Mifepristone-induced nitric oxide release and expression of nitric oxide synthases in the human cervix during early pregnancy

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BACKGROUND: Nitric oxide (NO) is a factor in cervical ripening, perhaps under the control of progesterone. We studied the effects of the antiprogesterone mifepristone on the release of NO and on the expression of inducible NO synthase (iNOS) and endothelial NO synthase (eNOS) in the uterine cervix of women in early pregnancy. METHODS: Thirteen women were treated with oral mifepristone (200 mg), and 15 women were studied as controls. Cervical fluid samples were collected before treatment then hourly up to 3 h, and the samples were assayed for the concentration of nitric oxide metabolites (NOx). In addition, cervical biopsy samples from six women treated with mifepristone and from six controls were assessed for iNOS and eNOS by immunohistochemistry and Western blotting. RESULTS: In 1–3 h, mifepristone induced 7.4- to 17.2-fold elevations in cervical fluid NOx concentrations; no change was seen in the controls. The expression of both iNOS and eNOS was detected in the cervical cells. The expression of cervical iNOS was strong in five of the six women treated with mifepristone but was not strong in any of the six control women. CONCLUSION: This is the first study to show that mifepristone stimulates the release of NO and the expression of iNOS in cervical cells of women in early pregnancy. This may be one mechanism by which mifepristone initiates cervical ripening.

Key words: antiprogestin/cervical ripening/eNOS/iNOS/nitrate/nitrite

Introduction

It is well established that uterine cervical cells produce nitric oxide (NO) via three different NO synthases (NOS): endothelial, inducible and neuronal NOS (Tschugguel et al., 1999; Ledingham et al., 2000; Maul et al., 2003). Both endogenous (Chwalisz and Garfield, 1997; Maul et al., 2003) and exogenous (Chwalisz et al., 1997; Ekerhovd et al., 2003; Arteaga-Troncoso et al., 2005) NO may cause cervical ripening, because it may loosen cervical connective tissue by inducing various matrix metalloproteinases (MMPs) that degrade cervix-stabilizing glycosaminoglycans and collagens (Maul et al., 2003; Stjernholm-Vladic et al., 2004a). Inducible NOS (iNOS) appears to be more important than endothelial NOS (eNOS) in this process (Tschugguel et al., 1999; Maul et al., 2003), where sex steroids (Al-Hijji et al., 2001; Batra et al., 2003; Maul et al., 2003; Väisänen-Tommiska et al., 2004a), cytokines (Sennstrom et al., 2000; Kelly, 2002) and prostaglandins (Hausman et al., 2003; Stjernholm-Vladic et al., 2004a) are predominantly involved.

Because progesterone is essential for the maintenance of pregnancy, progesterone antagonists, such as mifepristone, may be used for termination of pregnancy or for induction of labour (Wing et al., 2000; Chia and Ogbo, 2002; Ben-Chetrit et al., 2004; Kulier et al., 2004). Mifepristone induces this effect by blocking progesterone receptors in the endometrium/myometrium (Olive, 2002). Mifepristone also causes cervical softening both in animals (Ali et al., 1997; Maul et al., 2003) and in humans (Olive, 2002; Kulier et al., 2004), but the mechanisms of this action are poorly understood. It has been shown that antiprogestins increase cervical iNOS expression in pregnant rats (Ali et al., 1997; Maul et al., 2003), and thus, it is plausible that mifepristone could induce cervical NOS expression and/or NO release also in women. Therefore, we studied the effect of mifepristone on cervical NO release in women in early pregnancy. Furthermore, we assessed the expression of iNOS and eNOS in the cervical cells of these women.

Materials and methods

With the approval of the Ethics Committee of Helsinki University Central Hospital, 28 women seeking termination of pregnancy for socioeconomic reasons between 8 and 12 weeks of gestation were included in the study (Table I). All women were parous because it was regarded unethical that nulliparous women would not receive medical ripening. The study protocol was approved by the Institutional Review Board. Each of the pregnancies was found to be single and viable in transvaginal ultrasonography, and none of the women had...
experienced bleeding nor had signs of threatened abortion or infection. Papanicolau (PAP) smears were all normal, and Chlamydia tests were all negative. Written informed consent was obtained from each woman before recruitment.

