AKT-independent Phosphorylation of TSC2 and Activation of mTOR and Ribosomal Protein S6 Kinase Signaling by Prostaglandin F2α*

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Prostaglandin F2α (PGF2α) is an important mediator of corpus luteum (CL) regression, although the cellular signaling events that mediate this process have not been clearly identified. It is established that PGF2α binds to a G-protein-coupled receptor (GPCR) to stimulate protein kinase C (PKC) and Raf-MEK-Erk signaling in luteal cells. The present experiments were performed to determine whether PGF2α stimulates the mammalian target of rapamycin (mTOR)/ribosomal protein S6 kinase 1 (S6K1) signaling pathway in steroidogenic luteal cells. We demonstrate that PGF2α treatment results in a time- and concentration-dependent stimulation of the phosphorylation and activation of S6K1. The stimulation of S6K1 in response to PGF2α treatment was abolished by the mTOR inhibitor rapamycin. Treatment with PGF2α did not increase AKT phosphorylation but increased the phosphorylation of Erk and the tumor suppressor protein tuberous sclerosis complex 2 (TSC2), an upstream regulator of mTOR. The effects of PGF2α were mimicked by the PKC activator PMA and inhibited by U0126, a MEK1 inhibitor. The activation of mTOR/S6K1 and putative downstream processes involving the translational apparatus (i.e. 4EBP1 phosphorylation, release of 4EBP1 binding in m7G cap binding assays, and the phosphorylation and synthesis of S6) were completely sensitive to treatment with rapamycin, implicating mTOR in the actions of PGF2α. Taken together, our data suggest that GPCR activation in response to PGF2α stimulates the mTOR pathway which increases the translational machinery in luteal cells. The translation of proteins under the control of mTOR may have implications for luteal development and regression and offer new strategies for therapeutic intervention in PGF2α-targeted tissues.

The corpus luteum (CL) is a transient endocrine gland derived from an ovulated follicle within the ovary. The steroidogenic cells of the CL produce progesterone, a prerequisite for normal maintenance of pregnancy in mammals. In the event of pregnancy, the CL retains its role in progesterone synthesis in support of early pregnancy. In the absence of pregnancy luteolysis or corpus luteum regression occurs, a physiologic process associated with a reduction in progesterone secretion (functional regression) followed by death of endothelial and steroidogenic cells (structural regression) (1–4). Prostaglandin F2α (PGF2α) was identified as a luteolytic factor over 35 years ago (5, 6). Recent genetic studies in mice lacking the PGF2α receptor (FP) further highlight the role for PGF2α in CL regression. Mice lacking FP receptors experience defects in CL regression and consequently parturition does not occur because of the maintenance of progesterone secretion at the end of pregnancy (7).

The FP receptor is a member of the large class of heterotrimeric G-protein-coupled receptors (GPCRs) (8) and is present on the surface of steroidogenic luteal cells. PGF2α binding to its Gq-coupled receptor results in activation of phospholipase β (PLCβ) and consequent generation of the second messengers; diacylglycerol and inositol trisphosphate (9). The resultant increase in protein kinase C (PKC) activity in luteal cells contributes to the activation of other downstream protein kinases. PGF2α stimulates the activity of the extracellular signal-regulated kinase (Erk) family of mitogen-activated protein kinases (MAPK) through a mechanism that involves the PKC-dependent phosphorylation and activation of Raf (10, 11). These events result in the induction of early response genes that code for transcription factors that in turn, alter the synthesis of proteins that regulate progesterone synthesis (12–14). Little is understood regarding other signaling mechanisms initiated by PGF2α that account for the actions of PGF2α in the CL.

The mammalian target of rapamycin (mTOR) protein is a key regulator of protein translation via mechanisms involving the phosphorylation of the translation regulator eukaryotic initiation factor 4E (eIF4E)-binding protein (4EBP1) and the 70-kDa ribosomal protein S6 protein kinase 1 (S6K1) (15, 16).
The activity of mTOR is regulated by inputs from multiple signaling pathways that appropriately increase or decrease protein synthesis. Although the activation of mTOR has been well characterized in response to growth factors acting via receptor tyrosine kinase-mediated stimulation of phosphatidylinositol 3-kinase (PI3K)/AKT signaling, less information is available on the mechanisms used by GPCRs to stimulate mTOR and the phosphorylation of 4EBP1 and S6K1 (17). In quiescent cells, 4EBP1 acts as a translational repressor by binding tightly to the 7-methylguanosine (m7G) cap-binding protein. The phosphorylation of 4EBP1 by mTOR promotes its release from eIF4E (18) and allows recruitment of mRNAs with a m7G cap to the ribosome (15–21). Once activated by mTOR, S6K phosphorylates the small ribosomal protein S6 in a step that is postulated to further enhance the efficiency of functional ribosomes (21). Evidence for the role of mTOR/S6K1 signaling in translational control has come largely from studies involving the macrolide antibiotic rapamycin (22). Rapamycin binds directly to FKBP12 (FK506-binding protein 12 kDa) (23), and the FKBP12-rapamycin complex binds to and deactivates the mTOR enzyme. Through its highly effective and specific effects on mTOR, rapamycin has proven to be an invaluable tool for investigating mTOR function, S6K1 function, and translation (reviewed in Refs. 15 and 24). The effects of rapamycin include inhibition of cap-dependent translation, inhibition of 4EBP1 phosphorylation and S6K1 activity, as well as inhibition of cell proliferation and cell growth.

