Pregnancy-Specific Changes in Uterine Artery Endothelial Cell Signaling in Vivo Are Both Programmed and Retained in Primary Culture

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Ovine uterine artery (UA) endothelial cells (UAEC) maintained in culture to passage 4 retain pregnancy-specific changes in vasodilator production, which in turn is associated with differences in Ca\(^{2+}\) and ERK 1/2 signaling. The question remains whether this is an accurate portrayal of the situation in vivo, or more simply whether these same signaling responses seen at passage 4 accurately reflect those functioning in the cells in vivo. Small groups of endothelial nitric oxide synthase-positive cells from both pregnant and nonpregnant ewes were freshly isolated and used to change in the intracellular free calcium concentration ([Ca\(^{2+}\)]\(_i\)) in primary culture and to detect ERK 1/2 phosphorylation by immunocytochemistry. Furthermore, detailed comparisons of mRNA species were made between freshly isolated and cultured (passage 4) cells using cDNA microarray analysis and verified, where possible, using PowerBlot analysis. Freshly isolated cells showed no detectable [Ca\(^{2+}\)]\(_i\) elevation in response to angiotensin II, epidermal growth factor, basic fibroblast growth factor, vascular endothelial growth factor but did respond to ATP in a dose-dependent (1–300 μM) manner. At higher doses of ATP, [Ca\(^{2+}\)]\(_i\) elevation was sustained longer and showed a high incidence of regular oscillations in cells from pregnant compared with nonpregnant ewes. Also, ATP and basic fibroblast growth factor treatment caused activation of ERK 1/2 in significantly greater numbers of freshly isolated cells from pregnant than from nonpregnant ewes. cDNA microarray analysis showed results consistent with endothelium but revealed few differences in mRNA species and levels between freshly isolated and passage 4 cells or between the pregnant and nonpregnant ewes. In conclusion, our data show for the first time that pregnancy-specific changes in Ca\(^{2+}\) and ERK 1/2 signaling are indeed observed in freshly isolated UA endothelium. This suggests in turn that such pregnancy-specific changes in UA endothelial function in vivo in response to a variety of agonists during pregnancy are both programmed at the level of cell signaling and retained in culture. (Endocrinology 144: 3639–3650, 2003)
and preeclamptic women. When maintained and expanded for 14–28 d in culture, (a similar time to our fourth passage or beyond for UAEC), HHVE had significantly larger ATP-stimulated increases in $[\text{Ca}^{2+}]_i$, than occurred in cells isolated from nonpregnant or preeclamptic women. Previous studies on the UAEC model have also shown that although ATP caused an increase in $[\text{Ca}^{2+}]_i$, (17) that is enhanced by pregnancy, AII, bFGF, EGF, and VEGF all induce NO and PGIL production without an associated increase in $[\text{Ca}^{2+}]_i$. However, ERK 1/2 activation is induced by AII, ATP, bFGF, and VEGF to a higher extent in P-UAEC than in NP-UAEC, and this correlated closely with increased vasodilator production (18). Thus, enhanced coupling to alternate signaling pathways during pregnancy may activate eNOS and cPLA2 independently of an increase in $[\text{Ca}^{2+}]_i$.

One limitation of the HHVE or UAEC model is that the data are derived from cells expanded in primary culture. As a consequence, not all of the cells come directly from the in vivo state, and underlying cellular and physiological properties of the cells may have been altered by culture conditions. To determine whether culture conditions altered cellular function, experiments were performed on freshly isolated and passage 4 cell preparations to compare agonist effects on cell signaling pathways. In addition, cDNA microarray and protein screening were used to fingerprint the cellular phenotype of both preparations. Ex vivo results consistent with the passage 4 cells would validate the UAEC model and provide evidence supporting our hypothesis that a cell programming event occurs during pregnancy, which leads to increased coupling of receptor activation to the ERK cascade. Alternatively, if ex vivo cells did not respond similarly to passage 4 UAEC, then the culture conditions may have altered cellular function and the model would be of limited value. Our results indicate that the UAEC model accurately reflects in vivo UA endothelium and pregnancy-associated changes in both $\text{Ca}^{2+}$ and ERK signaling events investigated to date, but the underlying cause remains unclear.

