Estradiol-mediated ERK phosphorylation and apoptosis in vascular smooth muscle cells requires GPR 30

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Estradiol-mediated ERK phosphorylation and apoptosis in vascular smooth muscle cells requires GPR 30. Am J Physiol Cell Physiol 297: C1178–C1187, 2009. First published September 9, 2009; doi:10.1152/ajpcell.00185.2009.—Recent studies suggest that the rapid and nongenomic effects of estradiol may be mediated through the G protein-coupled receptor dubbed GPR30 receptor. The present study examines the role of GPR30 versus a classical estrogen receptor (ERx) in mediating the growth regulatory effects of estradiol. GPR30 is readily detectable in freshly isolated vascular tissue but barely detectable in cultured vascular smooth muscle cells (VSMC). In freshly isolated aortic tissue, estradiol stimulated extracellular signal-regulated kinases (ERK) phosphorylation. In contrast, in cultured VSMC, where GPR30 expression is significantly reduced, estradiol inhibits ERK phosphorylation. Transfer of the genes encoding GPR30 led to estradiol stimulation of ERK phosphorylation, which is opposite the effects of estradiol in the primary culture of VSMCs. Transduction of the mineralocorticoid receptor (MR) had no effect on estradiol effects on ERK. Estradiol-mediated stimulation of ERK subsequent to heterologous GPR30 expression was pertussis toxin sensitive and phosphoinositide 3-kinase (PI3 kinase) dependent; under these conditions, estradiol also inhibited protein kinase A (PKA). In contrast, in the absence of GPR30 expression in cultured VSMC, estradiol stimulated PKA activity and inhibited ERK phosphorylation. To determine the functional effect of GPR30 (vs. estrogen receptor expression), we assessed estradiol-mediated apoptosis. In the absence of GPR30 expression, estradiol inhibited apoptosis. This effect was enhanced with ERx expression. In contrast, with GPR30 expression, estradiol stimulated apoptosis in an ERK-dependent manner. Thus the effect of estradiol on vascular smooth muscle cell apoptosis is likely dependent on the balance between ER-mediated PKA activation and GPR30-mediated PKA inhibition in mediating the effects of estradiol on regulation of ERK activity in VSMCs, using time in culture and gene transfer to modulate variable levels of GPR30 expression. In addition, we have shown that the divergent effects of steroids between species and cell types might relate, at least in part, to the existence of multiple receptors/receptor mechanisms.

METHODS

VSMC primary cultures. Isolation of rat aortic VSMC was performed as described previously (17). VSMC were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 4.5 g/l glucose supplemented with 10% FBS, gentamicin (0.05 mg/ml), and amphotericin B (2.5 μg/ml). Cells were cultured until confluence. Serum was withdrawn 24 h before ERK phosphorylation assays. VSMC were utilized between passages 4 to 8 and 2–4 wk after isolation. The rats were cared for in accordance with the Canadian Council on Animal Care guidelines. The study protocol was reviewed and accepted by the Animal Use Subcommittee/University Council on Animal Care of the University of Western Ontario.

Assessment of ERK content and EKR1/2 phosphorylation. The effect of estradiol on ERK1/2 phosphorylation in VSMC was assessed...
GPR30-MEDIATED ERK PHOSPHORYLATION AND APOPTOSIS

by immunoblotting. VSMC were treated either with estradiol (E2), followed by two washes with cold PBS. Except in time-course studies estradiol treatments were for 15 min. Cells were then lysed in a buffer containing 20 mM Tris, pH 8.0, 1% Nonidet P-40, 0.1% SDS, 140 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride. Resultant whole cell lysates were resolved by SDS-PAGE and transferred electrophoretically onto Immobilon PVDF membranes (Bio-Rad, Hercules, CA). Membranes were blocked overnight at 4°C with 5% skim milk powder and then incubated with either anti-phospho ERK1/2 or anti-ERK1/2 (1:1,000, Upstate Biotech and Cell Signaling). Blots were washed in Tris-buffered saline for 1 h followed by incubation of anti-mouse (1:1,000) or anti-rabbit antibody (1:5,000, Sigma, Oakville, Ontario, Canada) for 1 h at room temperature. Proteins were detected by chemiluminescence as described by the manufacturer’s protocol (NEN, Boston, MA). Under all conditions studied, total ERK content (as assessed by immunoblotting) was not altered with altered with gene transfers or treatments. Thus phospho-ERK content was expressed as a proportion of control phospho-ERK expression normalized for total ERK content.