After a 12 h fast, 13 women received 200 mg of mifepristone orally (Mifegyne®; Exelgyn Laboratories SA Exelgyn Nordic, Lidingö, Sweden), whereas 15 women were followed without treatment as a control group. Cervical fluid samples were collected at baseline before the intake of mifepristone and 1, 2 and 3 h later. These samples were collected as described before (Väisänen-Tommiska et al., 2003). Briefly, a Dacron swab (DuPont, Wilmington, DE, USA) introduced into the cervix under visual control was kept there for precisely 20 s and then flushed in 1.5 ml of physiological saline for 2 min. The saline samples were stored frozen at −21°C until assayed in duplicates for nitrate/nitrite (NOx) spectrophotometrically using the Griess reaction (Väisänen-Tommiska et al., 2003). The detection limit of the assay was 0.8 μmol/l, and the intra- and inter-assay coefficients of variation were 1.6 and 2.4%, respectively. To reduce the impact of inter-assay variation, we assayed all samples from a given individual in the same batch.

To study NOS expression, two cervical biopsies were collected from 12 women (six from each group) immediately after collection of the last cervical fluid sample by using Shumaker punch biopsy forceps (Stifle, Wooburn Green, Bucks, UK). This sampling from the anterior lip of the cervix (between 10 a.m. and 2 p.m.) took place under general anaesthesia before vacuum curettage and always before any artificial cervical dilation. One biopsy was fixed in formalin and embedded in paraffin for immunohistochemical staining, and the other was snap frozen in liquid nitrogen and stored at −70°C for subsequent Western blotting.

Before the vacuum curettage and 3–4 h after the intake of mifepristone, cervical ripening was semi-quantitatively assessed by one investigator (M.V.-T.) with the aid of Hegar dilators. The size of the Hegar dilator that could be introduced without force into the cervix was recorded.

**Immunohistochemistry**

A standard immunohistochemical technique [horse-radish peroxidase (HRP)-linked antibody conjugates method] was carried out to visualize iNOS and eNOS [with 3,3′-diaminobenzidine tetrahydrochloride (DAB)]. Briefly, sections were deparaffinized in xylene, rehydrated and pretreated by heating in a microwave oven at 700 W in 0.01 M citric acid (pH 6.0) for 10 min, and endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide in methanol for 30 min at room temperature. All tissue sections were analysed by utilizing a Power Vision+™ poly-HRP IHC Detection Kit (Immun-Vision Technologies, Brisbane, CA, USA) and a Lab Vision Autostainer (Lab Vision, Fremont, CA, USA), and they were counterstained for 10 s with Mayer’s hemalum solution (Merck 1.09249). A polyclonal rabbit anti-iNOS antibody (RB-1605-P1; Neo Marker, Fremont, CA, USA) was used for the detection of the iNOS C-terminal domain. The antibody was diluted to a concentration of 20 μg/ml (1:50), and the sample was incubated for 60 min at room temperature. A rabbit polyclonal antibody (RB-1711-P1; Neo Marker) was used for the detection of eNOS. Positive controls for iNOS and eNOS were sections of umbilical cord, and negative controls were slides incubated without primary antibody. Three observers blind to the identity of the slides evaluated the staining semi-quantitatively, and the intensity was graded as follows: (0) no staining, (1) weak, (2) moderate and (3) strong staining. The cellular localization of the staining and the proportion of stained/unstained cells were also recorded.

**Western blot analysis**

Western blot analyses were carried out to detect iNOS and eNOS protein in the cervical tissue samples. Total protein was extracted from the cervical tissue biopsy samples using the TriPure Isolation Reagent method, according to the manufacturer’s instructions (Roche Applied Science, Indianapolis, IN, USA). Protein was quantified using the Bio-Rad protein assay method (Bio-Rad Laboratories, Hercules, CA, USA) and spectrophotometry at 750 nm. Samples containing 25 μg of protein were prepared with application buffer, separated by means of Novex® 3–8% Tris-acetate gel electrophoresis (NuPage™) and transferred to a polyvinylidene fluoride (PVDF) membrane (pore size 450 μm) (Immobilon-P; Millipore, Bedford, MA, USA) by wet blotting (30 V for 2 h). The membranes were blocked in 3% bovine serum albumin (Sigma, St Louis, MO, USA) in 0.05% (v/v) Tween–Tris-buffered saline (TBS-T) for at least 1 h before antibody application. The antibodies and concentrations were iNOS (iNOS/NOS Type II; BD Transduction Laboratories Pharmingen, San Diego, CA, USA) at 1:2000 and eNOS (eNOS/NOS Type III; BD Transduction Laboratories, Pharmingen) at 1:2000. Lysates of interferon-γ/LPS-treated mouse macrophages (BD Transduction Laboratories Pharmingen) and human endothelial cells (BD Transduction Laboratories Pharmingen) were used as the controls for iNOS and eNOS, respectively. Immunoreactivity was visualized using peroxidase-conjugated secondary antibodies and stained with DAB (Fluka Chemie GmbH, Buchs, Switzerland, Germany). Stained molecular weight markers (Bio-Rad and Fermentas, Ontario, Canada) were transferred to the PVDF membranes and used to identify and characterize the molecular weights of the NOS isoforms examined.