Materials and Methods

Materials

Monoclonal antibodies to eNOS, bradykinin (BK) type 2 receptor (B2-R), Jun, inositol 1,4,5-triphosphate receptor 3 (IP3R3), protein kinase C (PKC)ζ, PKCβ, A-Raf, and C-Raf were purchased from Transduction Laboratories, Inc. (Lexington, KY). Polyclonal antibodies to PKCa and PKCγ were purchased from PanVera Corp. (Madison, WI). Polyclonal antibody to phosphorylated ERK (P-ERK) 1/2 was purchased from New England Biolabs, Inc. (Beverly, MA). ATP (disodium salt) was purchased from Sigma-Aldrich Corp. (St. Louis, MO), and thapsigargin was purchased from Calbiochem (San Diego, CA). Unless noted other wise, MEM α-Val and all other cell culture reagents were purchased from Life Technologies, Inc. (Grand Island, NY), glass-bottom microwell dishes for Ca$^{2+}$ imaging studies were purchased from MatTek Corporation (Ashland, MA), and eight-well glass chamber slides were purchased from Nunc, Inc. (Naperville, IL).

Isolation of UAEC

UAECs were obtained from Polypay and mixed Western breed nonpregnant and pregnant ewes at 120–130 d gestation during nonsurvival surgery. Procedures for animal handling and protocols for experimental procedures were approved by the University of Wisconsin-Madison research animal care committees of both the Medical School and the College of Agriculture and Life Sciences, and they follow the recommended American Veterinary Medicine Association guidelines for humane treatment and euthanasia of laboratory farm animals. Briefly, UAs were dissected free of connective tissue, fat, and veins. The arteries were thoroughly rinsed free of blood using medium 199 before tying off arterial branches, clamping off the larger diameter end, and inflating with medium 199 containing 5 ml collagenase B (Roche Applied Science, Indianapolis, IN) and 0.5% BSA through a luerlock three-way tap. Digestion was allowed to proceed for 55 min at 37°C before flushing the collagenase solution and endothelial cell sheets from the inner surface of the vessels. Cells were used immediately for RNA extraction or plated directly to experimental slides or dishes, and incubated overnight for attachment or plated to 35-mm dishes (Falcon Primaria, through Fisher Scientific, Pittsburgh, PA) and expanded by passing three times before freezing as described (17). Cells were then thawed as needed and plated to similar experimental dishes as for freshly isolated cells.

Fura 2 Ca$^{2+}$ imaging studies

Groups of freshly isolated cells were incubated in 35-mm glass-bottom dishes overnight to allow attachment before being used for experiments. Immediately before use, the cells were washed twice with 2 ml prewarmed (37°C) Krebs buffer [125 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 1 mM KH₂PO₄, 6 mM glucose, 25 mM HEPES, 2 mM CaCl₂ (pH 7.4)]. The cells were then covered with 1 ml Krebs buffer and loaded with 5 μM of membrane permeable fura 2-AM (Molecular Probes, Eugene, OR) for 45 min. The cells were washed with Krebs buffer as described above, covered in 1 ml of Krebs buffer, and incubated for 30 min to allow complete ester hydrolysis. At the end of the 30-min incubation period, the cells were again washed and covered with 1 ml of Krebs buffer. Fura 2 loading was verified by viewing at 380 nm UV excitation on a Nikon inverted microscope (InCyt Im2, Intracellular Imaging, Inc., Cincinnati, OH). A group of cells was placed in the field of view, and 5-min recordings commenced using alternate excitation at 340 and 380 nm at 50-sec intervals and measuring emitted light using a photomultiplier (Intracellular Imaging, Inc.). From the ratio of emission at 510 nm detected at the two excitation wavelengths and by comparison to a standard curve established for the same settings using buffers of known free $[\text{Ca}^{2+}]_i$, the $[\text{Ca}^{2+}]_i$ was calculated in real time using the InCyt Im2 software. ATP was added at 1, 5, 10, 30, 100, and 300 μM to determine Ca$^{2+}$ response of the cells to ATP (17, 18). Cells were treated with 10 μM thapsigargin to empty intracellular stores of Ca$^{2+}$, as required.

Immunocytochemistry (ICC)

After plating to eight chamber slides and incubation overnight, cells were washed and incubated in serum-free medium for 4 h. In some cases, cells were pretreated with U0126 (20 μM) for 20 min before agonist stimulation. Cells were stimulated with the agonists for the reported time at the appropriate doses (AII 100 nM, ATP 100 μM, bFGF 10 ng/ml, EGFl 10 ng/ml, and VEGF 10 ng/ml) in a final volume of 400 μl serum-free media. After treatment, cells were washed with 300 μl of ice-cold PBS to terminate the reaction and fixed (4% formaldehyde, 0.1 M sodium cacodylate buffer) for 1 h at room temperature. After fixation, cells were washed in distilled water and stored in 70% ethanol until ICC was performed. Anti-phospho p42/p44 MAPK polyclonal antibody (New England Biolabs, Inc.) was used at 1:125 dilution, and secondary antibody [horseradish peroxidase (HHR)-linked antirabbit IgG; Vector Laboratories, Burlingame, CA] was used at the manufacturer's suggested dilution. Anti-eNOS polyclonal antibody (Transduction Laboratories, Inc.) was used at 0.5 μg/ml, and secondary antibody (HRP-linked antirabbit IgG, Vector Laboratories) was used at the manufacturer's suggested dilution. Cells were briefly counterstained with hematoxylin. After staining, cells were dehydrated, mounted, and scored.