The activity of mTOR is negatively controlled by the tumor suppressors tuberous sclerosis complex 1 (TSC1, also known as hamartin) and TSC2 (also known as tuberin) (25–27). Epistatic analysis and biochemical studies indicate that AKT-dependent phosphorylation of TSC2 prevents the TSC2-dependent inhibition of mTOR signaling. Whereas PI3K/AKT appears to be the major growth factor-mediated signal that controls TSC2 phosphorylation and mTOR activation, TSC2 is also phosphorylated on other residues by the activation of PKC, MEK1, Erk, and p90 ribosomal S6 kinase (RSK1), which apparently contributes to AKT-independent mTOR activation (16, 28). Notably, the tumor promoter PMA stimulates the phosphorylation of TSC2 (Ser1798) and the activation of mTOR/S6K1 by an Erk-mediated and RSK1-dependent process (29). However, specific GPCR ligands that induce TSC2 phosphorylation and mTOR/S6K activity by AKT-independent mechanisms have not been identified.

The present experiments were performed to determine if activation of PGF2α receptors stimulates the phosphorylation of TSC2 and activation of mTOR/S6K1 signaling. Herein, we provide the first evidence that PGF2α stimulates the phosphorylation of TSC2 and the activation of S6K1 by an AKT-independent, Erk-dependent mechanism. We show that stimulation of S6K1 by PGF2α treatment is rapamycin-sensitive. Furthermore, we demonstrate that PGF2α stimulates the phosphorylation of mTOR substrates and regulates proteins involved in the initiation of translation in luteal cells. Taken together, our data suggest that PGF2α signaling promotes activation of the mTOR/S6K1 pathway leading to activation of the translational apparatus.

### EXPERIMENTAL PROCEDURES

#### Reagents—Rabbit polyclonal anti-phosphorylated S6K1 (Thr389); anti-phosphorylated S6K1 (Thr421/Ser424); anti-phosphorylated Erk1/2 (Thr202/Tyr204); anti-4EBP1; anti-S6; anti-phosphorylated ribosomal protein S6 (Ser235/236); anti-phosphorylated Akt (Ser473); phospho-TSC2 (Thr1462); phospho-4EBP1 (Thr37/46); and the phospho-TSC2 (Ser1798) RXXRXXpSer motif antibody (29) were purchased from Cell Signaling Technology (Beverly, MA). Antibodies recognizing TSC2 (sc-893) and eIF4G were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-4EBP1 was purchased from Bethyl Laboratories. The S6K1 antibody was from Upstate (Charlottesville, VA). Monoclonal anti-eIF4E, MEK1, and pan-Erk were purchased from BD Transduction Laboratories (San Diego, CA). Monoclonal anti-β-actin, horseradish peroxide-conjugated anti-rabbit and anti-mouse, BSA (fraction V), PGF2α and Ponceau stain were from Sigma-Aldrich. Immobilon™-P polyvinylidene difluoride (0.45-μm pore size) membrane was purchased from Millipore (Bedford, MA). Rapamycin was purchased from Calbiochem (La Jolla, CA). U0126 was obtained from BIOMOL (Plymouth Meeting, PA). The recombinant adenovirus Ad.dnMEK1 was obtained from Dr. J. Han (The Scripps Research Institute, La Jolla, CA). In the Ad.dnMEK1 vector, the MEK1 cDNA has been altered and two crucial serine residues located in the catalytic domain (Ser217 and Ser221) were replaced by alanines. The resulting MEK1 mutant has dominant negative activity and can be used to block the activation of ERK1/2 by wild-type MEK1 as described (30).

#### Isolation and Culture of Bovine Luteal Cells—Ovaries from cows were obtained from a local abattoir and processed as previously described (31). Luteal tissue was dissociated with type II collagenase (103 units/ml) (Atlantic Biologicals, Lawrenceville, GA) in basal medium (M199 (Cambrex, Walkersville, MD) containing 0.1% bovine serum albumin, 100 units/ml penicillin-G-Sodium, 100 μg/ml streptomycin sulfate, and 10 μg/ml gentamycin sulfate). Cell viability was determined by trypsin blue exclusion, and only those cell preparations with viability exceeding 90% were used. Enriched bovine steroidogenic luteal cells (bLcs) were plated (~1 × 10^7 cells/cm²) in basal medium and 5% FBS (Invitrogen, Carlsbad, CA) for 24 h at 37 °C in a humidified incubator with 5% CO_2. Cells were serum-starved in basal medium for 24 h. Following replacement with fresh medium, the cells were allowed to equilibrate for 3–4 h prior to initiation of treatments.

#### Experimental Conditions and Preparation of Cell Extracts—Cells were treated as described in the figure legends. Experiments were stopped by rapidly rinsing the cells with ice-cold phosphate-buffered saline and treatment for 20 min on ice with lysis buffer A (10 mM KPO_4, 1 mM EDTA, 5 mM EGTA, 10 mM MgCl_2, 50 mM β-glycerophosphate, 10% glycerol, 0.5% Nonidet P-40, 0.1% deoxycholate, and DVPLA (2 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and 10 μg/ml aprotinin)). The bLcs were scraped from the plates and briefly vortexed. Cell lysates were sonicated with a 5-s pulse followed by centrifugation for 10 min at 14,000 × g. The protein concentration of the super-
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natant was determined using Bradford reagent (Bio-Rad). Samples were processed immediately for experiments involving immunoprecipitation and immunocomplex protein kinase assays or frozen at −80 °C until analysis.

