Detection of EETs and HETE-generating cytochrome P-450 enzymes and the effects of their metabolites on myometrial and vascular function

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THE PHYSIOLOGICAL IMMATURITY OF BABIES born preterm (before 37 completed weeks of gestation) exposes them to an increased risk of comorbidities, some of which exist throughout early childhood (32, 33). Recent evidence also indicates that even babies born at a late preterm gestation (34–36 wk), usually considered to have favorable health outcomes similar to those of term neonates, have an approximately threefold risk of cerebral palsy and an increased risk of developmental delay and mental retardation compared with term infants (39). Since 1981, there has been an increase in the incidence of preterm delivery (<37 completed weeks of gestation) in the United States from 9.5 to 12.7% (22). Worryingly, the spontaneous preterm birth rate also rose by 22% (1995–2004) in a low-risk States from 9.5 to 12.7% (22). Worryingly, the spontaneous preterm birth rate also rose by 22% (1995–2004) in a low-risk
sponding dihydroxyecosatrienoic acids (DHETs) in human term placenta, fetal membranes, and myometrial cultures obtained before labor (48). In addition, we have identified the presence of all four regiosomers of the EETs within term myometrium, showing 5,6-EET to be the most abundant (51).

In view of the high levels of these metabolites in the uterus, we investigated the protein expression and localization within the myometrium of the two families of cytochrome P-450 enzymes CYP2 and CYP4, specifically CYP2J2, -2C9/19 and -CYP4A11/22 and -F3. We hypothesized that laboring myometrium has altered levels of expression of these enzymes that would promote a more contractile phenotype compared with nonlaboring tissues. We also investigated the sensitivity of isolated myometrial tissue strips and myometrial arteries to both EETs and HETEs by use of isometric force recordings and wire myography, respectively. The presence of CYPs within the myometrium supports possible autocrine and paracrine roles for some of the EET and HETE metabolites in modifying myometrial and vascular excitability during pregnancy and parturition.

MATERIALS AND METHODS

Ethical approval. The study was approved by the Derbyshire Research Ethics Committee (Protocol no. 04/Q2401/139). In accordance with the Declaration of Helsinki, all patients providing biopsies for the research gave fully informed, written consent.

Tissue collection. Myometrial biopsies, collected at the time of cesarean section (CS), were excised from the upper midline of the lower uterine segment from women undergoing either elective or emergency CS (39–42 wk gestation) as described previously (27). Elective sections (nonlaboring group) were performed for a breech presentation, previous CS, or maternal request. Laboring samples were obtained from women who were having an emergency CS for failure of labor to progress and had cervical dilatation of >5 cm. Specimens were snap-frozen (within 10 min) and stored at −80°C for the research gave fully informed, written consent.

Fetal membranes were excised from the upper midline of the lower uterine segment from women undergoing either elective or emergency CS, were excised from the upper midline of the lower uterine segment from women undergoing either elective or emergency CS (39–42 wk gestation) as described previously (27). Elective sections (nonlaboring group) were performed for a breech presentation, previous CS, or maternal request. Laboring samples were obtained from women who were having an emergency CS for failure of labor to progress and had cervical dilatation of >5 cm. Specimens were snap-frozen (within 10 min) and stored at −80°C for the research gave fully informed, written consent.

Western blotting. Snap-frozen myometrial tissue (~500 mg) was hand-chopped on ice in homogenization buffer (Tris base 20 mM, EDTA 10 mM, NaCl 120 mM, KCl 50 mM, dithiothreitol 2 mM, Igepal 0.15%, protease inhibitor cocktail 1/400, phosphatase inhibitor cocktail 1/80), vortexed several times, and sonicated at 4°C for 15 min. The cold homogenate was then centrifuged at 600 g for 5 min at 4°C and the supernatant collected followed by further centrifugation at 3,000 g for 20 min at 4°C. The supernatant was removed to a clean tube and centrifuged at 100,000 g for 1 h at 4°C. This final supernatant had its protein content determined by the bicinchoninic acid (BCA) method. Equal amounts (50 μg) of protein were loaded onto and resolved by SDS-PAGE on 12% (wt/vol) gels. Proteins were then transferred by electroblotting onto a nitrocellulose (0.45 μm) membrane, after which blots were stained with Poncze S to check for transfer of proteins. Thereafter, blots were washed with TBS-T and blocked with 10% nonfat milk powder (Marvel) overnight at 4°C in TBS. Subsequently, blots were exposed to specific primary antibodies made in 6% nonfat milk powder (in TBS) for 2–3 h at room temperature, washed repeatedly, and exposed to an alkaline phosphatase-conjugated secondary antibody at room temperature for 2 h. Blots were then washed repeatedly and incubated with immune-STAR reagent, and protein was quantified by imaging densitometry (ChemiDoc, Bio-Rad). After viewing, blots were washed in TBS, stripped using a stripping buffer (Chemicon), and then reprobed with a primary antibody raised against β-actin (Abcam, 1:8,000) to check equal loading. This also enabled quantitation of the protein density of the two patient groups (laboring vs. nonlaboring) to identify any differences in the density of expression. The primary antibodies used were those previously used for immunocytochemistry: goat anti-CYP2C9/19 (1:1,300), rabbit anti-CYP4A11/22 (1:3,000), mouse anti-CYP4F3 (1:2,300), and an alternative monoclonal mouse anti-CYP2J2 (1:5,000).