Total RNA isolation

Either freshly dispersed cells were solubilized directly into 1 ml RNAzol B or 70–80% confluent cells in a T75 (passage 4) were rinsed twice in ice-cold PBS and lysed in 2 ml of cold RNAzol B (Tel-Test, Inc., Friendswood, TX). Samples were phase-separated with chloroform, and after brief centrifugation, the upper aqueous phase was removed twice with phenol/chloroform/isoamyl alcohol using heavy-
grade phase lock gel (5Prime-3Prime, Boulder, CO). After isopropanol precipitation of RNA for 1 h at −20 °C, pellets were recovered by centrifugation (12,000 × g, 30 min). RNA pellets were washed in 75% ethanol. Total RNA was solubilized in molecular biology grade water (5Prime-3Prime) and quantified by spectrophotometry. Northern analysis verified UAEC RNA quality recovered by this methodology.

**Total cellular protein lysate isolation and PowerBlot analysis**

At 70% confluency, cells were rinsed twice with ice-cold PBS and lysed in 500 μl phosphatase protein lysis buffer (4 mM sodium pyrophosphate, 50 mM HEPES, 100 mM NaCl, 10 mM EDTA, 10 mM NaF, 2 mM NaVO₄, with 1 mM phenylmethylsulfonyl fluoride, 1.0% Triton X-100, 5 μg/ml leupeptin, and 5 μg/ml aprotinin). Sonicated lysates were centrifuged at 10,000 rpm for 10 min to pellet cellular debris. Protein concentration was determined using the bicinchoninic acid assay. After verification of a small aliquot by SDS-PAGE for eNOS expression in each protein sample, NP-UAEC or P-UAEC lysates were pooled [−8 mg of pooled protein lysates, nonpregnant (n = 4 ewes) and pregnant (n = 4 ewes)] and sent to BD Biosciences (San Jose, CA) for PowerBlot analysis.

**cDNA microarray analysis**

RNA was pooled from three animals in each group for each cDNA microarray analysis. Total cellular RNA (2.5 μg from freshly isolated cells or 5 μg from passage 4 cells) was primed with oligo-dT primer, and cDNA probes were generated in 1× First Strand Buffer, 3.3 μM dithiothreitol, 1 mM deoxy (d)ATP, dGTP, dTTP, 300 U Superscript II reverse transcriptase, in the presence of 100 μCi ³²P α-dCTP. Probes were purified using Bio-Spin 6 Chromatography Column (Bio-Rad Laboratories, Inc., Hercules, CA). Incorporation of ³²P α-dCTP was routinely found to be 60–65% per 5 μg. The amount of probe used for hybridization was normalized to the lowest incorporated disintegrations per minute. Human cDNA arrays (GF211; Research Genetics, Inc., Huntsville, AL) were prehybridized in 10 ml of MicroHyb buffer with Cot-1 DNA and Poly dA at 42 °C for at least 2 h. After overnight hybridization in fresh MicroHyb/Cot-1/PolydA buffer with radiolabeled probe, membranes were washed twice at 50 °C in 2 standard saline citrate, 1% sodium dodecyl sulfate for 20 min, and once at room temperature in 0.5× standard saline citrate, 1% sodium dodecyl sulfate for 15 min. The arrays were analyzed by autoradiography. Exposure times were normalized (24, 48, and 96 h for each 2.5 × 10⁶ dpm/ml of incorporated probe) to allow direct comparison, and images were analyzed using Pathways2.0 (Research Genetics, Inc., Excel 97 (Microsoft, Redmond, WA) and SigmaPlot 6.0 (SPSS, Inc., Chicago, IL). Data from GF211 arrays presented here were also entirely consistent with that for cultured cells and showed regular oscillations over the 30–300 μM ATP dose range in 12 of 23 observations for cells from pregnant ewes (Fig. 2, inset), whereas oscillations were only seen in 1 of 25 observations at these same doses for cells from nonpregnant ewes. Our previous studies implied a strong dependence of the [Ca²⁺]i response on intracellular Ca²⁺ pools in UAEC at passage 4 (18). Thapsigargin (10 μM) pretreatment of freshly isolated cells was able to dramatically impair the subsequent response to ATP in freshly isolated cells from both nonpregnant (n = 3) and pregnant (n = 1) ewes (data not shown). This suggests that the extent to which intracellular Ca²⁺ stores are involved is similar even if the mechanisms governing Ca²⁺ release and possible oscillations are subtly altered.

