β-Arrestin mediates oxytocin receptor signaling, which regulates uterine contractility and cellular migration

Chad A. Grotegut,1 Liping Feng,1 Lan Mao,2 R. Phillips Heine,1 Amy P. Murtha,1 and Howard A. Rockman2,3,4

Departments of 1Obstetrics and Gynecology, 2Medicine, 3Cell Biology, and 4Molecular Genetics and Microbiology, Duke University School of Medicine, Durham, North Carolina

Submitted 1 July 2010; accepted in final form 1 December 2010

Grotegut CA, Feng L, Mao L, Heine RP, Murtha AP, Rockman HA. β-Arrestin mediates oxytocin receptor signaling, which regulates uterine contractility and cellular migration. Am J Physiol Endocrinol Metab 300: E468–E477, 2011. First published December 7, 2010; doi:10.1152/ajpendo.00390.2010.—Desensitization of the oxytocin receptor (OXTR) in the setting of prolonged oxytocin exposure may lead to dysfunctional labor, which increases the risk for cesarean delivery, and uterine atony, which may result in postpartum hemorrhage. The molecular mechanism for OXTR desensitization is through the agonist-mediated recruitment of the multifunctional protein β-arrestin. In addition to its desensitizing function, β-arrestins have recently been shown to simultaneously activate downstream signaling. We tested whether oxytocin stimulation promotes β-arrestin-mediated OXTR desensitization in vivo and activates β-arrestin-mediated mitogen-activated protein kinase (MAPK) growth signaling. Uterine muscle strips isolated from wild-type mice exhibited diminished uterine contractility following repeated exposure to oxytocin; whereas uterine muscle strips from β-arrestin-1 and β-arrestin-2 knockout mice showed no desensitization. Utilizing siRNA knockdown of β-arrestin-1 and β-arrestin-2 in HEK-293 cells expressing the OXTR, we demonstrated oxytocin-mediated MAPK signaling that was dependent on β-arrestin-1 and β-arrestin-2. Wild-type and β-arrestin-1 and β-arrestin-2 knockout mice receiving intravenous oxytocin also demonstrated oxytocin-mediated MAPK signaling that was dependent on β-arrestin-1 and β-arrestin-2. Finally, to test the significance of β-arrestin-mediated signaling from the OXTR, HEK-293 cells expressing the OXTR showed β-arrestin-dependent proliferation in a cell migration assay following oxytocin treatment. In conclusion, β-arrestin is a multifunctional scaffold protein that mediates both desensitization of the OXTR, leading to decreases in uterine contractility, and MAPK growth signaling following stimulation by oxytocin. The development of unique OXTR ligands that prevent receptor desensitization may be a novel approach in the treatment of adverse clinical events secondary to prolonged oxytocin therapy.

Address for reprint requests and other correspondence: C. A. Grotegut, Div. of Maternal-Fetal Medicine, Dept. of Obstetrics and Gynecology, Duke University School of Medicine, DUMC Box 3967, Durham, NC 27710 (e-mail: chad.grotegut@duke.edu).

OXYTOCIN IS A NANOPEPTIDE synthesized in the hypothalamus and stored in and secreted from the posterior pituitary gland. Oxytocin is responsible for uterine contractions and milk letdown and has been identified more recently to play a role as a neurotransmitter. Clinically, oxytocin infusion is used for induction and augmentation of labor and for the prevention and treatment of postpartum hemorrhage. In the US, the rate of labor induction has increased from 9.5 per 100 live births in 1990 to 22.3 per 100 live births in 2005 (30). Increasing induction rates expose more women to prolonged, continuous infusions of oxytocin, which is associated with dysfunctional labor and an increased rate of cesarean delivery (23). Furthermore, oxytocin infusion is a recognized cause of uterine atony (40).

Uterine atony is the cause of more than 80% of primary postpartum hemorrhage cases, leading to more than 140,000 peripartum deaths worldwide each year (1). Recent evidence suggests that an important predictor of uterine atony is prolonged oxytocin exposure, which likely leads to oxytocin receptor (OXTR) desensitization (40). Indeed, we postulate that the molecular basis for both dysfunctional labor and uterine atony may be desensitization of the OXTR mediated through the actions of β-arrestin.