Isometric contractility. Longitudinal myometrial strips, approximately 3 × 3 × 10 mm, were cleaned of adherent myometrial tissue and mounted in an organ bath (Letica; AD Instruments, Oxford, UK) under 2 g of tension in PSS at 37°C bubbled with 95% O2-5% CO2. Contractility for each channel was recorded via an isometric force transducer (0–25 g range) connected to a bridge amplifier and subsequently connected to a dedicated acquisition system (Powerlab/8SP, AD Instruments) on a PC. After 20–30 min of equilibration, myometrial contractions were stimulated using oxytocin (0.5 nM; Sigma Aldrich, Pool, UK). The experiment proceeded upon regular phasic contractions being achieved (between 30 and 90 min). Thereafter, the channels were designated as control, antagonists alone, or antagonists plus drug of interest, being the EETs or HETEs. All experiments were conducted in the presence of a cocktail of inhibitors designed to block other pathways that might influence contractility so that the effect of exogenously applied EETs and HETEs alone could be isolated from de novo vasoactive modulators. This cocktail consisted of 1 μM indomethacin, 10 μM l-NAME, 10 μM 17-octadecynoic acid (17-ODYA), 1 mM 1-aminobenzotriazole (1-ABT), 10 μM AA-861, and 1 mM ascorbic acid to collectively inhibit COX enzymes, nitric oxide production, ω-hydroxylation, and epoxidation leading to EET and HETE production. The concentrations used were based on previous reports attempting to isolate specific HETE or EET effects (2, 16, 43, 44). After ~30 min of tissue exposure to the antagonists, concentra-
tion-response experiments for EETs/HETEs were conducted over a 1–1000 nM range with additions to the bath every 25 min. A resultant 20-min activity integral (area under the time-force curve) was determined (Chart v. 4.2, AD Instruments) for each drug addition, normalized to the control integral, and plotted on a concentration-response curve. The end of each experiment was validated by washout of drug additions and reexposure to the same initial oxytocin stimulus. If the subsequent contraction activity integral over a 40-min period was reduced by more than 20% of activity integral at the start of the experiment, the data were discarded, as changes in activity over the experiment were likely due to other factors than the applied drug of interest. Following this validation, a final 40 mM KCl bolus dose was applied to identify the maximal contractile ability of the tested tissue. Contractility chambers were protected from light throughout the experiment.

**Wire myography.** Small (475 ± 21.04 μm, range 238–599 μm) myometrial resistance arteries (n = 96 from 24 patients) were carefully dissected from a myometrial biopsy and then cut into 2-mm segments before being mounted onto 50 μm wires connected to an isometric force transducer for myography (M610; DMT, Aarhus, Denmark). Arteries were bathed in PSS warmed to 37°C and gassed with 95% O2-5% CO2. Following normalization and determination of length-tension relationships, arteries were set to 0.9 L100 before being depolarized with 20 mM KCl. The role of the BKCa and the ATP-sensitive potassium (KATP) channel in EET- and HETE-induced effects was tested by preincubation of vessels with either the BKCa channel blocker paxilline at 1 μM or KATP inhibitor glibenclamide, also at 1 μM, based on our earlier experiments (10, 35). In some experiments, the endothelial layer was removed by gentle abrasion of the vessel lumen with a horse hair to distinguish endothelial- and nonendothelial-dependent effects. Concentration-response curves were derived by expressing responses relative to the maximum AVP contraction.