Increased vasodilator production is associated with not only elevated [Ca²⁺]i, but also enhanced ERK 1/2 activity in passage 4 UAEC. Therefore, we investigated whether P-ERK 1/2 was increased in response to the same agonists in freshly isolated cells. Because of extremely limited cell numbers, ICC was used instead of the Western analysis used previously (17, 18). First, an agonist time course on passage 4 P-UAEC was performed to compare the sensitivity of ICC to the immunoblotting performed in the previous study (17) (Fig. 3). Random subsets of stained cells were photographed and then scored as positive or negative for P-ERK 1/2. The percentage of positive cells at 5, 10, and 20 min were compared with unstimulated controls. ATP, the most potent agonist tested, robustly increased P-ERK 1/2 immunostaining at 5 min and sustained P-ERK 1/2 staining through 10 and 20 min. Stimulation with bFGF also evoked increased P-ERK 1/2 staining at 5 min, which was sustained to 10 min and then fell to a level below significance at 20 min. VEGF and All showed a similar pattern to that of ATP with peak stimulation occurring at 5 min. Both VEGF and All maintained increased P-ERK 1/2 staining at 10 but not 20 min. EGF treatment induced a different pattern of stimulation compared with that of any of the other agonists used. EGF stimulation of P-ERK 1/2 only occurred at 10 min of treatment. In light of these data, the 10-min time point was chosen for subsequent experiments on freshly isolated cells. This time point was also consistent with our previous Western analysis from passage 4 UAEC studies.

In subsequent experiments with both passage 4 and freshly isolated cells, additional controls were used. First, at least two chambers of unstimulated cells were reserved for

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**Results**

On the basis of our previous studies in passage 4 UAEC, freshly isolated UAEC loaded with fura 2 were treated with All (100 nM), ATP (30 μM), bFGF (10 ng/ml), EGF (10 ng/ml), or VEGF (10 ng/ml) (17, 18). Only ATP evoked an increase in the [Ca²⁺]i (Fig. 1) in freshly isolated cells from either pregnant or nonpregnant animals. Cells treated with 1–300 μM ATP showed a progressive dose-dependent elevation in [Ca²⁺]i, with 100–300 μM ATP causing a maximal response for pregnant and nonpregnant derived ex vivo cells (Fig. 2). The [Ca²⁺]i response observed in cells from pregnant ewes was similar at lower doses (Fig. 1; 30 μM ATP), but the response in cells from pregnant ewes was more consistently sustained above basal for a longer time when higher doses of ATP were used (above 30 μM; Fig. 2, inset). The cells also showed a similar pattern to that of ATP with peak stimulation occurring at 5 min. Both VEGF and AII maintained release and possible oscillations are subtly altered.

Increased vasodilator production is associated with not only elevated [Ca²⁺]i, but also enhanced ERK 1/2 activity in passage 4 UAEC. Therefore, we investigated whether P-ERK 1/2 was increased in response to the same agonists in freshly isolated cells. Because of extremely limited cell numbers, ICC was used instead of the Western analysis used previously (17, 18). First, an agonist time course on passage 4 P-UAEC was performed to compare the sensitivity of ICC to the immunoblotting performed in the previous study (17) (Fig. 3). Random subsets of stained cells were photographed and then scored as positive or negative for P-ERK 1/2. The percentage of positive cells at 5, 10, and 20 min were compared with unstimulated controls. ATP, the most potent agonist tested, robustly increased P-ERK 1/2 immunostaining at 5 min and sustained P-ERK 1/2 staining through 10 and 20 min. Stimulation with bFGF also evoked increased P-ERK 1/2 staining at 5 min, which was sustained to 10 min and then fell to a level below significance at 20 min. VEGF and All showed a similar pattern to that of ATP with peak stimulation occurring at 5 min. Both VEGF and All maintained increased P-ERK 1/2 staining at 10 but not 20 min. EGF treatment induced a different pattern of stimulation compared with that of any of the other agonists used. EGF stimulation of P-ERK 1/2 only occurred at 10 min of treatment. In light of these data, the 10-min time point was chosen for subsequent experiments on freshly isolated cells. This time point was also consistent with our previous Western analysis from passage 4 UAEC studies.