To assess the effects of estradiol on ERK phosphorylation in freshly isolated tissue, aortas were excised, the endothelial layer was removed by flushing with distilled water, and 3- to 4-mm rings were cut as we have previously described (19). Rings were treated with estradiol (10 nM) for 15 min in Krebs buffer at 37°C, washed with ice-cold PBS, transferred into lysis buffer, chopped into small pieces, and ground and centrifuged at 16,000 g for 10 min at 4°C. The resultant supernatants were collected. Proteins were separated by SDS-PAGE and immunoblotted as described above. Phospho-ERK1/2 expression in aortic rings was normalized to the expression of α-actin.

Assessment of PKA activity by VASP phosphorylation. Rat smooth muscle cells were infected with MR, ERα, GPR30, or green fluorescent protein (GFP, control). Infected cells were cultured in serum-free DMEM media for 24 h and incubated in the absence or presence of estradiol (10 nM) for 15 min. The effect of estradiol on cAMP-mediated vasodilator-stimulated phosphoprotein (VASP) phosphorylation was determined by Western blotting as described previously (17). Under all conditions studied, total VASP content (i.e., phosphorylated + nonphosphorylated VASP as assessed by immunoblotting) was not altered with altered with gene transfers or with drug treatments (data not shown).

Generation of adenoviral constructs. Adenoviral constructs were generated with AdMax adenovirus vector creation kit as per manufacturer instructions (Microbix BioSystems, Toronto, Canada) as previously described (17). Briefly, cDNAs encoding, GFP, MR, GPR30, or ERs were generated by PCR using plasmid templates of GFP cDNA (Clontech), hGPR30 (ATCC), hMR (kindly provided by Dr. Marc Lombes, INSERM, Paris, France), or ERs (kindly provided by Dr. Murray Huff, Robarts Research Institute). The resultant cDNAs were subcloned into shuttle vector pDC516 and purified. The recombinant plasmid was then cotransfected into human embryonic kidney (HEK) 293 cells with adenoviral DNA pBHGlox (delta E1, E3Cre. Recombinant adenoviruses were harvested by lysis of transected HEK293 cells using three freeze-thaw cycles.

Assessment of receptor expression in endothelium-denuded aortic tissue. Aortic rings were isolated as described above. Isolation of total RNA was performed utilizing the one-step RNA isolation reagent TRIzol (Invitrogen). One microgram of RNA from cultured VSMC or aorta was utilized for analysis of GPR30, MR, ERα, or GAPDH (control) mRNA expression using SuperScript one-step RT-PCR kits (Invitrogen) with primers specific to GPR30, MR, ERα, or GAPDH (for GPR 30: forward: 5′-GCACGGTCTTCTTCTCTCACC-3′, reverse: 5′-ACAGCTGAGCTTGCCCT-3′; for MR: forward: 5′-CCAAGGCTACACGATCTT-3′, reverse: 5′-TCCAGACGACTATTGTCT-3′; for ERα: forward: 5′-TCCTAAACTTGGCTGTGAGG-3′, reverse: 5′-ATCTTTGCAGACTGCTG-3′; for GAPDH: forward: 5′-TGAACGGGAAGCTTCACTGG-3′, reverse: 5′-TCCACCACCTGTTGCTGA-3′).