**Electrophysiology.** Whole cell recordings using the patch-clamp technique were made using cultured myometrial cells. Free intracellular Ca2+ (pipette) concentrations were calculated to be 50 nM as described in previous publications (27, 28). The bath solution consisted of (in mM): NaCl 135, MgCl2 5, CaCl2 5, HEPES 10, pH 7.4. Outward current was generated by holding the membrane voltage at −60 mV and stepping to +60 mV in 10-mV increments for 200 ms through a Multiclamp 1B amplifier with pClamp v. 9.0 software (Molecular Devices). Automatic series-resistance compensation was performed routinely and monitored continuously. Recordings were terminated if the access resistance changed by more than 25% during the recording period. Steady-state current-voltage (IV) curves were obtained following leak subtraction by use of an automated positive/ negative (P/N) protocol. Drug effects were tested by gravity perfusion. All experiments were carried out at room temperature (20–24°C).

**Reagents.** AVP, BK, oxytocin, ascorbic acid, indomethacin, 1-aminobenzotriazole (ABT), paxilline, protease inhibitor [with 4-(2-aminoethyl)benzenesulfonfonylfluoride (AEBSF), pepstatin A, E-64, bestatin, leupeptin, aprotinin] cocktail and phosphatase inhibitor with sodium vanadate, sodium molybdate, sodium tartrate, and imidazole cocktail were all from Sigma Aldrich. 17-ODYA, AA-861, and L-NAME were obtained following leak subtraction by use of an automated positive/ negative (P/N) protocol. Drug effects were tested by gravity perfusion. All experiments were carried out at room temperature (20–24°C).

**Abstract.** The synthesis and pharmacological effects of the EETs and HETEs were assessed in human myometrium and its resistance arteries. Myometrial resistance arteries were dissected from nonlaboring (NL; A) and laboring (L; B) myometrium. Large inset: α-actin smooth-muscle staining in cultured myocytes. Anti-CYP2J2 immunofluorescence observed in NL (C) and L (D) myocytes. Immunoreactivity is localized to cytosol and nucleus in A and C but either absent or punctuate within the nucleus in B and D. A and C, insets: lack of immunoreactivity when primary antibody was replaced with control IgG. Scale bar, 20 μm.
Electrophysiology data were analyzed using Student’s two-tailed t-test. Group comparisons were noted at significance levels of 0.05. Control lane (C) in this and subsequent figures to follow, is clear.

from Cayman Chemicals. EETs and HETEs were shipped in ethanol. Prior to use, ethanol was evaporated off with pure nitrogen gas, and then the lipids were resuspended in 10% EtOH-PSS ready for use (final vehicle concentration used in experiments was 0.25% inclusive of all antagonist vehicles). All drugs were stored on ice and protected from light until used. Anti-CYP2C9/19 was purchased from Santa Cruz Biotechnology (Calne, UK). Anti-CYP4A11/22 and β-actin loading control antibodies were purchased from AbCam (Cambridge, UK). Anti-CYP2J2 and anti-CYP4F3 were from Abnova (Heidelberg, Germany). Alkaline-phosphatase-linked rabbit and mouse secondary antibodies and anti-smooth muscle α-actin antibody were purchased from Dako (Glostrup, Denmark). All cell culture reagents, including DMEM, HBSS, FBS, and 10,000 U/ml penicillin-10,000 μg/ml streptomycin were bought from Invitrogen (Paisley, UK), and Immunstar reagent for chemiluminescence and molecular weight markers were purchased from Bio-Rad (Hemel Hempstead, UK).

Data and statistical analysis. Protein expression for the CYP enzymes of interest was quantitated against β-actin. For isometric recording studies, concentration-response curves were analyzed by fitting experimental data to the equation

\[ Y = (y_{\text{min}}) + (y_{\text{max}} - y_{\text{min}})/(1 + (x/x_0)^p) \]

where \( y \) is the observed response, \( y_{\text{min}} \) and \( y_{\text{max}} \) are the lowest and highest relaxations achieved, \( p \) is the exponent, \( x \) is the drug concentration, and \( x_0 \) is the EC_{50} giving half-maximal response. Thus, data are expressed as a percentage of the maximum oxytocin response for myometrial contractility or the maximum AVP response when vascular reactivity is assessed. All data are presented as mean ± SE, and the one-way ANOVA statistical test was applied to compare differences among groups with significance being noted at \( P < 0.05 \). Electrophysiology data were analyzed using Student’s two-tailed t-test.