In subsequent experiments with both passage 4 and freshly isolated cells, additional controls were used. First, at least two chambers of unstimulated cells were reserved for
FIG. 1. Agonist-induced rise in intracellular free Ca\(^{2+}\) concentration in freshly isolated cells. Cells were preloaded with fura 2-AM and treated as described. Representative tracings of [Ca\(^{2+}\)]\(_i\) elevation in response to AII (100 nM), ATP (30 μM), bFGF (10 ng/ml), EGF (10 ng/ml), or VEGF (10 ng/ml) in a representative cell group derived from a pregnant ewe (A) or nonpregnant ewe (B) are shown. Results are representative of those from five or six similar observations, each using cells from separate animals.
FIG. 2. ATP dose-response in freshly isolated cells from pregnant (A) and nonpregnant (B) ewes. Cells were loaded with fura 2-AM as described and were then stimulated with 1, 3, 10, 30, 100, or 300 μM ATP. The response for each dose was calculated as the area under the curve for the first 3 min after the addition of ATP. All responses were normalized to the 100-μM response. Data are the mean ± SEM of data from four to six independent animals/experiments each. Significance from control is as indicated (*, P < 0.05).
eNOS and IgG immunostaining. In every case, all of the cells immunostained positive for eNOS and negative with mouse IgG control (Fig. 4). These data confirmed that the freshly isolated cells and the cultured cells were pure populations of endothelial cells. Second, unstimulated cells were immunostained for P-ERK 1/2 to determine the basal level of P-ERK 1/2 within the cell population. In addition, one chamber of cells was stimulated with bFGF and then stained using IgG to control for antibody specificity. For one further control, cells were pretreated with 20 μM U0126 (an MAPK kinase antagonist) for 20 min before agonist stimulation; U0126 entirely eliminated P-ERK 1/2 staining, further validating the specificity of the P-ERK 1/2 antibody (Fig. 4).

A comparison of agonist treatments on cells freshly isolated from nonpregnant or pregnant ewes showed that the P-ERK 1/2 response was largely similar to that observed at passage 4 (Fig. 5). In unstimulated cells alone there were no significant differences between the percentage positive for nonpregnant vs. pregnant derived freshly isolated cells or for UAEC at passage 4. In the initial time courses in P-UAEC, AII stimulated a significant increase in P-ERK 1/2 positive cells at 5 and 10 min, but in these experiments (Fig. 5), in which a 10-min stimulation was routinely used, no significant difference was detected, probably because of the choice of a suboptimal stimulation time for AII responsiveness. ATP stimulated a significant response in all cell preparations over the respective unstimulated controls but also achieved a higher level for pregnant compared with nonpregnant in freshly isolated cells and in P-UAEC compared with NP-UAEC. Similar findings were also observed for bFGF. Different results were obtained, however, for EGF and VEGF. For EGF, the responses of nonpregnant freshly isolated and NP-UAEC were significant above the respective unstimulated controls, whereas the pregnant freshly isolated and P-UAEC did not reach significance. In addition, although VEGF consistently showed a response significantly above...
unstimulated control in all cell preparations, there was no significant difference between pregnant and nonpregnant derived cells for both freshly isolated cells and for UAEC at passage 4. As far as changing responsiveness of cultured cells vs. freshly isolated cells, for ATP and bFGF the responses were comparable, with the exception that ATP responsiveness in NP-UAEC was significantly reduced, but by no means abolished, relative to fresh cells. For EGF, the UAEC at passage 4 responded somewhat more than freshly isolated cells, although the relative nonpregnant vs. pregnant differ-

TABLE 1. Comparison of GF211 microarray and PowerBlot results with previously investigated findings

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<thead>
<tr>
<th>Accession no.</th>
<th>Gene description</th>
<th>MA</th>
<th>PB</th>
<th>Previously investigated</th>
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<td>H16958</td>
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UAEC at passage 4 were previously investigated for RNA, protein, or activity as indicated in the far right column. The presence (+) or absence (-) of the gene or protein signal on the cDNA microarray (MA) or PowerBlot (PB) is indicated. IP, Immunoprecipitation.
ence was similar in each case. For VEGF, the UAEC showed a significantly bigger response than that for freshly isolated cells, but again the relative change of nonpregnant vs. pregnant was not altered between freshly isolated and UAEC preparations.

