.

**IL-15 assay**
Anti-human IL-15 capture antibody (R&D Systems) was used diluted in 0.1 mol/l NaHCO3 pH 8.4 and incubated overnight at 4°C. Capture antibody was removed, plates were blocked [10% fetal calf serum (FCS) in PBS at 200 μl/well at 37°C for 2 h] washed (Ã©2 in PBS/ Tween) and standards (diluted in 10% FCS in PBS with 1.5 pg/ml to 0.04 ng/ml) were added. Plates were incubated (37°C for 2 h) washed ×4 as before and detection antibody added [R&D Systems]; diluted at 200 ng/ml; 100 μl/well and incubated at 37°C for 2 h]. Plates were washed ×6 and streptavidin–peroxidase (SAPU 1/10000) diluted in 10% FCS in PBS added at 100 μl/well. Plates were detected and read as described previously. The intra- and inter-assay coefficients were 3.9% and 9.1% respectively. Detection limit of the assay was 1.0 pg/ml.

**TNF-α**
Paired capture (4 μg/ml) and detection antibodies (100 ng/ml) (both R&D Systems) were used to detect bound standards and samples. Standards (R&D Systems) were added with 5000 pg/ml as top standard. The intra- and inter-assay coefficients were 5.0% and 7.3% respectively. Detection limit of the assay was 4.4 pg/ml.

**PGE2 assay**
Prostaglandin E2 assay was performed as previously described (Denison et al., 1999). The intra- and inter-assay coefficients were 7.8% and 15% respectively and the ED50 was 195 pg/ml.

**Prostaglandin E metabolite (PGEM) assay**
A similar protocol was used to detect PGEM. Peroxidase-conjugated PGEM was added at 1 in 50 000 diluted in ELISA buffer and antisera at 1.0×10^5 in assay buffer. Standard range of the assay was 1280 to 2.5 pg/ml. Methyloximating solution was present in all standards and samples at a final concentration of 12.5%. The intr-assay coefficient was 4.1% and ED50 was 195 pg/ml.

**6-OXO-PGFα**
6-OXO-PGFα was detected using a similar protocol. Peroxidase conjugate was added at 1 in 2.0×10^5 and antisera added at 1 in 10 000. The standard range of the assay was 10 240 pg/ml to 5 pg/ml. The intra-assay coefficient was 4.8%. Methyloximating solution (25%) was present in all samples and standards.

**TXB2**
Assay was performed using the same protocol. Peroxidase conjugate was used at 1 in 1.25×10^5 and antisera at 1 in 25 000. Standard range of the assay was 327.7 ng/ml to 0.04 ng/ml. The intra-assay coefficient was 7.3%. Methyloximating solution was present in all standards and samples at a final concentration of 12.5%.

**PGF2α**
Peroxidase conjugated PGF2α was added at 1 in 1.0×10^6 and antisera at 1 in 20 000. Standard range of the assay was 5120–10 pg/ml. The intra- and inter-assay coefficients were 18.3% and 5.2% respectively. ED50 was 220 pg/ml.

**PGFM**
Peroxidase-conjugated prostaglandin F metabolite (PGFM) was added at a concentration of 1 in 40 000 and antisera at 1 in 50 000 diluted in ELISA buffer. The standard range of the assay was 327.7 ng/ml to 0.04 ng/ml. The intra- and inter-assay coefficients were 14.6% and 6.8% respectively.

**RNA extraction**
Total RNA was isolated from cervical tissue explants using an adaptation of a previously published method (Slater et al., 1995). Briefly, 1ml trizol (Gibco Life Technologies, Paisley, UK) was added to tissue samples and incubated overnight. RNA was isopropyl alcohol–chloroform (BDH, Glasgow, UK)-precipitated and the supernatant removed. The pelleted RNA was washed in 75% ethanol and resuspended in diethyl-pyrocarbonate (DEPC)-treated water. The RNA yield was determined spectrophotometrically at 260/280 nm.

Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis
Reverse transcription was used to identify expression of COX-1 and COX-2 in cervical tissue explants (Slater et al., 1995). Total RNA (3 μg) was reverse transcribed into cDNA using Superscript II reverse transcriptase (Gibco Life Technologies) in 20 μl of reaction buffer ([10×PCR buffer, 25 mmol/l MgCl2, 0.1 mol/l DTT (Gibco Life Technologies), 10 mmol/l dNTP and 50 ng/ml random hexamers (Boehringer Mannheim)]. Five μl cDNA was used for PCR amplification with either COX-1, COX-2 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (Gibco Life Technologies). Primer sequences were: COX-1 (sense) 5'-TGCCCAGCTCCTGGCCCGC CGCTT-3', COX-1 (antisense) 5'-CCATGGCCAAAGCCCTTG-3' (Slater et al., 1998); COX-2 (sense) 5'-TCTAAATGAGATGTTGG GAAATTTGCT-3', COX-2 (antisense) 5'-CCACCATGGCAAA TTCCCATGGA-3' (Iniguez et al., 1998); GAPDH (sense) 5'-CC ACCATGCGAAATCTGGA-3', GAPDH (antisense) 5'-T CAGACGGCGAGTAGTGGT GTCCACC-3' (Slater et al., 1998). PCR was performed in a 50 μl volume of reaction buffer containing 10×PCR buffer, 25 mmol/l MgCl2, 2 mmol/l dNTP, 1.3 μl primer 1, 1.0 μl primer 2, 5% dimethylsulphoxide (DMSO) and 0.1 μl Taq polymerase (Gibco Life Technologies). The reaction was amplified by 35 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. Products were run on agarose gels and bands visualized using ethidium bromide.

**Immunohistochemistry**
Immunohistochemistry protocols for the detection of IL-1β, IL-6, IL-10, IL-15, MCP-1, TNFa, COX-1, COX-2, and prostaglandin dehydrogenase (PGDH) were established to determine the correct conditions for optimal staining (Table I). PGDH is a nicotinamide adenine dinucleotide (NAD+)–dependent 15-hydroxy-PGDH responsible for prostaglandin metabolism.

**COX-2, IL-1β, IL-6, IL-8, IL-10 and TNFa**
Sections 5 μm thick were cut from paraffin-embedded cervical samples and mounted on silane-coated slides, heated to 60°C for 35 min, deparaffinized in xylene and rehydrated in graded alcohol series. Sections were placed in 0.5% hydrogen peroxide in methanol to block endogenous peroxide activity. If required, sections were pretreated to
retrieve the antigen by microwaving at full power for 5 min in 0.01 mol/l citrate buffer pH 6.0. Sections were washed in PBS (PBS + 0.1% saponin for microwaved sections) then blocked in 20% rabbit serum with 20% human serum for 30 min at room temperature. Slides were then incubated overnight at 4°C with the primary antibody diluted in 2% normal rabbit serum with 5% normal human serum. Sections were washed in PBS (± 0.1% saponin) before incubation with biotinylated anti-goat (Vector Laboratories, Peterborough, UK) diluted 1:200 in 2% normal rabbit serum with 5% normal human serum added. The sections were washed as before in PBS (± 0.1% saponin) then incubated for 30 min with avidin/biotin horseradish peroxidase reagent (Vector Laboratories) in PBS before final washing.

The antigens were localized by incubating slides for 10 min with 1 mg/ml diaminobenzidene tetrahydrochloride (DAB), 0.02% H2O2 in 50 mmol/l Tris–HCl, pH 7.6 and appeared as a brown end product. Negative controls included sections incubated without the primary antibody. Kidney and endometrium (Jones et al., 1997) were used as positive controls for COX-2 and monosialic tissue was used as a positive control for IL-1β, IL-6, IL-8, IL-10 and TNFα. To assess the specificity of the staining for COX-2 and TNFα representative slides were included where the primary antibody was preabsorbed with the appropriate peptide (COX-2 blocking peptide from Santa Cruz Biotechnology, sc-1745P; recombinant human TNFα from R&D Systems, 210-TA-010) (Van Noorden, 1993).