Western Blot Analysis—Proteins (40 μg/lane) were separated on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes prior to immunoblot analysis. Equivalent loading was verified by Ponceau staining. Membranes were blocked in 5% fat-free milk in TBST (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween-20) for 1 h and incubated overnight at 4 °C with primary Ab in TBST plus 5% bovine serum albumin. After three 5-min washes with TBST, membranes were incubated in appropriate horseradish peroxidase-conjugated secondary Ab for 1 h at room temperature. Membrane-bound antibodies were detected with the Western Lightning™ ECL detection system (PerkinElmer Life Sciences Inc.) and signals visualized using x-ray film or a Kodak Digital Sciences Image Station 440.

Immunoprecipitation and Immunocomplex Protein Kinase Assays—Cells (7.5 × 10⁶) were lysed in 0.5 ml of buffer A. Tuberin was immunoprecipitated from lysates (500 μg of protein) overnight at 4 °C in a final volume of 500 μl containing anti-tuberin antibody (1:100). After washing extensively, the immune complex was boiled in 2× sample buffer and fractionated on 7% SDS-PAGE gels. Immunoprecipitation with Ab for 4EBP1 (1:100) was performed as above. Immune complexes were boiled in 2× sample buffer and fractionated on 13% SDS-PAGE gels.

The activity of S6K1 was determined as previously described (32, 33). Lysates were pre cleared by tumbling for 1 h at 4 °C with 40 μl of packed volume protein A-Sepharose beads (Amersham Biosciences). The samples were centrifuged briefly, and the supernatant was removed to new tubes containing 20-μl packed volume protein A-Sepharose beads. Total S6K1 was immunoprecipitated from lysates (500 μg of protein) overnight at 4 °C in a volume of 500 μl containing polyclonal anti-S6K1 antibody (1:100). Immunocomplexes were washed by resuspending in detergent wash (1% Nonidet P-40, 0.5% sodium deoxycholate, 100 mM NaCl, 10 mM Tris, pH 7.2, 1 mM EDTA, and DVEPLA), followed by a high salt wash (1 M NaCl, 0.1% Nonidet P-40, 10 mM Tris, pH 7.2, and DVEPLA). Immune complexes were washed a third time in Tris-saline buffer (150 mM NaCl, 50 mM Tris-HCl, 5 mM Tris-Base, and DVEPLA). The immune complexes were resuspended in 20 μl of 1.5× kinase buffer (30 mM HEPES, pH 7.2, 15 mM MgCl₂, 0.15 mg/ml bovine serum albumin, 0.325 μl/ml freshly added β-mercaptoethanol (0.011% final)). To each reaction, 10 μl of H₂O containing 2 μg of GST-S6 fusion protein (32), 5 μCi of [γ-^32P]ATP (4500 Ci/mmol) (ICN, Irvine, CA) and 50 μM ATP were added. Reactions were vortexed briefly and incubated for 5 min at 30 °C, followed by vortexing and incubating at 30 °C for another 5 min. Reactions were stopped by the addition of 6 μl of 6× Laemmli sample buffer. Proteins were resolved by 10% SDS-PAGE. Gels were dried, exposed to Kodak X-OMAT AR film, and quantified using a Kodak Image Station 440.

Phosphatidylinositol 3-Kinase (PI3K) Assays—Following treatment, bLCs were washed twice in ice-cold phosphate-buffered saline, lysed, and PI3K assays were performed essentially as previously described (31). Reaction products were separated by TLC, scraped from the plates, and quantified by scintillation counting.

7-Methyl-guanosine (m7G) Pulldown Assays—Following treatment, bLCs were washed twice in ice-cold phosphate-buffered saline followed by lysis in buffer B (150 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml apronitin). The lysate was pre cleared by tumbling for 1 h at 4 °C with 40 μl of washed protein A-Sepharose beads. Aliquots of lysate (500 μg) were added to a 20-μl packed volume of 7-methyl-guanosine (m7G)-conjugated Sepharose (Amersham Biosciences). Samples were tumbled at 4 °C for 18 h followed by three washes in 500 μl of buffer B. Samples were boiled in 20 μl of 2× Laemmli sample buffer, separated on 12% SDS-PAGE gels, and Western blot analysis was performed to determine co-precipitating 4EBP1, eIF4E, and eIF4G proteins.

32P and 35S Metabolic Labeling—For 32P metabolic labeling studies, bLCs (2.5 × 10⁶) were incubated in PO₄-free Dulbecco’s modified Eagle’s medium (Invitrogen) for 1 h followed by a prelabeling period of 2 h in the presence of 1 mCi/ml [32P]HPO₄ (ICN, Irvine, CA). The cells were pretreated for 30 min with or without rapamycin (20 μM) prior to 15 min of treatment with control media, PGF2α or FBS. Immunoprecipitation with Ab for either 4EBP1 (1:100) or S6K1 (1:100) was performed as described above. For 35S metabolic labeling studies, bLCs (2.5 × 10⁶) were incubated for 2 h in 1 ml of cysteine/methionine-free Dulbecco’s modified Eagle’s medium (Invitrogen) followed by a prelabeling period of 2 h with 1 mCi/ml [35S]Cys/Met Trans-label (ICN, Irvine, CA). The cells were pretreated for 15 min with or without rapamycin (20 μM) prior to treatment for 240 min with control media, PGF2α, or FBS. Ribosomal protein S6 was immunoprecipitated (1:100), radioactive products were separated by SDS-PAGE and exposed to x-ray film.