β-Arrestin is a multifunctional scaffold protein that causes homologous desensitization of G protein-coupled receptors (GPCRs) in response to agonist stimulation (28, 39). Following agonist stimulation of a GPCR, a family of enzymes known as GPCR kinases (GRKs) phosphorylate the COOH-terminal tail of the receptor, leading to the recruitment of β-arrestin to the agonist-occupied receptor (28, 38). β-Arrestin binding to the phosphorylated receptor sterically interdicts further G protein coupling, resulting in diminished second messenger signaling. Although β-arrestin causes OXTR desensitization and internalization in tissue culture (20, 33), its role in in vivo uterine physiology is unknown.

β-Arrestin also activates signaling pathways independent of G protein coupling, such as the mitogen-activating protein kinase (MAPK) pathway (15). An emerging concept in GPCR signaling is that different ligands can stimulate a signal through the G protein-coupled pathway, the β-arrestin pathway, or both, leading to the concept of signaling bias (18, 32).

In this study, we tested the hypothesis that in vivo desensitization of the OXTR is mediated by β-arrestin to cause blunted uterine contractility to repeated oxytocin exposure. Furthermore, we tested whether β-arrestin mediates OXTR signaling through the MAPK pathway and tested the role of β-arrestin in cellular proliferation and migration following oxytocin stimulation.

MATERIALS AND METHODS

Peptides and reagents. Oxytocin and the protein kinase C inhibitor Ro-31-8425 were obtained from Calbiochem (La Jolla, CA). Atosiban and pertussis toxin (PTX) were obtained from Sigma (St. Louis, MO). FuGENE 6 transfection reagent was obtained from Roche Applied Science (Indianapolis, IN), and Gene-Silencer siRNA transfection reagent was obtained from Genentech (San Diego, CA). Antibodies were obtained as follows: anti-phospho-p42/p44 (pERK1/2) and anti-p42/p44 (total ERK1/2) were obtained from Cell Signaling Technology (Danvers, MA), anti-OXTR was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-β-arrestin-1/2 was a gift.
from Dr. Robert Lefkowitz (Duke University Medical Center). Horseradish-peroxidase-linked anti-rabbit secondary antibody was obtained from Cell Signaling Technology. The HA-OXTR plasmid [in pcDNA3.1(+) vector; Invitrogen] was a gift from Dr. Marc Caron (Duke University Medical Center).

**Cell culture.** Human embryonic kidney (HEK)-293 cells were obtained from the Duke University Institutional Animal Care and Use Committee approved the use of mice for this project. Control wild-type (WT) C57/B6, β-arrestin-1 knockout (β-arrestin1KO) (12), and β-arrestin-2 knockout (β-arrestin2KO) (6) mice were kindly provided by Dr. Robert Lefkowitz (Duke University Medical Center). The animals were anesthetized and euthanized, and then a vertical midline abdominal incision was made, and each of the two uterine horns were removed. The uterine horns were immediately immersed in modified Krebs buffer (118 mM NaCl, 4.8 M KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, 2.5 mM CaCl2, 25 mM NaHCO3, and 11 mM glucose, pH 7.4). A 1-cm-length muscle strip was isolated and suspended using 4-0 silk suture between a stainless steel wire hook connected to a Grass GR-03 force displacement transducer (Grass Technologies, West Warwick, RI) and a glass hook inside the organ bath that served as an anchor. The bath was filled with modified Krebs buffer, maintained at 37°C, and constantly bubbled with a premixed gas consisting of 20% O2, 5% CO2, and balance N2. The strips were then allowed to equilibrate at 0.5 g of tension for 30 min. All uterine muscle strips demonstrated a spontaneous contraction pattern. The strips were then stimulated with 1 nM oxytocin, and the contraction pattern was recorded for 9 min. The strips were then washed with Krebs solution twice and allowed to equilibrate for 3 min prior to the next stimulation. This was repeated, stimulating with 10, 50, and 100 nM oxytocin sequentially, each for 9 min, followed by a 3-min washout. Following the washout after the initial 100-nM treatment, a new spontaneous pattern was obtained for 15 min, and then the strips were rechallenged with increasing oxytocin doses of 1, 10, 50, and 100 nM under the same protocol as the first. Contraction tracings were recorded using PolyVIEW (Grass Instruments). The area under the curve for each contraction response was calculated for the final 5 min of each 9-min contraction tracing using GraphPad Prism (GraphPad Prism for Macintosh version 5.00; GraphPad Software, San Diego, CA; www.graphpad.com). The area under the curve for each response was then normalized to the area under the curve for the spontaneous contraction pattern over the 5-min period that immediately preceded the challenge.