**Statistics**

Categorical data were analysed by linear regression, the chi-square test or Fisher’s exact probability test. Statistical calculations were carried out by analysis of variance (ANOVA) on ranks (Kruskal–Wallis test) or by the Mann–Whitney U-test as a post hoc test, and significances were evaluated by Dunnet’s test. Values of P < 0.05 were considered statistically significant. A concentration below the detection limit (0.8 μmol/l) was given an arbitrary value of 0.75 μmol/l. To better describe treatment-induced changes in cervical fluid NOx levels, we also present the NOx data as percentages of pretreatment values.

**Results**

The study groups were comparable as regards age, gestational weeks and pretreatment levels of cervical fluid NOx (Table I). The age of the subject and the duration of gestation were not determinants of baseline NOx levels. No woman reported any uterine contractions or bleeding during the 3 h study period.

The administration of mifepristone was followed by 7.4- to 17.2-fold elevations in cervical fluid NOx concentrations after 1–3 h (Figure 1). The rise was already significant after 1 h but was more pronounced after 2 and 3 h. The absolute median NOx values after mifepristone (1 h 12.1 μmol/l 95% CI 4.2–31.0),

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**Table I. Clinical characteristics of the study population**

<table>
<thead>
<tr>
<th></th>
<th>Mifepristone group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of women</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Years (mean ± SD)</td>
<td>28.8 ± 5.9</td>
<td>32.2 ± 9.6</td>
</tr>
<tr>
<td>Range</td>
<td>20–35</td>
<td>20–42</td>
</tr>
<tr>
<td>Gestational age (weeks) (mean ± SD)</td>
<td>9.3 ± 1.9</td>
<td>8.2 ± 0.7</td>
</tr>
<tr>
<td>Cervical nitric oxide metabolites before treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Detectable (%)</td>
<td>69</td>
<td>67</td>
</tr>
<tr>
<td>Median (μmol/l) (95% CI)</td>
<td>4.2 (&lt;0.8–16.2)</td>
<td>11.7 (&lt;0.8–19.3)</td>
</tr>
</tbody>
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P = 0.04; 2 h 18.1 μmol 95% CI (12.4–59.8), P = 0.005; and 3 h 45.3 μmol 95% CI (15.5–82.2), P = 0.0008) were signifi-
cantly higher than pretreatment levels (4.2 μmol/l, 95% CI <
0.8–16.2). Cervical NOx levels remained stable in the controls
during the entire follow-up period.

Three to four hours after treatment, a size 7 Hegar dilator
could be introduced in five of the 13 mifepristone-treated
women (38%) and in five of the 15 controls (33%) (P = 0.78).
Neither the level of NOx nor the NOx responses to mifepris-
tone were correlated with the size of the Hegar dilator before
vacuum curettage.

**Immunohistochemistry**

**iNOS**

All women except one treated with mifepristone showed strong
cervical iNOS staining, while none of the women in the control
group showed strong iNOS staining (83 versus 0%; Figure 2),
cervical iNOS staining was considered to be weak in five of six
of these control women. Inducible NOS was detected in the vas-
cular endothelium, pericytes and fibroblasts, and also in the cer-
vical glands after mifepristone treatment but was not as obvious
in the controls (Figure 2). The ratio of iNOS expression in the
endothelium compared with that in the pericytes was low. The
positive control sections showed appropriate staining for iNOS,
whereas the negative control sections showed no staining.

**eNOS**

eNOS was present mainly in the vascular endothelium but was
also in the parabasal cells of the surface epithelium and the cer-
vical glandular epithelial cells, and there was no difference in
the localization or intensity of eNOS between the groups. The
endothelium/pericyte ratio of staining for eNOS expression
was high. The pattern of immunostaining was similar in both
study groups (Figure 2). The positive control sections showed
appropriate staining for eNOS, and the negative control sec-
tions showed no staining.

**Western blot analysis**

Western blot analysis confirmed the presence of protein for
both iNOS (130 kDa) and eNOS (140 kDa) isoforms in the cervix (Figure 3).