In addition to functional signaling assays, changes in UAEC as a result of pregnancy and/or exposure to cell culture conditions were assessed. To achieve this, expression of approximately 4000 mRNA species (cDNA microarray, Research Genetics, Inc.) and 500 receptors and cell-signaling associated proteins (PowerBlot, BD-Transduction Laboratories, Inc.) were investigated with the expectation that a gene may be switched on or off during pregnancy. The Human Named Genes GeneFilters Release I (GF211) cDNA array contains cDNA targets of 3’ sequence of human IMAGE/LLNL clones that are approximately 1000 bp in length. The specificity of the cDNA microarray and the PowerBlot for ovine tissue was initially verified by comparing the results with those previously obtained in UAEC using conventional means (Western, RT-PCR, and functional data). These data are summarized in Table 1. From this analysis, the combined approach correctly identifies the presence and absence of previously examined proteins and mRNAs. Comparisons of data from freshly isolated cells to passage 4 UAEC revealed no significant differences for either nonpregnant or pregnant ewes (Fig. 6). Likewise, comparison of nonpregnant vs. pregnant freshly isolated endothelium showed few, if any, differences beyond 2-fold (Fig. 6), a level that is also seen when comparing RNA from individual NP-UAEC with NP-UAEC or individual P-UAEC with P-UAEC preparations on GF211 arrays (data not shown). Thus, no differences are seen between nonpregnant and pregnant derived endothelium or UAEC that exceed the level of individual variations between animals in a given nonpregnant or pregnant state.

Although no differences in mRNA or protein expression were detected, the data are consistent with cells of endothelial origin, and a summary of positive signals is presented (see the table published as supplemental data on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org). A subset of the cDNA array and PowerBlot findings were then independently confirmed in NP-UAEC vs. P-UAEC where other methods or reagents were available. The cDNA array demonstrated positive signals for PLCβ3 and IP₃R (type 1), and the presence of the corresponding protein in UAEC was validated in each case by Western analysis (Fig. 7). The cDNA array also reported the existence of the type 1 ryanodine receptor, but we have been unable to elicit a Ca²⁺ response using ryanodine in UAEC (data not shown). Of the PKC isoforms available for detection on the cDNA array, subtypes I and II were clearly detected. A suitable antibody that reacts with ovine PKCII is not available; however, the presence of PKCII was substantiated in UAEC by Western analysis (Fig. 7). PowerBlot analysis demonstrated the presence of A-Raf, and immunoblotting established for comparison of cDNAs between freshly isolated cells and cultured cells derived from nonpregnant ewes. B. Intensities are plotted for comparison of cDNAs between freshly isolated cells and cultured cells derived from pregnant ewes. C. Intensities are plotted for comparison of cDNAs generated from freshly isolated cells derived from pregnant vs. nonpregnant ewes.
lished the presence of both A- and C-Raf isoforms in UAEC (Fig. 7). The cDNA array also detected the presence of the BK B2-R. This was verified by Western blot (Fig. 7) and functionally confirmed by treating passage 4 NP-UAEC and P-UAEC with BK. BK treatment caused a rise in \([\text{Ca}^{2+}]_i\), stimulated phosphoinositol production, and increased ERK 1/2 phosphorylation (Fig. 8). Although the magnitude of the phosphoinositol response was not significantly different between P-UAEC and NP-UAEC, the P-UAEC consistently showed higher phosphoinositol production to all of the agonists tested (Fig. 8). Furthermore, after AII, BK, or ATP stimulation, the ERK 1/2 response as measured by Western analysis was clearly greater in the P-UAEC than in NP-UAEC (Fig. 8).

Intracellular \([\text{Ca}^{2+}]_i\) responses to BK were robust at any concentration used; however, no further \([\text{Ca}^{2+}]_i\) response could be evoked with additional BK treatments (Fig. 8). The rapid loss of response by homologous desensitization and receptor internalization is a feature of the BK B2-R (20). We estimate that BK B2-R internalization is complete in less than 5 min in UAEC. In addition, a BK response is not recovered even after as much as 60 min of incubation under basal conditions before the next treatment. Our finding that the subsequent response to ATP was unaffected further implied homologous desensitization.