**Preparation of cell lysates and Western blot analysis for MAPK signaling.** HEK-293 cells at 50% confluence were transfected with HA-OXTR and either control scramble siRNA or siRNA directed against β-arrestin1 and β-arrestin2 and split to fibronectin-coated six-well plates. Seventy-two hours after transfection, a scratch in the cell monolayer was made using a p200 pipette tip. The cells were washed twice with PBS to remove the cellular debris, and then the medium was replaced. Using an inverted Zeiss Axio Observer microscope, 10 sequential images were obtained as described (27). Once the initial scratch was imaged, the cells were then treated with PBS or 100 nM oxytocin and returned to the incubator. Twelve hours later, the plates were reimaged, taking 10 images/scratch, equally spaced as previously. For each scratch image, 10 measurements were made across the scratch, equally spaced, for each of the 10 images. This provided 100 measurements across the length of each scratch before and after treatment. Metamorph (Molecular Devices, Silicon Valley, CA) was used for all image analysis. A similar procedure was used utilizing hTERT-HM cells (11).

**Statistical analysis.** For uterine muscle strip experiments, the area under the contraction curve for spontaneous contractions showed good correlation (r2 = 0.90) with the area under the contraction curve following treatment with 1 nM oxytocin, using a linear regression goodness of fit model (Supplemental Fig. S1; Supplemental Material for this article can be found online at the AJP-Endocrinology and Metabolism web site). Therefore, all contraction responses following oxytocin stimulation were reported as the percentage of the spontaneous contraction response immediately preceding the oxytocin challenge. The area under the contraction curve for the spontaneous contraction pattern was measured for the 5 min immediately before the first oxytocin dosing challenge (initial challenge 1, 10, 50, and 100 nM) and then again immediately before the second oxytocin dosing challenge (rechallenge 1, 10, 50, and 100 nM). The area under the contraction curve for each oxytocin dose was measured during the final 5 min at each dose and then reported as the percentage of the spontaneous contraction pattern that immediately preceded the dosing challenge. To account for repeated measurements recorded for individual strips at increasing oxytocin dosing, a repeated-measures ANOVA model was used to compare the dose-response curves (1, 10, 50, and 100 nM) for the WT and β-arrestin-2KO mice for the initial and then subsequent rechallenge. Next, a nonlinear regression curve was fit for each of the dose-response curves and the mean max-dose contraction response

**siRNA transfections.** The sequence of the double-stranded siRNA directed against β-arrestin-1, β-arrestin-2, and control scramble siRNA has been used by our group and described previously (2, 22, 32). Briefly, HEK-293 cells at 50% confluence were transfected simultaneously with 1 μg of HA-OXTR plasmid and 1.44 nmol combined total of β-arrestin-1 and β-arrestin-2 siRNA or control scramble siRNA using Gene Silencer siRNA transfection reagent (GenLantis). Following 72 h of incubation, cells were serum starved for 1 h, stimulated with 100 nM oxytocin, and processed on an SDS-PAGE gel per standard Western protocol.

**In vivo MAPK signaling assays.** An in vivo assay was designed to determine whether MAPK signaling from the OXTR was dependent on β-arrestin. WT, β-arrestin1KO and β-arrestin2KO mice were anesthetized using intraperitoneal ketamine and xylazine. A central venous catheter was inserted through the right internal jugular vein. Oxytocin (1 μM) or normal saline was infused for 10 min at a rate (μL/min) of twice the mouse body weight (20 g/mouse, infusion ran at 40 μL/min). Following infusion, the mice was euthanized, the abdomen was opened, and the uterus was then homogenized in cell lysis buffer (Cell Signaling), processed on an SDS-PAGE gel per standard Western protocols, and probed for phosphorylated ERK.

**Cell migration assay.** Given the known role of β-arrestin in activating cell survival pathways (25), we postulate that β-arrestin-dependent signaling may be important for cell growth in pregnancy. To determine the implications of β-arrestin-mediated signaling from the OXTR, a cell migration assay was utilized and is described elsewhere (27). Briefly, HEK-293 cells were transfected with HA-OXTR and either control scramble siRNA or siRNA directed against β-arrestin1 and β-arrestin2 and split to fibronectin-coated six-well plates. Seventy-two hours after transfection, a scratch in the cell monolayer was made using a p200 pipette tip. The cells were washed twice with PBS to remove the cellular debris, and then the medium was replaced. Using an inverted Zeiss Axio Observer microscope, 10 sequential images were obtained as described (27). Once the initial scratch was imaged, the cells were then treated with PBS or 100 nM oxytocin and returned to the incubator. Twelve hours later, the plates were reimaged, taking 10 images/scratch, equally spaced as previously. For each scratch image, 10 measurements were made across the scratch, equally spaced, for each of the 10 images. This provided 100 measurements across the length of each scratch before and after treatment. Metamorph (Molecular Devices, Silicon Valley, CA) was used for all image analysis. A similar procedure was utilized using hTERT-HM cells (11).