RESULTS

Protein expression of CYP enzymes. Myocytes from pregnant nonlaboring and laboring women were identified on the basis of positive immunoreactivity for α-actin (Fig. 1A, inset) in all patient samples tested (n = 12), confirming the predominance of a smooth muscle phenotype within dispersed samples (data not shown).

Immunoreactivity for CYP2C9/19 and CYP2J2 was present respectively in cells from both nonlaboring (Fig. 1, A and C) and laboring (Fig. 1, B and D) states. Staining with 2C9/19 was apparent in both myocyte types and the nuclear membrane and cytosol (Fig. 2B) states. Staining with 2C9/19 was apparent in both myocyte types. Strongest immunoreactivity for the CYPs tested was found in myometrial cells prepared from both nonlaboring (n = 3; Fig. 2A) and laboring (n = 3; Fig. 2B) myometrium, where CYP4A11/22 expression was present in cells obtained from laboring women (Fig. 1D).

Myometrial samples from pregnant laboring (n = 6) and nonlaboring (n = 7) women were used throughout the Western blotting studies for all four CYPs.

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The expression of CYP4A11/22 proteins in laboring and nonlaboring myometrium (Fig. 2C) showed no significant difference in densitometry at 29.4 ± 1.8 (n = 6) in the laboring state compared with 26.4 ± 1 (n = 7, P = 0.17) in the nonlaboring state (Fig. 2C). Figure 3A depicts blots for CYP2C9/19, where there was no significant difference. A comparison of band intensity (Fig. 3B), although illustrating some differences between laboring (53.5 ± 10.6) and nonlaboring (39.9 ± 6.3) tissues, failed to achieve significance (P = 0.14). However, analysis of the densitometric expression of CYP2J2 protein (Fig. 4A) did show a significant difference (P = 0.01) in expression between laboring (25.0 ± 3.0, n = 6) and nonlaboring (12.4 ± 2.6, n = 7) patients (Fig. 4B).

In addition, there was no significant difference in the densitometric signal comparing CYP4F3 in laboring (5.0 ± 0.7, n = 6) with nonlaboring (3.2 ± 0.7, n = 7, P = 0.12) samples, although the expression level was very low and arguably at the limit of detection (data not shown). This low expression of CYP4F3 was consistent with faint expression in immunocytochemistry (data not shown). In all cases, the expression level determined by densitometric analysis of the loading control β-actin was not significantly different between the laboring and nonlaboring groups (t-test, P > 0.05; Figs. 2C, 3, and 4).

Table 1. IC$_{50}$ values for effects of EET regioisomers on myometrial contractility

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Log IC$_{50}$ Myo</th>
<th>y$_{max}$</th>
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<tbody>
<tr>
<td>5.6-EET</td>
<td>−7.66 ± 0.17</td>
<td>49.82 ± 2.90*</td>
</tr>
<tr>
<td>5.6-EET + 1 μM Pax</td>
<td>−8.44 ± 0.30</td>
<td>67.21 ± 2.52</td>
</tr>
<tr>
<td>8.9-EET</td>
<td>−8.38 ± 0.25</td>
<td>54.88 ± 4.02*</td>
</tr>
<tr>
<td>11.2-EET</td>
<td>−8.11 ± 0.30</td>
<td>77.90 ± 3.37</td>
</tr>
<tr>
<td>14.15-EET</td>
<td>−7.81 ± 0.33</td>
<td>72.90 ± 2.82</td>
</tr>
<tr>
<td>Pax alone</td>
<td>−8.07 ± 0.36</td>
<td>67.49 ± 3.07</td>
</tr>
<tr>
<td>Vehicle control</td>
<td>−7.97 ± 0.22</td>
<td>77.91 ± 1.44</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4–12 observations. EET, epoxyeicosatrienoic acid; Pax, paxilline; y$_{max}$, highest relaxations achieved. *Significantly different from vehicle control (P < 0.05).

β-actin was not significantly different between the laboring and nonlaboring groups (t-test, P > 0.05; Figs. 2C, 3, and 4).

Effect of exogenous HETEs and EETs on myometrial contractility. Over 95% of strips were responsive to 0.1 nM oxytocin at the start of an experiment. All but a further 5% reproduced the initial oxytocin response on secondary exposure to the hormone.