RESULTS

PGF2α Stimulates Time- and Concentration-dependent Phosphorylation of S6K1—We observed a rapid induction of S6K1 phosphorylation on Thr⁴²¹/Ser⁴²⁴ and Thr⁴³⁹ residues (Fig. 1A) in response to a maximally effective concentration of PGF2α (1 μM). Maximal phosphorylation (comparable to 15-min treatment with 10% FCS as a positive control) was observed at each site in as little as 5–15 min. Phosphorylation on Thr⁴²¹/Ser⁴²⁴ and Thr⁴³⁹ residues of S6K1 persisted beyond 90 min, albeit to a lesser extent. The temporal pattern of S6K1 phosphorylation at Thr⁴²¹/Ser⁴²⁴ was similar to the temporal pattern observed for Erk1/2 phosphorylation (Fig. 1A). Maximal phosphorylation of Erk1/2 and S6K1 (Thr⁴²¹/Ser⁴²⁴) was observed within 2–5 min following PGF2α treatment, whereas maximal phosphorylation of S6K1 (Thr⁴³⁹) was observed only after 15–30 min of treatment.

Unlike treatment with FCS, treatment bLCs with PGF2α did not induce phosphorylation of AKT (Fig. 1A). Furthermore, treatment of bLCs for 0–60 min with PGF2α (1 μM) did not result in an increase in PI3K activity (data not shown). Similar to previous observations (31) treatment of bLCs for 15 min with
IGF-I (50 ng/ml) stimulated increases (4-fold, n = 3) in PI3K activity.

Treatment of bLCs for 15 min with increasing concentrations of PGF2α (0–1 μM) (Fig. 1B) induced concentration-dependent phosphorylation of S6K1 on Thr421/Ser424 and Thr389 residues, and maximal phosphorylation was observed with 10–100 nM PGF2α. As little as 1 nM PGF2α was sufficient to stimulate S6K1 phosphorylation on Thr421/Ser424 and Thr389 residues. PGF2α stimulated a similar concentration-response pattern of Erk1/2 phosphorylation (Fig. 1B). The phosphorylation of AKT was not observed following treatment with any concentration of PGF2α (not shown).

PGF2α Stimulates S6K1 Activity—Immune complex protein kinase assays were performed to determine S6K1 enzymatic activity in response to PGF2α treatment (Fig. 1C). The time course of S6K1 activity in response to treatment with PGF2α (1 μM) is shown as an individual experiment (Fig. 1C) and as a summary of three experiments in Fig. 1D. A 2.5-fold (p < 0.05, n = 3) increase in S6K1 activity was observed following 5 min of PGF2α treatment. Maximal increases in S6K1 activity were observed within 15 min of treatment with PGF2α and S6K1 activity returned to basal levels after 60 min of treatment. Treatment of bLCs with FCS (10%, 15 min) also increased S6K1 activity, which was consistently greater than S6K1 activity observed in response to treatment with PGF2α (Fig. 1E).

RESULTS

PGF2α-stimulated S6K1 Phosphorylation and Activation Are Rapamycin-sensitive—Experiments were conducted to determine if S6K1 phosphorylation in response to PGF2α (1 μM), PMA (20 nM, a PKC activator), and FCS (10%) was sensitive to the mTOR inhibitor rapamycin. Pretreatment with rapamycin abrogated phosphorylation at the Thr389 site (Fig. 1F). However, phosphorylation of S6K1 on Thr421/Ser424 residues was only partially reduced by rapamycin pretreatment. The mobility of S6K1 in SDS-PAGE gels, as observed with the phospho-S6K1(Thr421/Ser424) antibody, was increased by the presence of rapamycin. Each of the three treatments stimulated a robust response in terms of Erk1/2 phosphorylation, but rapamycin did not reduce their stimulatory effect on Erk1/2 phosphorylation (Fig. 1F). Furthermore, treatment with rapamycin did not reduce the phosphorylation of AKT in response to FCS (Fig. 1F).

As determined in immune complex protein kinase assays (Fig. 1G), levels of S6K1 activity in response to both PMA and PGF2α were comparable, with a level of activation exceeding 4-fold (p < 0.05) over untreated control levels. Stimulation of S6K1 activity by each of the agents tested was completely sensitive to rapamycin pretreatment. Additionally, rapamycin treatment increased the mobility of immunoprecipitated S6K1, consistent with the reduced phosphorylation of S6K1 shown in Fig. 1F.
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**FIGURE 2.** PGF2α treatment promotes time-dependent and concentration-dependent phosphorylation of the ribosomal protein S6 by a rapamycin-sensitive process. A, bLCs were treated with 1 μM PGF2α for up to 60 min. B, bLCs were treated for 15 min with increasing concentrations of PGF2α (0.01 nM to 1 μM). C, bLCs were treated with control media or rapamycin (20 nM) for 60 min prior to treatment for 15 min with control media (CTL), FCS (10%), PMA (20 nM), or PGF2α (1 μM). Western blot analysis was performed using phosphospecific S6 Ab (Ser235/236; 1:5000) and polyclonal S6 Ab (dilution 1:1000). Results are representative of three experiments.