**IL-15, COX-1 and PGDH**

Paraffin-embedded sections were prepared as before and pretreated in order to retrieve the antigen if necessary (Table I). Sections were then preincubated in 20% goat serum and 20% human serum for 30 min at room temperature. They were then incubated with the appropriate monoclonal antibody diluted in 2% normal goat serum in PBS (± saponin) with 5% human serum added and left overnight at 4°C. Primary antibody was omitted from the negative control slides. Sections were then washed in PBS (± saponin) and incubated with biotinylated goat anti-mouse (Dako) diluted 1:200 in 2% normal goat serum in PBS (± saponin) with 5% normal human serum added for 30 min at room temperature in a humidified box. Sections were washed and incubated again with streptavidin peroxidase (Dako, Cambridge, UK) diluted 1:400 in PBS (± saponin) before washing and final treatment with DAB as before.

**MCP-1**

MCP-1 was localized in frozen tissue sections as described previously (Jones et al., 1997). Tonsil tissue was used as positive control and negative control slides were set up with either no primary antibody or non-immune rabbit IgG.

**Table I. Immunohistochemistry protocols for optimal staining of prostaglandins and interleukins**

<table>
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COX = cyclooxygenase; PGDH = prostaglandin dehydrogenase; IL = interleukin; rh = recombinant human; TNF = tumour necrosis factor; MCP = monocyte chemotactrant protein.

**Figure 1.** The effect of in-vivo treatment with the nitric oxide (NO) donor isosorbide mononitrate (IMN) on cytokine levels in cervical tissue in the first trimester of pregnancy. Concentrations of interleukin (IL)-6, IL-8, IL-10, IL-15, monocyte chemotactrant protein (MCP)-1, tumour necrosis factor-α (TNFα) from cervical explants of 10 control and 10 patients treated with the NO donor IMN 40 mg in vivo were measured by enzyme-linked immunosorbent assays. The increase in IL-8 concentrations in the NO donor-treated group was not statistically significant. Values are expressed as pg/ml ± SEM.

**Statistical analysis**

Statistical analysis of IL-1β, IL-6, IL-8, IL-10, IL-15, MCP-1, TNFα, PGF2α, PGE2, PGFM, PGE2, 6-OXO-PGF2α and TXB2 concentrations in culture media was performed using analysis of variance (Statview SE + Graphics v.1.04; Abacus Inc, Berkley, CA, USA). Significance was determined using Scheffé’s F-test as a post-hoc test. Results are expressed as mean level pg/ml ± SEM with P < 0.05 taken to indicate significance.

**Results**

**Effect of NO donors on pregnant first trimester human cervix**

IL-1β, IL-6, IL-8, IL-10, IL-15, MCP-1 and TNFα release

Tissue explants of first trimester cervix released IL-6, IL-8, IL-10, IL-15, MCP-1 and TNFα (Figure 1). IL-1 was not released. In-vivo treatment with the NO donor IMN did not
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Figure 2. Immunostaining of interleukin (IL)-1 (a), IL-6 (b), IL-8 (c), IL-10 (d), IL-15 (e), tumour necrosis factor-α (f), monocyte chemotactant protein-1 (g) in cervical tissue biopsy specimens from early pregnant subjects. bv = blood vessels; e = epithelium; s = stroma. Original magnification: (a, b, d) ×94; (c, f) ×48; (e) ×60; (g) ×75.

significantly alter the release of these cytokines from the first trimester cervix in culture.