The CYP450 metabolite 20-HETE elicited myometrial contractions (n = 6; Fig. 5) that were similar to those induced by oxytocin. In addition, in the presence of 20-HETE, a small increase in baseline tension was apparent (Fig. 5). No significant effect of either 12-HETE (n = 10) or 20-HETE (n = 6) at the concentrations tested (1–1,000 nM) on spontaneous or oxytocin-induced myometrial contractions was found (P > 0.05; Fig. 5). Results are summarized in Table 1.

Exposure of myometrial strips to 11,12-EET and 14,15-EET produced little change in oxytocin-induced myometrial contractions, y$_{max}$ and IC$_{50}$ (n = 6–12; Table 1). However, 8.9-EET (n = 7; Table 1) and 5.6-EET (n = 15, Fig. 5), although showing no overall significant difference from control contractions at concentrations between 10$^{-10}$ and 3 × 10$^{-7}$ M (n = 10, P > 0.05), elicited a significant respective relaxation (y$_{max}$) to 54.88 ± 4.02 and 49.82 ± 2.90% of maximum at 10$^{-6}$ M (Table 1). Preincubation with 1 μM paxilline (n = 6) significantly reduced 5,6-EET-mediated myometrial relaxation to 67.21 ± 2.52% at 10$^{-6}$ M (Fig. 6), which was itself not significantly different from the effect produced by incubating tissue strips in paxilline alone (n = 7, P > 0.05; Table 1).

Fig. 4. (A) Anti-CYP2J2 detects protein bands of ~47 kDa in laboring (L) and nonlaboring (NL) myometria. (B) Average band density for CYP2J2 between laboring and nonlaboring samples was significantly different (P < 0.05), whereas no significant difference was found between levels for β-actin (P > 0.05).

Fig. 5. Application of 20-HETE (hydroxyeicosatetraenoic acid; 10$^{-9}$–10$^{-6}$ M) induced myometrial contractility, which was accompanied by an increase in baseline tension.

Fig. 6. Effect of 5.6-EET (epoxyeicosatrienoic acid) on myometrial contractility. Experiments were conducted in the presence of the BKCa channel blocker paxilline (Pax; 1 μM), which inhibited contractility significantly (P < 0.05) at the highest concentrations tested. *Significance at P < 0.05 vs. control and 5.6-EET + Pax.
Effect of EETs and HETEs on isolated myometrial arteries.

Myometrial arteries responded by vasoconstriction to the application of increasing concentrations of AVP (10^{-10}–10^{-8} M; 13.13 ± 0.46 kPa). Addition of increasing concentrations (10^{-10}–10^{-6} M) of BK to preconstricted vessels caused endothelium-dependent vasorelaxation (n = 6), which was fully inhibited in arteries stripped of endothelium (n = 5). Similar to our findings for isometric recordings of myometrial contractility, we observed no significant inhibition of vascular reactivity by 11,12- or 14,15-EET (n = 5–6; data not shown); nor did 8,9-EET have any effect on vascular activity (n = 5). Interestingly, 5,6-EET relaxed preconstricted arteries to a maximal value of 42.32 ± 2.65%, but the effect was not blocked by 1 μM paxilline (n = 6; Fig. 7). Denuded arteries produced a diminished relaxant maximal response to 5,6-EET (\( \gamma_{\text{max}} \)) 55.62 ± 7.23% and a corresponding rightward shift in the concentration-response curve, which contrasted with the complete lack of effect of BK in arteries stripped of their endothelium (n = 6; Fig. 7 and Table 2).

Both 12- and 20-HETE failed to constrict arteries in normal PSS (n = 10). However, addition of 20 mM K^+ to the recording chamber evoked a maximal vasoconstriction of 72.62 ± 3.62 and 42.99 ± 9.99% relative to control for 1 μM 20-HETE (n = 6, P < 0.05; Fig. 8) and 12-HETE (n = 6, P < 0.05; Fig. 9), respectively.

Pretreatment of vessels with 1 μM paxilline had no effect on either the 20-HETE (n = 5; Fig. 8) or 12-HETE (n = 5; Fig. 9) response. However, preincubation of vessels with 1 μM glibenclamide enhanced vasoconstriction to 12-HETE, increasing \( \gamma_{\text{max}} \) from 45.38 ± 4.10 to 59.55 ± 1.89 (n = 5; Fig. 9) but had no effect on 20-HETE (Fig. 8).