**Discussion**

In freshly isolated endothelial cells, ATP was the only agonist that initiated a \([\text{Ca}^{2+}]_i\), increase in UA endothelium, as previously reported for passage 4 P-UAEC and NP-UAEC. In both the P- and NP-UAEC, a robust acute response was consistently seen after exposure to 10 \(\mu\text{M}\) ATP. For groups of freshly isolated cells, a significant \([\text{Ca}^{2+}]_i\) response was not reliably seen until cells were exposed to 30 \(\mu\text{M}\) ATP. It should be noted that the previous UAEC study used single isolated cells, whereas the freshly isolated cell study herein imaged the groups of 5–20 cells obtained by the protocol for UA endothelium dispersion. Therefore, if some cells in each imaged group did not respond to ATP, the \([\text{Ca}^{2+}]_i\), changes would appear blunted because the response reflects the average for the group instead of a peak response for a single cell. This is highly likely because in studies of individual UAEC at passage 4, typically 50–60% of cells respond to ATP (18). For this same reason, absolute quantitative comparison of peak \([\text{Ca}^{2+}]_i\) levels recorded from groups of cells from pregnant and nonpregnant ewes bear little meaning. Nonetheless, consistent with our previous studies, the response in freshly isolated cells from pregnant ewes was more sustained and prone to \([\text{Ca}^{2+}]_i\) oscillations at higher doses (30–300 \(\mu\text{M}\)) during the sustained phase as opposed to cells from nonpregnant ewes. Thus, although the dose responses are similar between cells from nonpregnant and pregnant ewes, the in vivo UA endothelium has a more sustained \([\text{Ca}^{2+}]_i\) response during pregnancy, likely using oscillations to protect against \([\text{Ca}^{2+}]_i\) toxicity. This also suggests that synchronization of oscillations may have occurred, most likely through gap junctions (21, 22). This raises the interesting possibility that during pregnancy UA endothelium may be able to propagate signals from the point of origin by means of \([\text{Ca}^{2+}]_i\) waves. Thus, primary vessel responses to ATP may influence lower level branches of the uterine vasculature. Likewise, if such wave propagation occurs, trigger cells within the monolayer may initiate generalized responses, as has been described in muscular tissues.
In addition to calcium mobilization, the phosphorylation of ERK 1/2 in cultured and freshly isolated ex vivo cells was examined because both [Ca\(^{2+}\)]\(_i\) and ERK activation can modulate vasodilation production (9–14, 23–27). Our previous studies used Western analysis to quantify the extent of ERK 1/2 phosphorylation in response to various agonist treatments; however, acute isolation procedures yield only limited numbers of cells, and therefore ICC was used. Detection of ERK 1/2 phosphorylation differs between ICC and Western analysis because the data collected with ICC reflect the number of cells stained and not a change in staining intensity, whereas Western analysis measures the total amount of P-ERK 1/2 for a population of cells, a function of both individual cell responsiveness and the number of cells responding. When detection of P-ERK 1/2 using ICC on passage 4 P-UAEC was compared with previous Western analysis of the same cells (17), the data from ICC did not achieve the same levels of sensitivity or statistical significance. Use of this assay technique did, however, show that the overall trends between nonpregnant and pregnant derived freshly isolated cell preparations were retained after prolonged culture. Thus, we can now conclude that UAEC are an accurate reflection of the situation observed in vivo. Furthermore, the current investigation also extends this prior finding because the data obtained by cell counting show further that such enhanced responses to many agonists (AII, ATP, bFGF, and VEGF) may in at least some cases (ATP and bFGF) involve recruitment of increasing numbers of responding cells.

Because the pregnancy-specific effect of agonists on Ca\(^{2+}\)/H11001 and P-ERK signaling pathways observed in freshly isolated cells was largely retained in cultured cells, this allows us to make one further important observation, namely that these changes in signaling are due to a programmed event rather than being due to pregnancy-specific tonic stimulation (shear stress, elevated estrogen, angiotensin or insulin, etc.) because such tonic stimuli would not be differentially retained in culture conditions. It was this finding that led us to attempt microarray analysis in the hope of identifying the mechanistic basis of this programming event.

cDNA array analysis combined with subsequent Western blotting and functional analysis further confirms our findings that the passage 4 UAEC model represents freshly isolated UA endothelium. Analysis of array data can be performed in two ways. The first is to simply identify the data points as a fingerprint of the status of the cells and compare the regression analysis, looking for data points outside predetermined confidence limits as an index of significantly altered expression between groups. A comparison of cDNAs, proteins, and cellular function demonstrates that passage 4

Fig. 8. Functional responsiveness of UAEC to BK. A, UAEC were loaded with fura 2-AM as described, and Ca\(^{2+}\) responses to BK (at the doses shown) and ATP (30 \(\mu\)M) were determined. Similar results were obtained in both P-UAEC and NP-UAEC. B, Phosphoinositol turnover was assayed in response to 30-min treatment with AII (100 nM), BK (10 \(\mu\)M), or ATP (100 \(\mu\)M) in the presence of 20 mM LiCl, as previously described (17; also see Materials and Methods). Results are the mean and SEM for four cell preparations each. C, Effects of AII (100 nM), BK (10 \(\mu\)M), and ATP (300 \(\mu\)M) on ERK 2P levels in P-UAEC and NP-UAEC are shown. Results are the mean and SEM for four cell preparations each. In all panels, significant differences relative to unstimulated controls are as indicated (*, \(P < 0.05\)).
cells maintain the characteristics of freshly isolated endothelium. Although our experiments were successful in providing a detailed fingerprint of the cell state, few differences in the more abundant mRNA species or corresponding protein expression were detected between pregnant and nonpregnant cell preparations for either freshly isolated or passage 4 cells that were beyond that of individual animal variation (2-fold) determined independently (data not shown). This may be due to a lack of sensitivity needed to detect the many less abundant mRNA species or the fact that the relevant cDNAs involved in pregnancy reprogramming of UAEC were absent from the gene filter. An alternative explanation is that altered UA endothelial function is not controlled at the level of mRNA or protein expression, but instead is an alteration of activity of existing proteins, perhaps through subtle changes in intracellular location that may facilitate or impede their participation in signaling events.