Assessment of apoptosis by annexin labeling. Rat smooth muscle cells were cultured 24 h before gene transfer and then infected with adenoviral constructs expressing MR, ERα, GPR30, or GFP (control) for 16 h. Infection medium was then replaced with DMEM medium without FBS. After 48 h serum starvation, cells were treated with estradiol for 24 h and then detached with trypsin and washed in PBS. Pooled cells were suspended in binding buffer (in mM: 10 HEPES, 140 NaCl, and 2.5 CaCl2, pH 7.4) containing FITC-conjugated annexin V (0.25 μg/ml) and propidium iodide (5 μg/ml) in the dark for 15 min and then acquired (20,000 cells/sample) by using a BD FACScalibur flow cytometer (BD Biosciences). A total of 20,000 events were analyzed for double-stained positive cells for each
sample with FlowJo software (Tree Star, Ashland, OR) by a blinded observer. Data were normalized relative to the control levels of annexin positive staining determined for each experiment (1.7 ± 0.3% of cells, n = 20).

Statistical analyses. For multiple group comparisons, initial analysis by ANOVA was followed by Dunnett’s multiple comparison tests. The significance of difference between paired groups was determined by Student’s test for paired data. *P < 0.05 on a two-sided test was taken as a minimum level of significance.

Materials. The AKT inhibitor LY-294005 (1L-6-hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-octadecylcarbonate), the ER antagonist ICI-182780 (7a,17b-[9-[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl] estra-1,3,5(10)-triene-3,17-diol), the PI3 kinase inhibitor LY-294002 (2–2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one), and the GPR30 agonist G1 (1-(4-(6-bromobenzo[1,3]dioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinolin-8-yl)-ethanone)(2) were purchased from Calbiochem-Novabiochem (San Diego, CA). All other reagents were of the highest chemical grade available and were obtained from Sigma-Aldrich (Oakville, Ontario, Canada).

RESULTS

GPR30, MR, and ER expression is detectable in freshly isolated aortic tissue, but GPR30 expression declines in primary culture of aortic VSMC. Endogenous GPR30, MR, and ER expression was detected by RT-PCR in freshly isolated, endothelium-denuded aortic tissue, as reported previously (18, 33) (Fig. 1A). Comparable expression of these receptors was also apparent in freshly isolated VSMC (data not shown). However, comparison of the extent of gene expression in freshly isolated aorta versus primary cultures of smooth muscle cells reveals that there is a decline in GPR30 expression in cultured VSMC when compared with the persistence of MR and ER expression following primary culture (Fig. 1A).

Fig. 2. Estradiol-mediated effects on extracellular signal-regulated kinase (ERK) phosphorylation. A: estradiol mediates ERK1/2 inhibition. Serum-starved VSMC were incubated with increasing concentrations of estradiol (0.1–100 nM) for 15 min. The effect of estradiol on phospho-ERK1/2 was assessed by Western blot analysis. B: estradiol-mediated inhibition of ERK phosphorylation and effects of ER antagonists. VSMC were preincubated with the nonspecific steroid receptor antagonist spironolactone (Spiro, 10 μM) or by the ER-directed antagonists tamoxifen (1 μM) or ICI-182780 (1 μM for 15 min), followed by the incubation with estradiol (10 nM for 15 min). The effect of these agents on estradiol-mediated inhibition of ERK1/2 phosphorylation was assessed by Western blot analysis. Data represent the means ± SE from 3 to 9 independent experiments performed under identical conditions. *P < 0.05 vs. control cells by one-way ANOVA followed by Dunnett’s multiple comparison test.