Table 2. IC_{50} values for effects of bradykinin and EET regioisomers on vasoconstriction of myometrial vessels

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Log IC_{50}</th>
<th>Vessels</th>
<th>( \gamma_{\text{max}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bradykinin</td>
<td>-7.94 ± 0.09</td>
<td>46.69 ± 2.05</td>
<td></td>
</tr>
<tr>
<td>Bradykinin stripped</td>
<td>ND</td>
<td>94.31 ± 8.96*</td>
<td></td>
</tr>
<tr>
<td>5,6-EET</td>
<td>-9.44 ± 0.17*</td>
<td>45.21 ± 3.13*</td>
<td></td>
</tr>
<tr>
<td>5,6-EET stripped</td>
<td>-8.56 ± 0.27</td>
<td>55.46 ± 4.55</td>
<td></td>
</tr>
<tr>
<td>5,6-EET + 1 μM Pax</td>
<td>-9.55 ± 0.14*</td>
<td>39.82 ± 2.59*</td>
<td></td>
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</table>

Values are means ± SE; n = 4–12 observations. ND, not determined. *Significantly different from bradykinin (P < 0.05).

DISCUSSION

Prostaglandins represent the most studied of the eicosanoids in relation to pregnancy, specifically their roles in cervical ripening and myometrial contractility. Much less clear is the contribution of non-prostanoid eicosanoids to uterine function during pregnancy. Real-time PCR studies confirm the presence of mRNA for CYP2C9 and -2J2 epoxygenases and the \( \omega \)-hydroxylase CYP4A11 in the nonpregnant human uterus (37), but there are no data currently available concerning corresponding

Fig. 7. 5,6-EET relaxes myometrial arteries at highest concentrations tested. This effect is diminished when the endothelium is stripped (5,6-EET-str). The endothelium-dependent vasorelaxant effect of bradykinin (BK) is abolished when the endothelium (BK-str) is denuded. Paxilline (1 μM) preincubation (Pax + 5,6-EET) has no significant effect on the magnitude of the 5,6 EET response.

Fig. 8. 20-HETE results in a concentration-dependent increase in vasoconstriction of partially depolarized (K^+) myometrial arteries, which is unaffected by 1 μM paxilline (K^+ + 20-HETE + Pax) and glibenclamide (K^+ + 20-HETE + Glib). Arteries display normal vasoconstriction to arginine vasopressin (AVP).

Results for the HETEs’ effects are summarized in Table 3. Further studies examining the separate application of 8,9-, 11,12-, and 14,15-EET on vessels preconstricted with 20 mM K^+ elicited no significant (P > 0.05) differences compared with control (n = 5–6; results not shown).

Whole cell outward currents recorded from cultured myometrial cells upon stimulation with a voltage step paradigm of step depolarizations from −60 to 40 mV showed an increase in outward current density upon exposure to 5,6-EET (1 μM) at membrane potentials positive to −20 mV (n = 4; Fig. 10). This increase in whole cell current induced by 5,6-EET was significantly different from control (P < 0.05) and reversible upon washout. The relatively small magnitude of this current supports the effect seen in Fig. 6 for the exogenous application of 5,6-EET (>700 nM) upon isometric myometrial contractions. 5 mM TEA blocked over 60% of this current (data not shown). None of the remaining EETs tested exhibited any effect on outward currents (n = 3–6).

Fig. 9. 12-HETE causes a concentration-dependent increase in vasoconstriction of myometrial arteries, which is increased further in the presence of 1 μM glibenclamide (K^+ + Glib + 12-HETE) but unaffected by 1 μM paxilline (K^+ + Pax + 12-HETE).
protein expression in the pregnant human uterus. We now provide evidence for expression of CYP2C9/19 and -2J2 and the ω-hydroxylase CYP4A11/22 in pregnant human myometrium, hence likely formation of EETs regiosomers and 20-HETE in myometrial and vascular components.