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  - **Vol 300 • March 2011 • www.ajpendo.org**
  - **AJP-Endocrinol Metab** • VOL 300 • MARCH 2011 • www.ajpendo.org

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(100 nM oxytocin) for each curve calculated. The mean max-fit response from the initial challenge was compared with the rechallenge mean max-fit for each mouse type using a t-test.

For the MAPK signaling experiments, the mean \( \pm SE \) intensity of the phosphorylated ERK band at each time point was determined and expressed as a percent of maximal stimulation (phosphorylated ERK signal at 5 min in cells without inhibitors) normalized to total ERK for each condition. The curves were compared using two-way ANOVA between oxytocin-stimulated cells without inhibitors and those with inhibitors, and Bonferroni posttests were used to compare values at each time point.

The cell migration assays were reported as the percent scratch closure (mean scratch distance following stimulation as a percent of the original mean scratch distance subtracted from 100). One-way ANOVA was used to compare the percent scratch closure between each group, and Bonferroni posttests were used for multiple comparisons. A \( P \) value <0.05 was considered significant for all comparisons.

RESULTS

Desensitization of the OXTR leads to decreases in uterine contractility that are mediated by \( \beta \)-arrestin. To test the hypothesis that desensitization of the OXTR leads to decreases in oxytocin-stimulated uterine contractility, we studied the contraction response of uterine muscle strips from WT, \( \beta \)-arr1KO, and \( \beta \)-arr2KO mice exposed to increasing concentrations of oxytocin. All strips were exposed to a dose escalation challenge of oxytocin, with increasing doses from 1 to 100 nM, followed by a rechallenge of the same dosing regimen. All uterine muscle strips from WT, \( \beta \)-arr1KO, and \( \beta \)-arr2KO mice exhibited spontaneous contraction patterns during suspension in the organ bath prior to oxytocin stimulation. A typical contraction pattern of WT uterine muscle strips to initial oxytocin-stimulated contraction was observed in uterine muscle strips from WT, \( \beta \)-arr1KO, and \( \beta \)-arr2KO mice (Supplemental Fig. S4).

Upon oxytocin rechallenges, a significant blunting of uterine contraction occurred in WT uterine muscle strips but not in uterine muscle strips from \( \beta \)-arr1KO and \( \beta \)-arr2KO mice (Figs. 1B and 2A). In response to maximal oxytocin stimulation (100 nM), uterine strips from \( \beta \)-arr1KO and \( \beta \)-arr2KO mice showed greater uterine contraction strength and lack of desensitization compared with uterine muscle strips from WT mice (Fig. 2B). There were no differences in the amount of OXTR protein as detected by Western blot among WT, \( \beta \)-arr1KO, or \( \beta \)-arr2KO mice (Supplemental Fig. S2).

\( \beta \)-Arrestin-dependent OXTR-mediated MAPK signaling. To test the hypothesis that \( \beta \)-arrestin mediates MAPK signaling from the OXTR, we measured p-ERK in uteri from WT, \( \beta \)-arr1KO, and \( \beta \)-arr2KO mice following treatment with intravenous oxytocin. WT mice demonstrated an increase in p-ERK signaling after a 10-min intravenous oxytocin infusion (Fig. 3A). In contrast, \( \beta \)-Arrestin activation was significantly blunted in the uteri from \( \beta \)-arr1KO and \( \beta \)-arr2KO mice after agonist stimulation (Fig. 3A), consistent with the concept that a component of oxytocin-stimulated ERK signaling is dependent on both \( \beta \)-arrestin-1 and \( \beta \)-arrestin-2.