**FIGURE 3.** PGF2α stimulates the phosphorylation and activation of S6K1 by a MEK1-sensitive process. A, bLCs were pretreated with control media or the MEK1 inhibitor U0126 (30 μM) for 30 min prior to treatment for 15 min with control media (CTL) or PGF2α (1 μM). Western blot analysis was performed to determine phosphorylation of S6K1 and Erk as in Fig. 1 and phospho-S6 as in Fig. 2. B, bLCs were pretreated with control media or U0126 (10 μM) for 30 min prior to treatment for 15 min with control media (CTL) or PGF2α (1 μM). S6K1 immunocomplex (IP) protein kinase assays were performed using GST-S6 as substrate as in Fig. 1 (mean ± S.E., n = 3).

**PGF2α-stimulated S6 Phosphorylation Is Rapamycin-sensitive**—To further confirm the activation of S6K1 in primary bLC cultures, immunoblot analysis was performed using a phospho-specific S6 Ab. Treatment with PGF2α induced time (Fig. 2A) and concentration (Fig. 2B)-dependent increases in the phosphorylation of S6 comparable to those observed for S6K1 activation. S6 protein levels were unchanged by these treatments (Fig. 2, A and B). Treatment with the PKC activator PMA (20 nM) and FCS (10%) also increased the phosphorylation of S6 (Fig. 2C), and pretreatment with rapamycin blocked the stimulatory actions of FCS, PMA, or PGF2α on S6 phosphorylation.

**PGF2α-stimulated S6K1 Activation Is Sensitive to the MEK1 Inhibitor U0126**—Because PGF2α increases Erk signaling, but does not induce AKT phosphorylation (Fig. 1), experiments were conducted to determine if PGF2α-stimulated S6K1 phosphorylation was sensitive to the MEK1 inhibitor U0126. Pretreatment for 30 min with U0126 (30 μM) inhibited the stimulatory actions of PGF2α on Erk1/2 phosphorylation and on S6K1 phosphorylation on Thr231/Ser235 and Thr389 residues (Fig. 3A). Treatment with U0126 (10 μM) resulted in a 60% decrease in S6K1 activity (p < 0.05, n = 3) (Fig. 3B) which correlated with the inhibition of S6 phosphorylation (Fig. 3A). Similar results were observed when bLCs were treated with the MEK1 inhibitor PD98059. To further verify the specificity of the response to U0126, we performed Western blot analysis with phosphospecific antibodies for p38 MAPK, Jun-N-terminal kinase (JNK) and Erk5 in bLCs treated with PGF2α (1 μM) or epidermal growth factor (EGF, 10 ng/ml) as a positive control. Treatment with U0126 (10 μM) did not inhibit the stimulatory responses to either PGF2α or EGF on the phosphorylation of p38, JNK, or Erk5 (data not shown).

**PGF2α-stimulated TSC2 Phosphorylation Is Sensitive to the MEK1 Inhibitor U0126**—Treatment with PGF2α and PMA increased phosphorylation of TSC2 as observed using the RXXRxxSer motif antibody (Fig. 4A), which recognizes phosho-TSC2 (Ser1798) (29). FCS, but neither PGF2α nor PMA, increased the phosphorylation of AKT and the AKT substrate recognition site on TSC2 (Thr1462) (Fig. 4B). The increase in TSC2 phosphorylation in response to PGF2α treatment was inhibited by pretreatment with U0126 (Fig. 4C) or by overexpression of dominant negative MEK1 (Fig. 4D).

**PGF2α Stimulates mTOR-regulated Translational Responses**—To determine mTOR activity in response to PGF2α in intact cells, the mTOR substrates, 4EBP1 and S6K1, were immunoprecipitated from metabolically labeled bLCs. The levels of 32P-labeled substrates (4EBP1 and S6K1) were greater in response to PGF2α compared with the untreated control (Fig. 5A). The stimulatory actions of PGF2α and FCS on 32P incorporation into 4EBP1 and S6K1 were abrogated by pretreatment with rapamycin (20 nM).
The phosphorylation of 4EBP1 was also monitored by phosphorylation site-specific 4EBP1 antibodies (Fig. 5B). Treatment with PGF2α/H9251 and FCS for 15 min increased phosphorylation of 4EBP1 on Thr70 and Thr37/46 residues. The responses to PGF2α/H9251 and FCS were sensitive to inhibition with rapamycin treatment. Levels of 4EBP1 protein were unchanged by the treatments, but both PGF2α/H9251 and FCS reduced the mobility of 4EBP1 in SDS-PAGE, a response indicative of increased phosphorylation. The levels of eIF4E were comparable in each sample (Fig. 5B).

To determine if PGF2α treatment regulates mTOR-mediated translational responses, we used 7-methyl-guanosine (m7G)-conjugated Sepharose to pull down eIF4E-4EBP1 protein complexes (Fig. 6A). Treatment with PGF2α or FCS for 15 min stimulated a marked reduction in the level of co-precipitating 4EBP1, which was fully sensitive to treatment with rapamycin (20 nM). The levels of 4EBP1 were elevated in m7G pulldowns from rapamycin-treated versus control bLCs, suggesting that basal mTOR-regulated translation was operational in bLCs. In another set of experiments, we observed that the decrease in 4EBP1 following treatment with PGF2α or FCS was associated with an increase in eIF4G in the m7G-cap complex (Fig. 6B).