**Immunohistochemistry for IL-1β, IL-6, IL-8, IL-10, IL-15, MCP-1 and TNFα**

Immunohistochemistry localized staining for IL-1 to the epithelium, glands and blood vessel endothelium (Figure 2a). IL-6 was present in the epithelium and in perivascular structures (Figure 2b). Staining for IL-8 was confined to the epithelium and blood vessel endothelium (Figure 2c). IL-10 stained strongly in the epithelium and weakly in the blood vessels (Figure 2d). IL-15 stained strongly in the epithelium and blood vessels and more weakly in the cervical connective tissue stroma (Figure 2e). Staining for TNFα was localized to the surface epithelium with a small amount of perivascular staining also being present (Figure 2f). MCP-1 staining was present strongly in perivascular structures and in the surface epithelium (Figure 2g).

**PGF2α, PGE2, PGFM, PGEM, 6-OXO-PGF2α and TXB2 release**

Cervical explants from first trimester cervix released PGF2α, PGE2, PGFM, PGEM, 6-OXO-PGF2α and TXB2 (Figure 3). Treatment with the NO donor IMN in-vivo stimulated PGF2α release ($P < 0.05$) and inhibited TXB2 release ($P < 0.01$). There was no significant effect of the NO donor IMN on the concentrations of PGE2, PGFM, PGEM and 6-OXO-PGF2α.

**Immunohistochemistry for COX-1, COX-2 and PGDH**

Immunohistochemistry was performed to localize the enzymes COX-1, COX-2 and PGDH to the cervical tissue in both NO-treated and control patients (Figure 4).

Staining for COX-1, COX-2 and PGDH was present in both NO-treated and control subjects. COX-1 was localized strongly to the superficial layers of the surface glandular epithelium and weakly to the connective tissue stroma (Figure 4a). COX-2 staining was also strong in the glandular epithelium and...
perivascularly with weaker staining in the stroma (Figure 4b). Staining for PGDH showed a similar pattern (Figure 4c).

**RT–PCR for COX-1 and COX-2**

RT–PCR was performed to identify the presence of mRNA for COX-1 and COX-2 in the cervix. The primer pairs yielded amplified products of the expected sizes: 304 bp for COX-1, 305 bp for COX-2 and 598 bp for GAPDH. Gel electrophoresis for COX-1 and COX-2 is shown (Figures 5 and 6). There was no contamination by amplified cDNA as assessed by appropriate negative controls. COX-1 was present in the pregnant cervix. Treatment with NO donors in vivo had no apparent effect on COX-1 expression. COX-2 was not present in cervical tissue samples obtained from pregnant women in the first trimester (n = 2) but was expressed in two of three samples obtained after treatment with the NO donor IMN.

**Effect of NO donors in vitro on non-pregnant human cervix**

**IL-6, IL-8, IL-10, IL-15, MCP-1 and TNFα release**

Non-pregnant cervical explants released IL-6, IL-8, IL-10, IL-15, MCP-1, TNFα as assessed by ELISA (Figure 7). The production of these cytokines was not affected by treatment with either the NO donor SNAP at concentrations of 100 or 200 µmol/l, by bacterial LPS and IFNγ in combination, by PGE1, by MPA or by mifepristone + MPA (data not shown).

**PGF2α, PGE2, PGFM, PGEM, 6-OXO-PGF2α and TXB2 release**

In contrast to the in-vivo pregnant group, non-pregnant cervical explants treated with the NO donor SNAP in vitro did not show any significant change in the release of PGF2α or TXB2 (Figure 8). RT–PCR for COX-1 and COX-2 confirmed the presence of mRNA in non-pregnant control cervical tissue and tissue treated with the NO donor SNAP (Figures 5 and 6).