To test the relative contributions of G protein- and \( \beta \)-arrestin-mediated MAPK signaling by the OXTR, we performed cell culture experiments. Oxytocin did not activate ERK in control HEK-293 cells, which lack the OXTR (data not shown). HEK-293 cells expressing the OXTR were stimulated with oxytocin ranging from 1 nM to 1 \( \mu \)M, and a dose-response curve was constructed (Supplemental Fig. S3A). From this, a dose of 100 nM was chosen for all cell culture experiments. HEK-293 cells expressing the OXTR were treated with control scramble siRNA and showed ERK phosphorylation that peaked at 5 min. Cells treated with siRNA directed against \( \beta \)-arrestin-1/2 showed a 50% decrease in phosphorylated ERK (Fig. 3B). The OXTR couples primarily to the G protein \( G_\beta \) but is also known to couple to the G protein \( G_\gamma \) (19). To determine the contributions of each, we utilized the \( G_\beta/\gamma \)-PKC inhibitor Ro-31-8425 (Ro-31) and the \( G_\gamma \) inhibitor PTX. HEK-293 cells expressing the OXTR pretreated with the PKC inhibitor Ro-31 demonstrated a 40–50% reduction in maximal phosphorylated ERK signaling at 5 min (Fig. 4A), whereas cells pretreated with the \( G_\gamma \) inhibitor PTX demonstrated a 30% reduction in phosphorylated ERK (Fig. 4B). Cotreatment with siRNA directed against \( \beta \)-arrestin-1, \( \beta \)-arrestin-2, PTX, and Ro-31, thereby inhibiting \( \beta \)-arrestin-, \( G_\gamma \), and \( G_\beta/\gamma \)-mediated signaling, abolished ERK activation with oxytocin stimulation (Supplemental Fig. S3B). Finally, the p-ERK signal remaining following cotreatment with PTX and Ro-31 represents \( \beta \)-arrestin-mediated ERK activation (Supplemental Fig. S3C). Taken together, these data demonstrate that activation of ERK by the OXTR results from both G protein- and \( \beta \)-arrestin-dependent signals and that there is likely dependence between \( G_\gamma \) and \( G_\beta/\gamma \) and \( \beta \)-arrestin-mediated MAPK signaling.

Inhibition of \( \beta \)-arrestin leads to altered oxytocin-induced cell migration. We next tested the role of \( \beta \)-arrestin signaling in OXTR-stimulated cellular growth by performing cell proliferation assays (27). To determine whether the oxytocin-mediated effects on cell growth and migration were mediated by \( \beta \)-arrestin, we performed the scratch assay (27), following \( \beta \)-arrestin-1/2 knockdown with \( \beta \)-arrestin-1/2 siRNA in HEK-293 cells expressing the OXTR. Oxytocin treatment induced a significant scratch closure in cells treated with control scramble siRNA (Fig. 5, A and C) that was completely abrogated in the presence of siRNA targeting \( \beta \)-arrestin1/2 (Fig. 5, B and C). Similar findings were seen when the in vitro scratch assay was replicated using an immortalized human myometrial cell line, hTERT-HM (11). Oxytocin increased cell migration compared with saline-treated control cells, which was reversed in the setting of \( \beta \)-arrestin depletion using siRNA (Supplemental Fig. S4).

DISCUSSION

In this study, we demonstrate that 1) uterine muscle strips exhibit OXTR desensitization that is mediated by \( \beta \)-arrestin-1 and \( \beta \)-arrestin-2, leading to decreases in uterine contractility, 2) the OXTR activates ERK in HEK-293 cells in both a G protein- and \( \beta \)-arrestin-dependent manner with dependency between the two signaling pathways, 3) the OXTR activates ERK in both a G protein- and \( \beta \)-arrestin-dependent manner within uterine muscle from mice receiving oxytocin infusions, and 4) OXTR-stimulated cell migration is \( \beta \)-arrestin dependent.

Clinically, OXTR desensitization in the setting of continuous oxytocin infusion is an important issue. Continuous, pro-
A prolonged infusion of oxytocin is associated with dysfunctional labor, labor dystocia, uterine atony, and postpartum hemorrhage. Clinical studies demonstrate a loss of uterine activity as measured by an intrauterine pressure catheter in women undergoing labor augmentation after a fixed oxytocin dose infusion of 90 min (14). This loss of uterine activity leads to dysfunctional labor patterns and labor dystocia, eventually increasing the risk for cesarean delivery. Moreover, oxytocin exposure is an important predisposing factor for uterine atony following delivery, resulting in postpartum hemorrhage (40). Together, these adverse clinical outcomes have in common prolonged oxytocin exposure leading to decreases in uterine contractility secondary to OXTR desensitization. In addition, experimental studies utilizing uterine muscle strips from WT pregnant rats also demonstrate decreases in uterine contractility in the setting of prolonged oxytocin stimulation, with contraction response curves similar to our data (29). Subsequent reports from this same group demonstrate that, despite decreases seen in uterine contractility following prolonged oxytocin exposure, the uterine muscle strips responded with normal uterine contractility to prostaglandin stimulation, demonstrating that the contractile apparatus remained intact despite OXTR desensitization (3).