Fig. 3. Divergent effects of transduction of GPR30 or ERα into VSMC on estradiol-mediated regulation of ERK phosphorylation. Concentration (A) and time (B) dependence VSMC infected with adenoviral GPR30, ERα, or GFP (control) and serum starved for 24 h were treated with increasing concentration of estradiol (0.1–100 nM for 15 min) (A) or treated (E2, 10 nM) for 1, 5, 10, 15, and 30 min (B). Data represent the means ± SE. from 3 independent experiments performed under identical conditions. *P < 0.05 vs. untreated VSMC by one-way ANOVA followed by Dunnett’s multiple comparison tests. #P < 0.05 vs. control. Control levels were defined as the extent of ERK phosphorylation in the absence of estradiol.
Environviral gene transfer led to successful expression of FLAG-tagged GPR30, MR, and ERα in cultured VSMC. These proteins, detectable upon Western blot analysis of cell extracts, migrated on SDS-PAGE with relative molecular mass corresponding to 44, 107, and 67 kDa, respectively, consistent with their previously reported molecular masses (1, 12, 15) (Fig. 1B).

Estradiol regulates vascular smooth muscle ERK1/2 phosphorylation. Estradiol mediated a time- and concentration-dependent inhibition of ERK phosphorylation in cultured VSMC (Figs. 2A, and 3). This estradiol-mediated inhibition of ERK phosphorylation was attenuated by the ER-selective antagonists tamoxifen and ICI-182780, as well as by the nonselective antagonist spironolactone (Fig. 2B). The findings with tamoxifen and ICI-182780 implicate a classical cytoplasmic/nuclear ER as the mediator of these estradiol responses.

Role of GPR30 in mediating gonadal steroid effects on ERK phosphorylation. Gene transfer of GPR30 into cultured VSMC reversed the estradiol-mediated inhibition of ERK phosphorylation, leading to time- and concentration-dependent estradiol-mediated stimulation of ERK phosphorylation (Figs. 3 and 4A). Furthermore, whereas the GPR30-specific agonist G1 (100 nM) had no significant effect on ERK phosphorylation in either GFP- or ERα-transduced cells (GFP: 91 ± 7% of control, not significant; ERα: 96 ± 2%, not significant), in the setting of GPR30 expression, G1 significantly increased ERK phosphorylation (142 ± 10%, P < 0.05, n = 4, Fig. 4C). Gene transfer of MR had no significant effect on estradiol-mediated inhibition of ERK phosphorylation. Estradiol/GPR30-mediated activation of ERK phosphorylation was not attenuated by either selective or nonselective ER antagonists (Fig. 4B). Notably, neither total ERK expression nor basal levels of phospho-ERK expression were altered by gene transfer of GPR30, ERα, or MR (Fig. 4A).

Fig. 4. GPR30 introduction into cultured VSMC leads to estradiol and G1 activation of ERK phosphorylation, reversing the estradiol-mediated inhibition of ERK phosphorylation in untransduced cells. A: role of GPR30 and ERα in estradiol-mediated ERK1/2 inhibition. VSMC were infected with adenoviral GPR30, MR, ERα, or GFP (control). The effect of receptor expression on estradiol (10 nM for 15 min)-mediated ERK1/2 phosphorylation was determined by Western blot analysis. ERα gene transfer further enhanced estradiol-mediated ERK1/2 inhibition, whereas GPR30 expression reversed estradiol-mediated ERK1/2 inhibition. MR gene transfer had no significant effects. Control levels were defined as the extent of ERK phosphorylation in the absence of estradiol, specific for each condition (i.e., each gene transfer). Receptor transduction had no significant effects on either total ERK content nor basal phospho-ERK expression. Effects of receptor gene transfer on total ERK content: GPR30 = 93 ± 7% of GFP-infected cells, n = 4; MR = 92 ± 7% of GFP-infected cells, n = 4; ERα = 98 ± 6% of GFP-infected cells, n = 4. Effects of receptor gene transfer on basal phospho-ERK content: GPR30 = 94 ± 8% of GFP-infected cells, n = 4; MR = 101 ± 14% of GFP-infected cells, n = 4; ERα = 100 ± 8% of GFP-infected cells, n = 4. Data represent the means ± SE from 4 independent experiments performed under identical conditions. *P < 0.05 vs. untreated VSMC by one-way ANOVA followed by Dunnett’s multiple comparison tests. B: ERα antagonists do not block estradiol/GPR30-mediated ERK phosphorylation. None of spironolactone (10 μM), tamoxifen (1 μM), nor ICI-182780 (1 μM) significantly attenuated estradiol/GPR30-mediated stimulation of ERK1/2 phosphorylation. Data represent the means ± SE from 6 independent experiments performed under identical conditions. *P < 0.05 vs. untreated VSMC by one-way ANOVA followed by Dunnett’s multiple comparison tests. C: GPR30 agonist G1 stimulates ERK phosphorylation only in VSMC expressing GPR30. Data represent means ± SE from 4 independent experiments performed under identical conditions. *P < 0.05 vs. untreated VSMC by one-way ANOVA followed by Dunnett’s multiple comparison tests.