**Discussion**

The data presented here show that the in-vivo administration of the NO donor, IMN, in the first trimester of pregnancy stimulates increased cervical production of PGF2α. Therefore our previously reported effects of IMN in inducing cervical ripening seem to be in part mediated through the production
Nitric oxide donors and cervical ripening

Figure 5. Reverse transcription–polymerase chain reaction showing COX-1 expression in cervical tissue biopsies from non-pregnant [± the NO donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP) in vitro] and pregnant subjects [± the NO donor isosorbide mononitrate (IMN) in vivo]. Lane 1: molecular weight markers. Lane 2: positive control. Lanes 3–6: non-pregnant control tissue. Lanes 7 and 8: pregnant control tissue. Lanes 9–11: pregnant tissue treated with IMN in vivo. Lanes 12–14: non-pregnant tissue treated with SNAP in vitro. Lanes 15 and 16: positive controls. Control lanes for GAPDH are also shown. COX-1 mRNA is expressed in all tissue samples.

Figure 6. Reverse transcription–polymerase chain reaction showing COX-2 expression in cervical tissue biopsies from non-pregnant [± the nitric oxide (NO) donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP) in vitro] and pregnant subjects [± the NO donor isosorbide mononitrate (IMN) in vivo]. Lane 1: molecular weight markers. Lanes 2–4: non-pregnant control tissue. Lanes 5 and 6: pregnant control tissue. Lanes 7–9: pregnant tissue treated with IMN in vivo. Lanes 10 and 11: non-pregnant tissue treated with SNAP in vitro. Lane 12: positive control. Control lanes for GAPDH are also shown. COX-2 mRNA was present in non-pregnant cervical tissue [the NO donor SNAP given in vitro]. In pregnant tissue COX-2 mRNA was not detected in the two control samples shown (lanes 5 and 6). COX-2 mRNA was detected in two of the three samples from pregnant women treated with the NO donor IMN in vivo (lanes 7 and 8).

Figure 7. The effect of in-vivo administration of the nitric oxide donor donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP) on cytokine production in the non-pregnant cervix. Interleukin (IL)-6, IL-8, IL-10, IL-15, tumour necrosis factor-α (TNFα) and monocyte chemotactant protein (MCP-1) concentrations were measured by enzyme linked immunosorbance assay in supernatant from cultured cervical tissue explants treated in vitro for 24 h with: lipopolysaccharide and interferon-γ (LPS + IFNγ); SNAP at 100 µmol/l; or SNAP at 200 µmol/l. Tissue treated with culture medium only acted as controls. Values are expressed as pg/ml ± SEM.

Our findings are in agreement with previously published reports where NO has been shown to activate PGF2α in human microglial cells (Janabi et al., 1996).

Cervical ripening in pregnancy is known to involve increased production of the prostanoids PGE2, PGF2α and PGI2 within the cervix (Ellwood et al., 1980). Although PGE2 is considered to be the most important of these (Calder and Greer, 1992), PGF2α may also be fundamentally involved. Animal studies have shown that the histological changes, which occur in the cervix after the administration of PGF2α, are comparable with the changes observed in control animals undergoing spontaneous labour. Studies in humans have also shown that PGF2α can be used to artificially induce cervical ripening in both the first trimester of pregnancy prior to suction termination (Rath et al., 1982; Arias, 1984) and at term (MacLennan and Green, 1979; MacLennan et al., 1994). PGE2 and PGF2α have similar effects on cervical ripening when used in equipotent doses (MacKenzie and Embrey, 1979; Keirse, 1993) but PGE2 remains the most commonly used agent for this purpose due to the reduced incidence of side-effects encountered using a clinically effective dose.

We postulated that any increase in PGF2α in the cervix might be mediated via either an increase in COX activity or expression. The NO and COX systems have often been shown to be present in concert in inflammatory conditions (Salvemini,
and NO may activate cyclooxygenase through a cGMP-independent mechanism (Salvemini et al., 1993; Uno et al., 1997). Immunohistochemistry localized COX-1 to the superficial epithelium and to the connective tissue stroma while COX-2 was localized in the cervix in a similar pattern. RT–PCR on first trimester cervical tissue was not intended to be quantitative and showed the presence of mRNA for COX-1 in both NO-treated patients and controls but only showed the presence of mRNA for COX-2 in two of the samples from the NO-treated group. The difference in the control and NO-treated groups should be interpreted with caution because of the small number of patients studied. NO may directly interact with COX-2 to cause an increase in PGF$_{2\alpha}$ either by an increase in enzyme activity via free radical stimulation of COX-2 or an increase in enzyme production. However, NO is an important second messenger in cell signalling pathways (Beck et al., 1999) and the effects seen in cervical tissue may be also mediated in part via a direct interaction with matrix metalloproteinases (Chatziantoniou et al., 1998), via apoptosis (Leppert, 1998) or by direct effects on other downstream pathways involved in the complex process of cervical ripening.