![Fig. 1. Representative contraction tracings of uterine muscle strips from wild-type (WT; A) and β-arrestin-1 knockout (β-ar1KO; B) mice. A: uterine muscle strips were isolated from WT mice and suspended in a tissue organ bath at 0.5 g of tension. Once the strips were allowed to equilibrate, they were treated with 1 nM oxytocin, and the contraction response was recorded for 9 min. The buffer and drug were washed away, and the strip was allowed to equilibrate for 3 min. This was followed by an increase in oxytocin dose to 10, 50, and 100 nM. For each dose, the contraction response was recorded for 9 min, a washout was performed, and the strip was allowed to equilibrate for 3 min prior to the next dose. Following the 100-nM dose of the initial challenge, the strips were allowed to equilibrate for 15 min (not shown) prior to the rechallenge with the same oxytocin dose escalation. All contraction responses were measured and reported as the area under the contraction curve (AUC) for the last 5 min of each response normalized to the AUC of the spontaneous contraction pattern that preceded the challenge. B: similar response seen in a representative β-ar1KO uterine muscle strip.](http://ajpendo.physiology.org/)
cesarean section in women in labor or induced labor compared with women not in labor (37). Together, these series of studies demonstrate that the OXTR undergoes desensitization leading to impaired uterine contractility, although the molecular mechanisms involved have yet to be fully elucidated. Our findings demonstrate that decreases in uterine contractility following prolonged oxytocin stimulation are dependent on both β-arrestin-1 and β-arrestin-2.

Oxytocin receptors, like other GPCRs, undergo desensitization and internalization in the setting of persistent agonist stimulation (35, 36) and are dependent on a clathrin-coated, pit-mediated mechanism (44). The OXTR recruits β-arrestin following agonist stimulation, and the OXTR colocalizes into endocytic vesicles with β-arrestin (20, 33), consistent with a class B receptor pattern (43). Similar to other GPCRs, β-arrestin recruitment to the OXTR is dependent on a highly conserved region within the carboxyl-terminal domain of the receptor (33, 44). This region contains a series of serine clusters that have been shown to be required for β-arrestin-GPCR interactions following agonist stimulation. Mutations of the OXTR in this highly conserved region of serine clusters within the carboxyl-terminal domain lead to unstable OXTR-β-arrestin interactions and failure of receptor internalization following agonist stimulation (33).

Fig. 2. Dose-response curves of uterine muscle strips from WT, β-arrestin-1 knockout (β-arrestin-1KO) and β-arrestin-2 knockout (β-arrestin-2KO) mice. A: nonlinear regression curves were fit to describe the dose response seen in the initial challenge (●) and rechallenge (○) for each of the 3 mouse types. Uterine muscle strips from WT mice exhibited a decrease in uterine contractility during the rechallenge, suggestive of oxytocin receptor (OXTR) desensitization as measured by repeated-measures 2-way ANOVA (†P < 0.05). This was not observed in the β-arrestin-1KO or β-arrestin-2KO mice. B: mean best-fit maximal contraction response at 100 nM during the initial and rechallenge for each of the 3 mouse types demonstrates that uterine muscle strips from WT mice exhibit a decrease in uterine contractility during the rechallenge (*P < 0.01, rechallenge compared with initial challenge). Uterine strips from β-arrestin-1KO and β-arrestin-2KO mice do not exhibit a decrease in uterine contractility during the rechallenge compared with the initial challenge. Both β-arrestin-1KO and β-arrestin-2KO uterine strips demonstrated greater uterine contractility at maximal oxytocin stimulation compared with WT (†P < 0.01, initial challenge knockout animal compared with initial challenge WT).
The molecular mechanisms of β-arrestin recruitment to GPCRs have been well studied (33). Following agonist stimulation of the OXTR, GRK phosphorylation of the receptor allows for the recruitment and binding of β-arrestin. β-Arrestin recruitment to the OXTR leads to receptor internalization, uncoupling the receptor from G proteins, interdicting further signaling. Following internalization, the OXTR has been shown to recycle back to the cell surface membrane via a short cycle mediated by Rab4 and Rab5 (13).