Differential pathways lead to estradiol regulation of ERK in freshly isolated aortic VSMC and in cultured VSMC. Given the correlation between attenuation of GPR30 expression in cultured VSMC and the inhibitory effect of estradiol on ERK phosphorylation in these cells, we wanted to determine the effect of estradiol on ERK phosphorylation in freshly isolated aortic vascular smooth muscle tissue. We postulated that, in freshly isolated tissue, the higher relative density of GPR30 (see Fig. 1) would result in estradiol-mediated stimulation of ERK phosphorylation, in a manner analogous to our findings that estradiol stimulated ERK phosphorylation in cultured VSMC heterologously expressing GPR30 following adenoviral transduction (Fig. 4A). Indeed, estradiol treatment of freshly isolated aortic ring segments resulted in increased ERK phosphorylation (123 ± 12% of control, n = 13, P < 0.05),
consistent with the much higher levels of GPR30 expression in freshly isolated tissue compared with cultured VSMC.

Since our data suggest that different receptors, namely GPR30 and ER, mediate the opposite effects of estradiol to stimulate versus inhibit ERK phosphorylation, respectively, we wondered whether different signaling pathways were responsible for these opposing effects. Thus we compared the signaling properties of estradiol in sham-infected and in GPR30-transduced VSMC. In sham-infected cultured VSMC where estradiol inhibits ERK phosphorylation, preincubation with either the PI3 kinase inhibitor LY-294002 or the AKT inhibitor LY-294005 did not block estradiol-mediated inhibition of ERK phosphorylation (Fig. 5A). In contrast, adenoviral infection of cultured VSMC with a cDNA encoding GPR30 led to an increase in ERK phosphorylation in response to estradiol, and this stimulation of ERK was blunted by the PI3 kinase inhibitor (LY-294002) or AKT inhibitor (LY-294005) (Fig. 5B).

It has been established previously that PKA activation in VSMC inhibits ERK activation (3, 17). To determine whether the inhibition of ERK phosphorylation by estradiol in cultured VSMC (in the absence of GPR30 gene transfer) might similarly occur via a cAMP-dependent mechanism, we assessed the effect of the PKA inhibitor Rp-cAMP (1 μM) on estradiol-mediated inhibition of ERK phosphorylation in cultured VSMC. Rp-cAMP pretreatment resulted in complete attenuation of the effect of estradiol on ERK inhibition in these cultured VSMC (Fig. 6). Interestingly, pretreatment with the PKA activator Sp-cAMP resulted in a reduction in ERK phosphorylation, which was further reduced in the presence of estradiol (Fig. 6).

Further evidence that estradiol activates PKA in cultured VSMC was provided by assessing phosphorylation of the PKA substrate VASP. Estradiol mediated a time- and concentration-dependent increase in VASP phosphorylation in cultured VSMC with a peak concentration (Cmax) at 10 nM to 184 ± 5% of control (n = 4, Fig. 7A) and a time to peak effect (Tmax) at 15 min (Fig. 7B). Interestingly, gene transfer of additional ERα into the primary cultures of VSMC resulted in a further increase in estradiol-mediated phosphorylation. In contrast, gene transfer of GPR30 resulted in estradiol-mediated inhibition of VASP phosphorylation (Fig. 8A). Furthermore, whereas the GPR30 agonist G1 had no effect on VASP phosphorylation in GFP-transduced cells (98 ± 7% of control, n = 4), in GPR30-transduced cells, G1 mediated inhibition of VASP phosphorylation (68 ± 2% of control, n = 4, P < 0.05).