The presence of CYP2C9/19 and -2J2 protein supports the EETs profiling determined by our LC-MS studies, demonstrating expression of all four EET regiosomers and 12-HETE in human myometria obtained at elective CS (51). Although no differential expression of CYP2C9/19 following the onset of labor was noted, immunoreactivity in cultured myometrial cells was evident albeit comparatively weak. A loss of mRNA and protein expression for CYP epoxygenases following endothelial cell dispersal has been observed previously (14), although these enzymes were inducible under certain conditions, including shear stress (15). It is probable that the protein signal that we observed by Western blotting in myometrial tissue reflects 2C9/19 expression in myometrial blood vessels as opposed to the predominantly nonvascular smooth muscle cells comprising the biopsy. Interestingly, 2C9/19 immunoreactivity appears to localize to spindle-shaped cells that appear to be vascular rather than myometrial in morphology but may also reflect a heterogeneity of the cell culture, as smooth muscle cells can dedifferentiate quickly in vitro. It is also likely that, although CYP2C9 is predominantly involved with EET synthesis, its widespread tissue distribution (2, 13, 30) implies that it may also have other roles associated with detoxification characteristic of CYP450 activity (13).

Raised levels of CYP2J2 protein noted in laboring samples compared with our nonlaboring cohort are interesting in that they imply a role for EETs in the transition to the labor state. Their role may involve maintaining vasodilatation and blood flow to the metabolically demanding laboring myometrium, possibly, but not exclusively, via EETs acting via BKCa channels (38, 52), although they have also been linked with TRPV channels (12) and thromboxane receptors (4). This unexpected contradictory observation of CYP2J2 also argues in favor of increased EET-generating capacity in laboring tissue. Others have reported CYP2J2 expression in a range of human tissue types, although the expression in myometrium was not studied (13). In common with other reports demonstrating at least two different band sizes for immunoreactive CYP2J2 (17), we suggest that the slightly differing band sizes that we observed possibly reflect isomers generated by alternative splicing of the CYP2J2 gene (49). Interestingly, our LC-MS studies found 5,6-EET followed by 8,9-EET to be present in larger amounts than either 11-, 12-, and 14,15-EET in pregnant human myometrium (51), a trend consistent with their observed effects on myometrial contractility reported here. We are aware of one study where microarray analysis of pregnant mouse myometrium identified clustering of the CYP2J6 gene (homolog of human 2J2), with the gene for COX-1, which in mice is essential to the timing of normal labor (6). This finding suggests that both genes are similarly regulated or that the CYP2J6 gene itself regulates COX-1 gene expression.

Intense immunoreactivity for CYP4A11/22 in myometrial cells indicates ω-hydroxylation and synthesis of 20-HETE in the human uterus. Although there was no apparent difference in protein expression of CYP4A11/22 between nonlaboring and laboring myometrium, we argue that expression of this enzyme is more widespread than the vasculature to include the contractile smooth muscle of the uterus. On this basis, we further propose a role for CYP4A metabolites in influencing the contractile status of the pregnant uterus, since levels of CYP4A4 (homolog of human CYP4A11) in rabbit lung appear upregulated with pregnancy compared with low expression in these organs from nonpregnant animals. CYP4A expression is also inducible by progesterone (34, 40), the classic propregnancy hormone.

There may be other explanations as to why CYP4A11 protein expression is unchanged in the pre- and parturient

Table 3. EC50 values for effects of AVP, 12-HETE, and 20-HETE on vasorelaxation of myometrial vessels

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Log EC50</th>
<th>Vessels</th>
<th>y_{max}</th>
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<tr>
<td>AVP</td>
<td>-9.50±0.10</td>
<td>100.7±1.72</td>
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</tr>
<tr>
<td>12-HETE</td>
<td>-9.43±0.60</td>
<td>45.38±4.10</td>
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<tr>
<td>12-HETE + Glib</td>
<td>-9.75±0.86</td>
<td>42.15±3.82</td>
<td></td>
</tr>
<tr>
<td>20-HETE</td>
<td>-8.05±0.15*</td>
<td>50.55±1.89*</td>
<td></td>
</tr>
<tr>
<td>20-HETE + Glib</td>
<td>-8.33±2.82*</td>
<td>71.96±2.82*</td>
<td></td>
</tr>
<tr>
<td>20-HETE + Pax</td>
<td>-8.01±0.11*</td>
<td>71.59±2.10*</td>
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</tbody>
</table>

Values are means ± SE; n = 4–12 observations. AVP, arginine vasopressin; HETE, hydroxyeicosatetraenoic acid; Glib, glibenclamide. *Significantly different from AVP, 12-HETE + Pax, 12-HETE (P < 0.05); *significantly different from AVP (P < 0.05).
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uterus. For example, 20-HETE is reported to be increased two- to threefold above basal levels by isometric stretch in porcine coronary arteries (42), an observation that is relevant to pregnancy in relation to the stretching of the uterus. Similar expression levels of CYP4A11/22 in biopsies of nonlabor and labor myometrium may reflect the maximally stretched status of the uterus in the third trimester. Unlike CYP4A11, a lack of expression of CYP4F3 due to poor antibody specificity made it difficult to draw any firm conclusions regarding its protein expression in human myometrium.