Uterine muscle strips from both β-arrestin-1 KO and β-arrestin-2 KO mice failed to exhibit decreases in uterine contractility with persistent oxytocin stimulation. This finding suggests that both β-arrestin-1 and β-arrestin-2 are necessary for physiological OXTR desensitization, since the presence of one β-arrestin isoform did not compensate for the loss of the other isoform. Consistent with our finding that uterine muscle strips from β-arrestin-1 KO and β-arrestin-2 KO did not undergo desensitization, we found greater uterine contractions in the knockout mice following oxytocin stimulation.

GPCRs as a class are known to undergo homologous desensitization mediated by GRK phosphorylation and β-arrestin recruitment. In addition, the GPCRs are known to undergo heterologous desensitization mediated by PKA or PKC (16). Indeed, Yue et al. (50) demonstrated that PKC activation by thymeleatoxin followed by oxytocin stimulation inhibited oxytocin-mediated increases in 1,4,5-triphosphate, demonstrating that the OXTR can undergo heterologous desensitization mediated by PKC. Moreover, the OXTR can associate with PKC following oxytocin stimulation, suggesting that PKC may also play a role in oxytocin-induced homologous desensitization, although the downstream effect of oxytocin signaling and its affect following PKC interactions was not measured (5).

Our work focused on the role of β-arrestin in mediating oxytocin receptor desensitization through loss-of-function experiments utilizing knockout animals. We did not appreciate decreases in uterine contractility in mice lacking β-arrestin following repeated oxytocin stimulation; however, we can...
not exclude the possibility that PKC may be involved in OXTR desensitization in the uterus in vivo.

In addition to its role in mediating desensitization of GPCRs, β-arrestin has also recently been identified to mediate GPCR-stimulated intracellular signaling independent of G protein activation (15, 18, 26, 32, 42, 49). Examples of GPCRs that show β-arrestin-mediated signaling are the parathyroid receptor (17), the angiotensin II type 1A receptor (49), the β1-adrenergic receptor (32), the β2-adrenergic receptor (42), and the vasopressin V2 receptor (9). The molecular mechanism by which β-arrestin transduces a signal appears to be through its scaffold function, which has been best characterized for the MAPK pathway (25).

To determine whether OXTRs activate MAPK in a β-arrestin-dependent manner, we performed β-arrestin loss-of-function experiments in vivo and in vitro. HEK-293 cells transfected with siRNA directed against β-arrestin-1/2 demonstrate decreased ERK phosphorylation following oxytocin treatment. Using inhibitors directed at Gq and Gi-mediated signaling, we demonstrated that Gq-mediated signaling contributed ~40–50% and Gi-mediated signaling contributed ~30% to ERK phosphorylation from the OXTR. β-Arrestin-1/2 alone seemed to contribute 50% of ERK phosphorylation signaling from the OXTR. In addition, uteri from both β-ar1KO and β-ar2KO mice receiving intravenous oxytocin also demonstrated marked decreases in ERK phosphorylation compared with WT mice. Together, our data suggest that both β-arrestin-1 and β-arrestin-2 are necessary for β-arrestin-dependent ERK signaling from the OXTR. In addition, our data suggest that there is a cooperative interaction between Gq- or Gi-mediated and β-arrestin-mediated signaling in activating ERK in response to OXTR stimulation, a similar finding to that observed for the GPR109A receptor. The GPR109A receptor is a GPCR that, when stimulated by nicotinic acid, activates Gi-mediated signaling. ERK activation by nicotinic acid from the GPR109A receptor is dependent on interactions between Gi- and β-arrestin-mediated signaling. Pretreatment with PTX essentially eliminates ERK activation after nicotinic acid stimulation. In addition, ERK activation is equally essentially eliminated in cells depleted of β-arrestin-1 and β-arrestin-2, demonstrating that β-arrestin-mediated signaling from the GPR109A receptor is PTX sensitive and dependent on Gi coupling (48). Similar interactions have been described with other Gi-coupled receptors, including the CCR7 and CXCR4 receptors (10, 24).