To determine whether the GPR30-mediated inhibition of VASP phosphorylation and consequent stimulation of ERK phosphorylation was via the inhibitory G protein G1/G0, we examined the effect of pertussis toxin on estradiol-stimulated ERK phosphorylation. Under control conditions, pertussis toxin preincubation (overnight, 200 ng/ml) did not affect the rapid effects of estradiol either to stimulate VASP phosphorylation (Fig. 8B) or inhibit ERK phosphorylation (Fig. 8C). However, following GPR30 gene transfer, pertussis toxin blocked both the estradiol-mediated inhibition of VASP phosphorylation (Fig. 8B) and the estradiol-mediated stimulation of ERK phosphorylation (Fig. 8C). These data suggest that estradiol stimulation of ERK via GPR30 is mediated via the inhibitory G protein G1/G0. In contrast, in cells where ER is mediating the effects of estradiol, e.g., cultured VSMC, estradiol-dependent inhibition of ERK phosphorylation occurs independent of the G1/G0 protein and thus is insensitive to incubation with pertussis toxin (Fig. 8, B and C).

Regulation of apoptosis evoked by serum starvation of VSMC in culture. ERK activation has been linked to apoptosis in vascular smooth muscle (39) [although others have questioned the role of ERK in regulation of vascular smooth muscle cell apoptosis (35)]. To determine the impact of expression of ERα or GPR30 on regulation of apoptosis in VSMC, we assessed the effect of estradiol on apoptosis of cultured VSMC.

Fig. 5. Estradiol mediates ERK activation through a different pathway in primary cultures of VSMC than in VSMC expressing heterologous GPR30, where estradiol stimulation by estradiol is phosphoinositide 3-kinase (PI3) kinase dependent. A: estradiol-mediated ERK inhibition in cultured VSMC is PI3 kinase independent. VSMC were preincubated with the PI3 kinase inhibitor (PI3K-I) (LY-29004, 0.1 μM) or the AKT inhibitor (AKT-I) (LY-29005, 0.1 μM) for 15 min, followed by the incubation with estradiol (10 nM for 15 min). B: estradiol-mediated ERK1/2 activation in GPR30-infected VSMC is PI3 kinase dependent. GPR30-infected VSMC were preincubated with PI3 kinase inhibitors as noted above, followed by the incubation with estradiol (10 nM). Data represent the means ± SE from 6 independent experiments performed under identical conditions. *P < 0.05 vs. untreated VSMC by one-way ANOVA followed by Dunnett’s multiple comparison tests.
GPR30-transduced VSMC (but not in GFP-infected cells) was co-incubation with U0126 but not by its analogue U0124 (Fig. 10A). Estradiol-mediated increase in apoptosis in VASP phosphorylation. VSMC were incubated with estradiol (10 nM)-mediated inhibition of ERK phosphorylation was assessed in cultured VSMC in the presence or absence of the PKA inhibitor Rp-cAMP (1 μM) or the PKA agonist Sp-cAMP. Data represents the means ± SE from 6 independent experiments performed under identical conditions. *P < 0.05 vs. untreated VSMC.