Experiments were performed to determine if a reciprocal relationship was observed between the phosphorylation status of 4EBP1 and eIF4G following treatment with rapamycin.
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The present study was performed to determine if PGF2α stimulates the translational machinery via mTOR/4EBP1/S6K1 signaling in steroidogenic luteal cells. We provide the first evidence that activation of the FP GPCR by treatment with PGF2α induces the phosphorylation of TSC2 and the activation of mTOR signaling in luteal cells. We also provide evidence that the phosphorylation of mTOR substrates (4EBP1 and S6K1) and the stimulation of the translational apparatus in response to PGF2α is a rapamycin-sensitive, mTOR-directed process. These results provide a better understanding of the intracellular signaling events that contribute to the response of the CL following treatment with PGF2α.

The PKC activator PMA mimicked the stimulatory actions of PGF2α on S6K1 activity. This finding is consistent with a PGF2α-initiated signaling mechanism in luteal cells involving binding to the FP GPCR, and subsequent activation of PKC. The S6K1 enzyme contains over 8 phosphorylation sites distributed over multiple structural domains (19, 21, 35, 36). Proline-directed protein kinases are thought to mediate the phosphorylation of Ser and Thr residues of the C-terminal pseudosubstrate domain (residues 400–436) of the S6K1 protein (37), including Erk, which is activated by a PKC-dependent mechanism in bLCs following treatment with PGF2α (10, 12, 14). These phosphorylation events are thought to promote activation of the S6K1 enzyme through a mechanism involving the release of the pseudosubstrate C terminus from the N terminus (35), which allows other protein kinases (e.g. mTOR) to phosphorylate S6K1. Indeed, we observed that interruption of PGF2α-induced Erk signaling by treatment with the MEK1 inhibitor U0126 abrogated the phosphorylation of S6K1 on Thr421/Ser424 residues, a response which was correlated with a reduction in S6K1 activity and S6 phosphorylation. Phosphorylation of S6K1 on Thr421/Ser424 residues was partially reduced in the presence of rapamycin, despite sustained elevations in Erk activity, suggesting that mTOR also contributes to the phosphorylation of these sites (35, 36). The rapamycin-insensitive phosphorylation of S6K1 on Thr421/Ser424 residues indicates that PGF2α and PMA catalyze the phosphorylation of S6K1 by protein kinases distinct from mTOR. The exact role of

FIGURE 6. PGF2α stimulates mTOR-mediated translational responses in bovine luteal cells. A and B, bLCs were pretreated with control media or rapamycin (20 nM) for 60 min prior to treatment for 15 min with control media (CTRL), FCS (10%), or PGF2α (1 μM). The eIF4E-containing complexes were precipitated using 7-mG-Sepharose (m7G) and separated on 13% SDS-PAGE gels. Western blot was performed using polyclonal Ab to detect co-precipitating 4EBP1 (dilution 1:100). B, bLCs were treated and m7G pulldown performed as in Fig. 6A. The m7G-binding complexes were also analyzed using an antibody against eIF4G (1:1000). C, bLCs were pretreated with control media or rapamycin (20 nM) for 60 min prior to treatment for 15 min with control media (CTRL), FCS (10%), or PGF2α (1 μM). 4EBP1 was immunoprecipitated, and Western blot analysis was performed to determine phosphorylation of 4EBP1 at Thr70 and Thr37/46 and levels of total 4EBP1 in the immunoprecipitates. Co-precipitating eIF4E was detected as in Fig. 6A. D, bLCs were metabolically labeled with 35S and pretreated for 15 min with or without rapamycin (20 nM) prior to treatment for 240 min with control media (CTRL), PGF2α (1 μM), or FCS (10%). The S6 protein was immunoprecipitated using a polyclonal S6 Ab (dilution 1:100). Immune complexes were separated on 10% SDS-PAGE gels, fixed, dried, and exposed to x-ray film.

of 4EBP1 and levels of eIF4E present in 4EBP1 immune complexes, thereby providing another indication of the activation of the translational apparatus in response to PGF2α. Following treatment for 15 min with PGF2α or FCS in the presence or absence of rapamycin, 4EBP1 was immunoprecipitated and the immune complexes interrogated for levels of phospho-4EBP1 and eIF4E. PGF2α and FCS increased the phosphorylation of 4EBP1. The treatments also reduced the amount of eIF4E complexed with 4EBP1 (Fig. 6C). Treatment with rapamycin inhibited the actions of PGF2α on 4EBP1 phosphorylation and prevented the dissociation of eIF4E from 4EBP1 (Fig. 6C).

The ribosomal protein S6 is a protein whose mode of translational regulation is dependent upon mTOR activation (19, 34). To determine if PGF2α could regulate S6 synthesis, we metabolically labeled luteal cells and analyzed 35S incorporation into S6 protein. Treatment with PGF2α or FCS increased the amount of 35S-labeled S6 protein recovered in S6 immune complexes (Fig. 6D) compared with controls. Metabolically labeled S6 protein was not detected in samples from rapamycin pretreated cells.

DISCUSSION

The present study was performed to determine if PGF2α stimulates the translational machinery via mTOR/4EBP1/S6K1 signaling in steroidogenic luteal cells. We provide the first evidence that activation of the FP GPCR by treatment with PGF2α induces the phosphorylation of TSC2 and the activation of mTOR signaling in luteal cells. We also provide evidence that the phosphorylation of mTOR substrates (4EBP1 and S6K1) and the stimulation of the translational apparatus in response to PGF2α is a rapamycin-sensitive, mTOR-directed process. These results provide a better understanding of the intracellular signaling events that contribute to the response of the CL following treatment with PGF2α.