The physiological role of β-arrestin-mediated signaling from the OXTR has not previously been explored. Prior work has shown that myometrial cells in culture subjected to mechanical stretch and uteri from rat pregnancy stretch-induced models

Fig. 4. The OXTR signals through the MAPK pathway through the Gq- and Gi-mediated pathways. To determine the relative component of MAPK signaling from G protein-mediated signaling, inhibitors of Gq and Gi-mediated signaling were utilized. A: HEK-293 cells expressing the OXTR were pretreated with the PKC inhibitor Ro-31, which inhibits Gq-mediated signaling. Cells were then treated with 100 nM oxytocin at 2, 5, 10, 30, or 60 min and the cells lysed and processed on a Western blot and probed for p-ERK. Maximal p-ERK signaling in control cells occurred at 5 min and was diminished by ~40–50% with Gq inhibition. Mean densitometry data from 6 independent experiments is illustrated. There is a significant decrease in maximal p-ERK signaling at 5 min (*P < 0.5) compared with control (CTL), and repeated-measures 2-way ANOVA demonstrates the 2 curves to be different (†P < 0.05). B: to determine the component of Gi-mediated signaling from the OXTR to the MAPK pathway, HEK-293 cells expressing the OXTR were pretreated with pertussis toxin (PTX) and then stimulated with an oxytocin time course. Mean densitometry data from 6 independent experiments illustrates an ~30% decrease in maximal p-ERK signaling at 5 min (*P < 0.05) compared with CTL, and repeated-measures 2-way ANOVA demonstrates that the 2 curves are different (†P < 0.05). IB, immunoblotting.
lead to MAPK activation, but whether stretch-induced MAPK activation in myometrial cells is OXTR or β-arrestin dependent is unknown (34). Prior work has shown that serum from women demonstrates detectable levels of oxytocin throughout gestation without clinical evidence of uterine contraction except at the onset of labor, whereas oxytocin was not detected in the serum of nonpregnant women (31). This suggests that oxytocin may play a role in uterine physiology beyond mediating uterine contraction. We propose that oxytocin may lead to myometrial cell growth that is mediated by β-arrestin. Given the known role of β-arrestin in activating cell survival pathways (25), we postulate that β-arrestin-dependent signaling is important for myometrial growth in pregnancy. Indeed, oxytocin has potent mitogenic effects on a number of cell types, and it has been postulated that these mitogenic properties are important for myometrial cell survival and maintenance (4, 7, 8, 41, 46, 47). In addition to its role as a mitogen, β-arrestin has also been implicated as mediating chemotaxis-related cell migration by other GPCRs (21, 45). Using an in vitro scratch assay, we demonstrate that β-arrestin mediates oxytocin-induced cell migration. The in vitro scratch assay is primarily a measurement of cell migration rather than cell growth or proliferation; therefore, it is difficult to determine whether there is a clinically significant correlation to cell migration within the myometrium that is oxytocin mediated.

In conclusion, we demonstrate that β-arrestin-1 and β-arrestin-2 mediate OXTR desensitization in murine uterine muscle strips subjected to increasing concentrations of oxytocin stim-
ulation, leading to decreased uterine contraction. These findings suggest that β-arrestin-mediated desensitization of the OXTR leading to decreases in uterine contractility may contribute to the clinical finding of dysfunctional labor patterns and uterine atony seen clinically following prolonged oxytocin stimulation. Based on our findings, we cannot exclude the role of other mechanisms involved in OXTR desensitization, such as PKC. In addition and consistent with the dual role of β-arrestin, we show that stimulation of OXTR-mediated MAPK signaling is in part dependent on β-arrestin, and β-arrestin mediates oxytocin-induced cell migration. The development of nondesensitizing OXTR agonists that stimulate only G-proteins and not β-arrestin may be a novel approach to the treatment of dysfunctional labor and uterine atony.

ACKNOWLEDGMENTS

We thank Dr. Robert Lefkowitz (Duke University Medical Center and Howard Hughes Medical Institute) for providing WT, β-ar1KO, and β-ar2KO mice. In addition, Dr. Lefkowitz provided antibodies against β-arrestin-1/2. We thank Dr. Jerry Eu (Duke University Medical Center and Durham Veterans Affairs Medical Center) for providing training on and use of the contraction organ bath bioassay. We thank Dr. Marc Caron (Duke University Medical Center) for the HA-OXTR plasmid and Dr. Jennifer Condon (Duke University and Durham Veterans Affairs Medical Center) for providing training on and use of the contraction organ bath bioassay. We thank Dr. Yasheng Gao of the Duke University Light Microscopy Core Facility for technical assistance with the in vitro scratch assay.

GRANTS

This work was supported by research grants from the National Institute of Child Health and Human Development (K12-HD-043446), the American College of Obstetricians and Gynecologists and Ortho Women’s Health and Urology Academic Training Fellowship in Obstetrics and Gynecology, and the Charles B. Hammond Fund of the Duke University School of Medicine to C. A. Grotegut.

DISCLOSURES

H. A. Rockman is the scientific cofounder of Trevena, a company that is developing G protein-coupled receptor-targeted drugs.

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