**Discussion**

In the present study, we show for the first time that orally
administered mifepristone induces cervical NO release and
expression of iNOS in the cervical cells of women in early
pregnancy. To our knowledge, this is also the first study dem-
onstrating concomitant NO release and NOS expression in the
human cervix.

We assessed NO release by measuring the levels of NOx in
cervical fluid and NOS expression by means of immunostain-
ing and Western blotting, all reproducible methods reflecting
the availability of NO (Tschugguel et al., 1999; Ledingham
et al., 2000; Väisänen-Tommiska et al., 2003, 2004a,b; Mitchell
et al., 2004). Mifepristone is rapidly absorbed following oral
ingestion, reaching peak serum levels in 1–2 h (Heikinheimo
et al., 1986). Therefore, we decided to collect cervical fluid
samples for up to 3 h, assuming that the effect of mifepristone
would be detectable during this time. Moreover, we did not
Cervical nitric oxide after mifepristone

Figure 3. Examples of the detection of inducible nitric oxide synthase (iNOS) (panel A) and endothelial nitric oxide synthase (eNOS) (panel B) by Western blotting in cervical samples of women in early pregnancy, given either no treatment (No treat) or treated with mifepristone (Mife).

exceed the 3 h follow-up, because the clinical effects of mifepristone, such as uterine contractions and bleeding, usually appear 4–6 h after mifepristone intake (Wing et al., 2000; Kulier et al., 2004; Shaamash and Zakhari, 2005), and these effects could have secondarily affected the cervical NO release. We could not conduct this study as a randomized, placebo-controlled trial, but in view of the objective parameters we assessed, this should not be considered as a major weakness. Moreover, the biochemistry of cervical ripening shows no difference between parous and nulliparous women (Kulier et al., 2004), and therefore, our findings should be applicable also to nulliparous women.

We observed a 7.4-fold increase in cervical NO release already at 1 h, whereas at 3 h, it was over 17 times higher than baseline release. This finding concurred with the maximal mifepristone serum levels observed within 1–2 h after oral administration (Heikinheimo et al., 1986). In contrast, we cannot deduce if a more prolonged study period would have resulted in an even further increase in NO release. However, because we detected a substantial rise in the cervical NO release without uterine contractions, we may presume that the cervical NO release is one of the initial effects of mifepristone during pregnancy. This may be followed by structural softening of the cervix. We were unable to detect a relationship between cervical ripening and NO release because our follow-up did not exceed 3 h when most clinical effects of oral mifepristone occur. Further studies are needed to investigate whether there is such a relationship.

The present data show for the first time that mifepristone induces iNOS in the human cervix. Although we also detected eNOS by means of immunostaining and Western blotting, mifepristone did not induce cervical eNOS expression. We acknowledge that these data should be confirmed with a larger number of cervical samples and with additional methods, such as PCR. However, our findings are consistent with the results of animal studies where the antiprogestin onapristone was used (Ali et al., 1997; Maul et al., 2003). Furthermore, our data demonstrate that after mifepristone treatment, iNOS appears in the cervical glands, where it has not been found in earlier studies (Tschugguel et al., 1999; Ledingham et al., 2000; Maul et al., 2003). This implies that the mifepristone-induced increase in cervical NO release could be derived in part from the cervical glands.

The exact mechanisms by which mifepristone induces cervical NO release are not known. Firstly, a progestosterone receptor-mediated pathway may be involved, as both A- and B-type progesterone receptors are found in the human cervix (Stjernholm-Vladic et al., 2004b). Local progesterone withdrawal in the cervix brought about by mifepristone may lead specifically to the stimulation of iNOS, as seen in our study. Secondly, mifepristone may trigger an influx of inflammatory cells, specifically neutrophils and monocytes, and thus induce cytokine secretion, monocyte chemotactic protein-1 and/or various MMPs (Denison et al., 2000; Maul et al., 2003; Stjernholm-Vladic et al., 2004a). Thirdly, the antiglucocorticoid effect of mifepristone may directly induce iNOS (Olive, 2002) and also stimulate the accumulation of inflammatory cells. Regardless of the mechanism of the NO release, it may soften the cervix by remodeling the extracellular matrix (Maul et al., 2003). NO may also act in concert with the COX pathway, especially with COX-II (Brune et al., 1998; Hapangama et al., 2002; Hausman et al., 2003; Maul et al., 2003; Marx et al., in press) or stimulate apoptosis (Brune et al., 1998; Maul et al., 2003).

In conclusion, the antiprogestin mifepristone accelerates NO release and iNOS expression in cervical cells in viable early pregnancy. This may be one mechanism by which mifepristone initiates cervical ripening.

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