The PKC activator PMA mimicked the stimulatory actions of PGF2α on S6K1 activity. This finding is consistent with a PGF2α-initiated signaling mechanism in luteal cells involving binding to the FP GPCR, and subsequent activation of PKC. The S6K1 enzyme contains over 8 phosphorylation sites distributed over multiple structural domains (19, 21, 35, 36). Proline-directed protein kinases are thought to mediate the phosphorylation of Ser and Thr residues of the C-terminal pseudosubstrate domain (residues 400–436) of the S6K1 protein (37), including Erk, which is activated by a PKC-dependent mechanism in bLCs following treatment with PGF2α (10, 12, 14). These phosphorylation events are thought to promote activation of the S6K1 enzyme through a mechanism involving the release of the pseudosubstrate C terminus from the N terminus (35), which allows other protein kinases (e.g. mTOR) to phosphorylate S6K1. Indeed, we observed that interruption of PGF2α-induced Erk signaling by treatment with the MEK1 inhibitor U0126 abrogated the phosphorylation of S6K1 on Thr421/Ser424 residues, a response which was correlated with a reduction in S6K1 activity and S6 phosphorylation. Phosphorylation of S6K1 on Thr421/Ser424 residues was partially reduced in the presence of rapamycin, despite sustained elevations in Erk activity, suggesting that mTOR also contributes to the phosphorylation of these sites (35, 36). The rapamycin-insensitive phosphorylation of S6K1 on Thr421/Ser424 residues indicates that PGF2α and PMA catalyze the phosphorylation of S6K1 by protein kinases distinct from mTOR. The exact role of
PKC, Erk and other mitogen-activated protein kinases in the phosphorylation, activation, and localization of S6K1 in bLCs is under investigation.

PGF2α treatment also stimulated the phosphorylation of S6K1 on Thr^{389}, a residue located just C-terminal of the catalytic domain. It is well established that mTOR phosphorylates the Thr^{389} residue on the S6K1 protein and that Thr^{389} phosphorylation is prevented by pretreatment of cells with rapamycin (35, 36). Mutagenesis experiments revealed that phosphorylation of the Thr^{389} residue is required for the activation of S6K1 (15), indicating that stimulation of S6K1 activity is mediated principally by a raptor/mTOR-catalyzed phosphorylation event (38). Erk and AKT signaling can stimulate mTOR by inhibiting the activity of the tumor suppressor protein TSC2 (tuberin), which represses mTOR/S6K1 signaling (39). Recent reports show that in response to PMA treatment, MEK1 (28), Erk (40) or the Erk effector p90 RSK1 (29) mediates the phosphorylation of TSC2 on Ser^{1798} and the resultant activation of mTOR/S6K1 signaling in HEK293 cells. Using an antibody which recognizes phospho-TSC2(Ser^{1798}) residues (29), the present studies provide the initial evidence that the activation of a GPCR (the FP receptor) in primary cells leads to Erk-dependent phosphorylation of TSC2. Importantly, treatment with the MEK1 inhibitor U0126 or overexpression of dominant negative MEK1 effectively inhibited PGF2α-stimulated phosphorylation of TSC2. The MEK1 inhibitor also prevented PGF2α-stimulated phosphorylation of S6K1(Thr^{389}). Thus, MEK1/Erk signaling in response to PGF2α may contribute to S6K1 activation directly by the phosphorylation of C-terminal sites, and indirectly by phosphorylation of TSC2, thereby repressing the TSC2 inhibitory constraints on mTOR activation. The actions of PGF2α and PMA in bLCs were independent of increases in PI3K/AKT signaling because PGF2α and PMA did not increase PI3K activity or AKT phosphorylation, nor did they significantly increase phosphorylation of TSC2 on the Thr^{1462} residue, a recognized AKT phosphorylation site (29). The ability of FCS to phosphorylate AKT and TSC2(Thr^{1462}), in addition to Erk and TSC2(Ser^{1798}), may contribute to the greater S6K1 activity observed in response to FCS as compared with PGF2α.

The protein kinase mTOR is a key regulator of translation in mammalian cells that acts to stimulate protein synthesis by phosphorylation of S6K1 and 4EBP1 (15). Using metabolically labeled cells, we demonstrated that PGF2α stimulates the phosphorylation of both S6K1 and 4EBP1. These studies also provide the first evidence that PGF2α stimulates the phosphorylation of the S6K1 substrate ribosomal protein S6, an event which has been shown to correlate well with increased translation of mRNAs containing a short tract of polyuridymines (4–14 nucleotides) immediately downstream of the 5’-cap (19). These mRNAs encode proteins that are components of the translation machinery, which includes ribosomal proteins, such as the ribosomal protein S6 and several other translation factors. Furthermore, PGF2α stimulated the synthesis of S6 protein in bLCs as evidenced by increased amounts of radioactive ribosomal protein S6 from ^35S pulse-labeled bLCs. These data support our idea that PGF2α activates the translational capacity of the luteal cell. Further experiments are required to determine the subset of mRNAs that are regulated by mTOR/S6K1-dependent signaling in the luteal cell.