Interestingly, the laboring phenotype of the cell was associated with apparently increased CYP2J2 and CYP4A11/22 immunoreactivity in the nuclear membrane and cytosol. This punctate immunoreactivity probably represents localization to the endoplasmic reticulum, where most CYPs are found. We suggest that this observation reflects a switch in physiological function of the myometrium in preparation for increased trafficking and export to other intracellular organelles of CYP enzymes to meet the increased demands of parturition, possibly by elevated production of EETs and HETEs. Shifts in subcellular localization of CYP enzymes have indeed been linked to modified cellular function (46). We found little functional evidence to support an obvious direct role for either 12-HETE or 20-HETE on myometrial excitability, although 20-HETE induced contractions that characteristically waned with time. Our results with the HETEs indicate that either the uterus is already contracting maximally in vitro or adding additional contractile agents does not enhance its activity much further. The lack of an obvious 20-HETE effect may also be explained by the fact that, if 20-HETE acts by blocking the BKCa channel (54), this may not be evident in isometric tension recordings, as it is argued that the BKCa channel plays no role in spontaneous rat myometrial contractility since preincubation with a range of BKCa channels blockers was without effect (1). However, this contrasts with our findings with 5,6-EET reported herein, where there does appear to be a small role for BKCa channels in oxytocin-induced myometrial contractions and whole cell outward current. Our results confirm earlier findings that showed myometrial contractility was unaffected by 12-HETE but was inhibited by 5-HETE (5), although we did not test 5-HETE in our system. However, given the interactions between the COX and CYP pathways, as well as the lipid mediators generated, further investigations of these pathways in relation to parturition seem warranted.

Myometrial resistance arteries from normal pregnancy display a robust endothelium-dependent response when challenged with BK (3). Our findings from normal myometrial arteries demonstrate that 5,6-EET causes vasodilation and that there is a rightward shift in this response when the endothelium is denuded, suggesting a reduced sensitivity to the eicosanoid. The underlying target for this effect is not the BKCa channel, as paxilline was without effect; however, a contribution from other endothelial mediators cannot be excluded.

Although the magnitude of the vasoconstriction observed in myometrial arteries with 12-HETE compared with AVP and 20-HETE is relatively small, our observation suggests that 12-HETE has vasoactive effects that appear to be mediated by blockade of KATP channels, known to play a key role in linking cellular metabolism with membrane excitability in vascular smooth muscle (41). However, the effects of eicosanoids on the KATP channel in the vasculature are unclear, given the reported nonspecific effects of inhibitors used as well as the possibility that suppressing CYP450 metabolite production unmask effects of the precursor molecule arachidonic acid, which has known effects at the KATP channel (8).

On the basis of the data presented, we postulate that altered CYP450 profiles and the downstream changes in synthesis and bioavailability of EETs and HETEs may, in part, regulate myometrial excitability as well as uterine vasoreactivity. It remains to be determined whether the CYP expression changes reported herein translate to metabolic activity that regulates levels of other mediators of labor, e.g., prostaglandins, given that 5,6-EET, 19-HETE, and 20-HETE are also substrates for COX enzymes (43), although our studies were undertaken in the presence of indomethacin, rendering this unlikely. Further investigations utilizing more effective and selective pharmacological agents and better antibodies to probe EETs and HETEs function in the uterus are required to unravel the complexity of these responses.

In summary, our findings indicate the presence of the major CYP enzymes involved in the formation of EETs and HETEs. Furthermore, 5,6-EET relaxed human myometrium and caused vasodilation of myometrial resistance arteries. Although clear effects of 12- and 20-HETE were difficult to discern in myometrium, they clearly caused vasoconstriction of myometrial arteries. We suggest that the effects of 20-HETE act to maintain cellular depolarization in a muscle that may already be contracting, such as may be found during labor.

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GRANTS

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