A second form of array analysis identifies each spot and considers the role of its gene product in cell signaling. This potentially useful analysis is limited by relative insensitivity, because cDNAs are reverse transcribed but not amplified. A lack of signal for a particular gene may have little meaning because it may simply reflect an expression level below the sensitivity of this reverse Northern analysis. Although only minor differences in mRNA or protein level were detected, the array data nonetheless provide potentially valuable information on the genes expressed in UA endothelium/UAEC. Many of the findings are as expected for endothelium, including the detection of the BK B2-R, endothelin receptors, adenosine receptors, γ-aminobutyric acidA receptor, insulin receptor, EGF receptor, several FGF receptors, connetive tissue growth factor, and VEGF-B. In addition, endothelial cells are known to respond to hypoxia, so the presence of hypoxia-inducible factor-1 was also not surprising. Furthermore, PKCα and PKCβ were among the numerous kinases detected in the cells. This may be important for deciphering the mechanism of vasodilator production in these cells because these isofoms may be activated by Ca2+ and be indirect mediators of ERK activation.

Many of the current findings have implications on future studies because endothelial function and signaling are widely published in the literature. For example, cDNA signals for the heterotrimeric G proteins Gsα, Gqα, and G12α were detected. Isoform Gqβ mediates activation of PLCβ (also detected) in a number of cell types (28). In endothelial cell models, Gα may mediate activation of ERK 1/2 in response to either heptahelical receptor agonists (29) or nongenomic estrogen receptor activation (30). Furthermore, Gαq and Gαq are reported to be caveolae-associated proteins, consistent with the finding that BK and ATP stimulation of Ca2+ wave initiation occurs in caveolae-rich regions of the cell (31, 32).

These results were further validated by independently verifying expression levels of key proteins of particular interest to our research goals. Our previous studies demonstrated the presence of C-Raf in UAEC (Sullivan, J., unpublished data), and PowerBlot analysis revealed the additional presence of A-Raf. Western analysis confirmed the presence of similar levels of both isofoms in P-UAEC and NP-UAEC. Raf is likely involved in the activation of MAPK kinase 1 and ERK 1/2 in UAEC and may be a key to the enhancement of this response during pregnancy. Raf signaling has proven to be a complex process (33), and further in-depth studies will be necessary to determine isoform-specific involvement in ERK 1/2 activation in UAEC and how this is altered during pregnancy.

The presence of the BK receptor B2-R, IP3R3, Jun, PKCα, PKCβ, and PKCγ was also confirmed by Western blot. The B2-R finding was further substantiated with BK mobilization of intracellular Ca2+ as well as phosphorylation of ERK 1/2 in a pregnancy-specific manner. Homologous desensitization of the B2-R was implied in imaging studies, consistent with the B2-R activities in many other tissues (20). Taken together, these results imply that our metanaalytical approach was valid and suggest that further investigation of mRNA transcripts using more complete arrays may yet identify the pregnancy-specific factors responsible for altered cell signaling and function.

In summary, the extensively characterized UAEC model was compared with freshly isolated UAEC using functional responses as well as cDNA array analyses. The findings support the assertion that our UAEC model and freshly isolated cells function similarly and that pregnancy is associated with increased downstream coupling of extracellular agonists to ERK 1/2 and a more sustained elevation of [Ca2+]i in response to ATP. Our data further support the notion that these changes in cell signaling are not the result of tonic stimulation but are in fact programmed events that are retained in culture. Several molecular components have been identified in UAEC that may couple heptahelical receptors and growth factor receptors to the ERK 1/2 pathway as well as components of Ca2+ signaling pathways. Further studies are still necessary to determine how UAEC are reprogrammed at the level of cell signaling during pregnancy, but the availability of a cell model that retains these in vivo characteristics will clearly facilitate such an investigation.

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