stimulated by serum starvation. In cultured VSMC transduced with the control GFP vector (control conditions), estradiol (10 nM) mediated a decrease in VSMC apoptosis, an effect that was marginally but significantly enhanced by gene transfer of ERα (Fig. 9A). The anti-apoptotic effect of estradiol was attenuated by co-incubation with the PKA inhibitor Rp-cAMP (1 μmol/l, overnight incubation), suggesting that the anti-apoptotic effect of estradiol was mediated via a cAMP-dependent mechanism (Fig. 9B). PI3 kinase inhibition with LY-294002 (0.1 μmol/l, overnight incubation) did not affect the anti-apoptotic actions of estradiol (Fig. 9C). In contrast, gene transfer of GPR30 into the VSMC resulted in enhancement of apoptotic rates following estradiol treatment (Fig. 9A), and this GPR30-mediated pro-apoptotic effect of estradiol was attenuated by the PI3 kinase inhibitor LY-294002 (Fig. 9C), but not by Rp-cAMP (Fig. 9B).

Gene transfer had no significant effect on basal apoptotic rates (control: 2.1 ± 0.4%, n = 6; GFP: 2.0 ± 0.4%, n = 23; ERα: 2.2 ± 0.2%, n = 10; MR: 2.2 ± 0.4%, n = 10; GPR30: 2.3 ± 0.2%, n = 12).

Role of ERK activation in estradiol-mediated effect on serum starvation induced-apoptosis in sham infected and GPR30-infected VSMC. To determine the role of ERK activation in regulation of apoptosis, we assessed the impact of ERK inhibitor U0126 on effect of estradiol on apoptosis in GPR30-infected VSMC. Estradiol mediated a decrease in apoptosis in GFP-infected VSMC, an effect that was not affected by co-incubation with ERK inhibitor U0126 (10 μM) nor by its chemically related (but inactive) analogue U0124 (10 μM). In contrast, the estradiol-mediated increase in apoptosis in VSMC expressing GPR30 was significantly attenuated by co-incubation with U0126 but not by its analogue U0124 (Fig. 10A). Furthermore, the G1-mediated increase in apoptosis in GPR30-transduced VSMC (but not in GFP-infected cells) was also blunted by co-incubation with ERK inhibitor U0126 but not by its analogue U0124 (Fig. 10B).

DISCUSSION

Relatively acute effects of steroids on the vasculature, including those of estradiol, have been suggested to be mediated both by “nonclassical” receptors as well as classical steroid receptors (e.g., ERs). Our data support this hypothesis and reveal that GPR30 activation is the predominant “nonclassical” receptor pathway mediating the stimulatory effects of estradiol on ERK activation and on regulation of VSMC apoptosis (Fig. 11). GPR30 leads to activation of PI3K to activate ERK and apoptosis and simultaneously to inhibit PKA activation, which suppresses ERK and attenuates apoptosis.

Our studies confirm previous reports that MR, ERs, and GPR30 are all expressed in VSMC (9, 16, 34) but also provide

Fig. 6. Protein kinase A (PKA) inhibition by Rp-cAMP blocks E2-mediated inhibition of ERK phosphorylation in cultured VSMC, whereas the PKA agonist Sp-cAMP enhances E2-mediated inhibition of ERK. Estradiol (10 nM)-mediated inhibition of ERK phosphorylation was assessed in cultured VSMC in the presence or absence of the PKA inhibitor Rp-cAMP (1 μM) or the PKA agonist Sp-cAMP. Data represents the means ± SE from 6 independent experiments performed under identical conditions. *P < 0.05 vs. untreated VSMC.

Fig. 7. Estradiol-mediated concentration and time-dependent increase in VASP phosphorylation. A: estradiol mediated a concentration-dependent increase in vasodilator-stimulated phosphoprotein (VASP) phosphorylation. VSMC were incubated with increasing concentrations of estradiol (0.01–10 nM) for 15 min. Estradiol effects on VASP phosphorylation were assessed by Western blot analysis. Data represents the means ± SE from 4 independent experiments performed under identical conditions. *P < 0.05 vs. untreated VSMC. B: estradiol mediated a time-dependent increase in VASP phosphorylation. VSMC were incubated with estradiol (10 nM) for 1, 5, 10, 15, and 30 min; estradiol effect on VASP phosphorylation was assessed by Western blot analysis; and estradiol mediated a significant increase in VASP phosphorylation at 15 and 30 min (15 min: 176 ± 12%; 30 min: 161 ± 18% of control). Data represents the means ± SE from 4 independent experiments performed under identical conditions. *P < 0.05 vs. untreated VSMC. Insets: representative immunoblot depicting nonphosphorylated VASP (bottom band) and phospho-VASP (top band).