Translation-initiating ribosome assembly at m^7G cap elements is mediated by a multiprotein complex (consisting of elf4A (RNA helicase), elf4E (cap-binding protein), and the scaffold elf4G) of which the initiation factor elf4E is a major constituent (15). Under quiescent conditions elf4E, which contains the m^7G cap binding domain, is negatively regulated by translational repressor 4EBP1. When activated, mTOR phosphorylates the 4EBP1 protein, an event which promotes dissociation of the 4EBP1-elf4E complex. As a result, elf4E is available to complex with other initiation factors to yield an active translation initiation complex (20). Three independent observations in the present study support our suggestion that PGF2α regulates the mTOR-dependent translational machinery in bLCs. First, treatment of luteal cells with PGF2α resulted in the phosphorylation of 4EBP1, a response which was abrogated by pretreatment with rapamycin. Second, m^7G-Sepharose pulldown assays provided evidence that PGF2α promotes dissociation of the 4EBP1-elf4E complex and the association of elf4G with elf4E. The release of 4EBP1 and association of elf4G with elf4E was also sensitive to rapamycin treatment, highlighting the requirement for mTOR activity in these processes. Third, we demonstrated a reciprocal relationship between the phosphorylation status of 4EBP1 and levels of elf4E present in 4EBP1 immune complexes. Other studies have shown that the RNA-binding protein elf4B is phosphorylated on Ser^{422} by S6K1 in a step that promotes its association with elf4A (41). The RNA helicase and ATPase activities of elf4A are specifically enhanced by association with elf4B. Because of its function in enhancing elf4A-mediated unwinding of RNA secondary structure, it has been suggested that elf4B phosphorylation by S6K1 may augment translation of mRNAs containing higher degrees of secondary structure at their 5’ terminus (42). More recently, Holz et al. (43) reported that mTOR/S6K1 interact with the elf3 translation preinitiation complex to regulate the phosphorylation of 4E-BP1, S6, and elf4B. Taken together, these results suggest that PGF2α-stimulated mTOR signaling may regulate multiple components of the translational initiation complex in luteal cells to promote the flow of information from genes to proteins.

mTOR is an evolutionarily conserved protein kinase that mediates cell growth and cell proliferation. The best characterized downstream targets of mTOR, S6K1, and 4EBP1, contain a TOS signaling motif which functions to receive mTOR signals and disruption of this motif mimics the effects of rapamycin on their phosphorylation and reduces cell growth (44). The induction of mTOR/4EBP1/S6K1 activity by PGF2α would seem counter-intuitive because the luteal cell is a terminally differentiated, non-dividing cell. However, luteal cells, in the course of cellular differentiation in vivo and in vitro, undergo a progressive hypertrophy (4, 45, 46). Given the selective induction of the FP receptor in ovarian cells following ovulation and the high levels of prostaglandins present during early CL development (1, 2, 47), it is plausible that mTOR/4EBP1/S6K1 signaling participates in the hypertrophy/differentiation of the luteal cell. In keeping with this theme, Alam et al. (48) recently suggested that gonadotropin stimulated mTOR signaling may play a role in
the differentiation of rat granulosa cells to the large luteal cell type. Further support for this argument is derived from studies demonstrating that PGF2α treatment induces hypertrophy of cultured cardiac myocytes (49). By analogy it is possible that other agents that activate mTOR via Erk signaling (for example phenylephrine, another potent hypertrophic agent in cardiac myocytes (50)) do so without stimulating the PI3K/akt pathway. These observations may have important implications for other FP target tissues such as the myometrium (51), eye (52), and bone (11).

During corpus luteum regression, the mTOR/S6K1 signaling may negatively influence the activity of survival signaling pathways. Survival of ovarian cells has been reported to be at least partially dependent upon a functional PI3K signaling pathway (53, 54). Activation of mTOR/S6K1 signaling has been shown to suppress growth factor-dependent PI3K/akt signaling through the phosphorylation and subsequent degradation of insulin receptor substrate proteins (55–58). At the end of the luteal phase, the ability of PGF2α to activate mTOR/S6K1 (without a requirement for PI3K activation) suggests a mode of action by which PGF2α could compromise survival in the luteal cell by reducing PI3K/akt signals and render the luteal cell susceptible to the actions of cytotoxic cytokines. This idea is supported by observations that PGF2α can reduce PI3K activity in HEK-293 cells expressing a FPB, a FP splice variant characterized by truncation of the last 46 amino acids of the C-terminal domain (59). The presence and/or activity of specific FP splice variants (60, 61) in the developing CL may influence the activity of survival signaling pathways and putative downstream processes involving the translational apparatus (52, 53).

Summary—The present experiments provide the first demonstration that PGF2α activates the mTOR/4EBP1/S6K1 signaling pathway in luteal cells. This process required an active Erk but not akt signaling pathway. The activation of mTOR and putative downstream processes involving the translational apparatus (i.e. 4EBP1 phosphorylation, release of 4EBP1 binding in m7G cap binding assays, phosphorylation of S6K1, and phosphorylation and synthesis of S6) were completely sensitive to treatment with rapamycin, strongly implicating mTOR in the actions of PGF2α. These studies provide the foundation for future studies to determine the role of mTOR signaling in CL development and regression. Moreover, these studies offer new approaches for disruption of specific actions of PGF2α in tissues expressing FP since clinical trials with various derivatives of rapamycin are underway.

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