In vitro, SNAP appeared to have no effect on prostaglandin production. We postulate that this may be due to lack of paracrine interaction in vitro. During cervical ripening, NO may act as an inflammatory mediator causing vasodilatation, changes in vascular permeability and activation of cytokines and other proinflammatory mediators. Although the role of NO in the process of lymphocyte trafficking is unclear, it has been suggested that high levels of NO produced in response to inducible nitric oxide synthase (iNOS) upregulation during acute inflammation contribute to leukocyte and platelet adhesion to the vascular endothelium (Clancy et al., 1998). NO is also involved in lymphocyte signalling through enhanced activation of a tyrosine kinase p56 (Clancy et al., 1998). Thus the lack of active tissue perfusion and hence the inability for such complex interactions to take place within the in-vitro tissue culture system may explain the lack of effect witnessed in the group treated with SNAP in vitro.

Alternatively, the difference between the groups could be related to the fact that the in vivo studies were carried out on pregnant cervix and may therefore reflect changes which may occur in the maternal immune response during pregnancy designed to prevent fetal allograft rejection (Wegmann et al., 1993).

Other previously published reports, however, show that NO donors in vitro are capable of stimulating prostaglandins in non-pregnant cervix (Denison et al., 1999). This may reflect the different NO donors used in these studies compared to those that we employed. Under different in-vitro experimental conditions it has also been shown that NO can either have no effect on prostaglandin release (Tsi et al., 1994; Curtis et al., 1996) or can actually inhibit prostaglandin production at high concentrations (Swierkosz et al., 1995). The discrepancies between our own and other reported studies may reflect differences in cell types, alterations in the active state of the cells examined and differences in the amount of iNOS and COX-2 present as well as variation in the type and doses of the NO donors used.

Our studies have also demonstrated that IMN administered to the first trimester cervix causes a decrease in TXB$_2$ release. TXB$_2$ is the metabolic breakdown product of the arachidonic acid derivative TXA$_2$ which plays a crucial role in platelet functioning. Following platelet activation, the release of TXA$_2$ causes vasoconstriction and stimulates platelet aggregation. Organic nitrates such as IMN are known to reduce platelet adhesion and aggregation as well as causing vasodilatation (Parker and Parker, 1998) and endogenous NO has similar effects (Radomski et al., 1987; Salvemini et al., 1990). Our studies suggest that the effect of NO in inhibiting platelet aggregation may be in part mediated by a decrease in thromboxane synthesis. Alternatively the decrease in thromboxane B$_2$ after treatment with NO may reflect substrate shift the arachidonic acid pathway being preferentially driven to increase production of PGF$_{2\alpha}$.

Our studies failed to show any significant effect of in-vivo or in-vitro administration of NO donors on cytokine production within the cervix. In-vivo administration of IMN to the pregnant cervix resulted in an increase in IL-8 release which was not statistically significant. Using other NO donors, NO has been shown previously to stimulate IL-8 production in both the cervix (Denison et al., 1997) and in peripheral blood monocytes (Cuthbertson et al., 1998). However, this relationship seems to vary with the NO donor used, as Cuthbertson et al. also showed that 3-morpholinosydnonimine (SIN), a combined NO and superoxide donor, was capable of decreasing IL-8 release from blood monocytes (Cuthbertson et al., 1998). Our results may be attributable to the specific effects of the NO donors used or to the small sample size studied.

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References


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