AJP-Cell Physiol • VOL 297 • NOVEMBER 2009 • www.ajpcell.org
additional evidence that expression levels of GPR30 decline in VSMC maintained in primary culture (vs. expression in freshly isolated tissue), which suggests that studies of the effects of estradiol and its responses in primary cultures of VSMC likely
We underestimate the role of GPR30 in mediating estradiol effects characteristic of native target cells.

Our studies also provide additional findings that the opposing effects of estradiol on ERK phosphorylation are a consequence of the different signaling cascades linked to ER versus GPR30 activation by estradiol (Fig. 10). In settings with relatively high GPR30 expression (i.e., freshly isolated aorta cells or after GPR30 transduction into VSMC primary cultures), estradiol stimulates ERK phosphorylation via a PI3 kinase-dependent pathway and simultaneously inhibits PKA activation. Notably, PI3 kinase-dependent, estradiol-mediated ERK phosphorylation has been previously described in adipocytes (7). Whether those effects as partial agonist are cell type-specific remains to be determined.

Fig. 10. Role of ERK activation in estradiol-mediated effect on apoptosis in GFP- or GPR30-infected VSMC. A: apoptotic effect of estradiol is ERK dependent. The effect of estradiol on apoptosis was assessed in either GFP- or GPR30-infected VSMC in the presence of ERK inhibitor U0126 (10 μM) or its chemically related compound U0124 (10 μM). Estradiol-mediated-anti-apoptotic effect in GFP infected was not affected by the co-incubation with either U0126 or U0124. In contrast, the apoptotic effect of estradiol in GPR30 cells was blunted by co-incubation with U0126 but not with U0124. B: G1-mediated apoptosis in GPR30-infected acts through ERK activation. The effect of G1 on apoptosis in GFP- or GPR30-infected cells was assessed in the presence of U0126 or U0124. G1 did not significantly affect the serum starvation-induced apoptosis in GFP-infected cells (104 ± 2%). In contrast, G1 increased apoptosis in GPR30-infected cells. This effect was blunted by the co-incubation with ERK inhibitor U0126 but not by its chemically related but ERK-inactive analogue U0124. Data represent the means ± SE from 4 independent experiments performed under identical conditions. *P < 0.05 vs. untreated VSMC.
GPR30 has been linked to rapid adenylyl cyclase-PKA activation in HEK 293 and MDA-MB-231 cells (10, 13), inferring a linkage via Gs. Our current findings of activation in HEK 293 and MDA-MB-231 cells (10, 13), and Stroke Foundation of Ontario.

Our studies demonstrate that GPR30 expression is an important determinant of the pro-apoptotic effects of estradiol. In VSMC, increased rates of apoptosis have been linked to the development of atherosclerosis (6). For estradiol, both anti- and pro-apoptotic effects have been reported (which have been linked to regulation of vascular inflammation in a range of pathological conditions) (5, 28). We would suggest that this balance of the pro- and anti-apoptotic effects of estradiol may be a reflection of balances in opposing signaling pathways determined, in part, by the level of expression of GPR30.

On balance, these data do confirm that estradiol is a potent ligand for GPR30 with effects in the nanomolar range as determined by the enhancement in both ERK activation and apoptosis mediated by nanomolar concentrations of estradiol.

In summary, these studies reveal GPR30 in VSMC may have an important role in mediating the effects of estradiol on ERK activation and apoptosis. We would propose that the relative levels of GPR30 expression/activity may be an important determinant in the balance of the deleterious/protective effects of estradiol in the vasculature.

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