

Activation of Enteroendocrine Membrane Progesterone Receptors Promotes Incretin Secretion and Improves Glucose Tolerance in Mice

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Glucagon-like peptide-1 (GLP-1) secretion is classically regulated by ingested nutrients. To identify novel molecular targets controlling incretin secretion, we analyzed enteroendocrine cell pathways important for hormone biosynthesis and secretion. We demonstrate that progesterone increases GLP-1 secretion and extracellular signal-related kinase 1/2 (ERK1/2) phosphorylation in enteroendocrine GLUTag cells via mechanisms sensitive to the mitogen-activated protein kinase inhibitor U0126. The stimulatory effects of progesterone (P4) or the synthetic progestin R5020 on ERK1/2 phosphorylation were independent of the classical progesterone receptor antagonist RU486. Furthermore, a cell-impermeable BSA-progesterone conjugate rapidly increased ERK1/2 phosphorylation and GLP-1 secretion. Knockdown of the membrane progesterone receptors Paqr5 or Paqr7 in GLUTag cells eliminated the stimulatory effect of R5020 and progesterone on GLP-1 secretion. Enteral progesterone administration increased plasma levels of GLP-1, glucose-dependent insulinotropic polypeptide (GIP), and insulin, and improved oral glucose tolerance in an RU486-insensitive manner in mice; however, systemic progesterone exposure did not improve glucose homeostasis. Unexpectedly, the glucoregulatory actions of enteral progesterone did not require classical incretin receptor signaling and were preserved in *Glp1r*^{-/-} and *Glp1r*^{-/-}:*Gipr*^{-/-} mice. Intestine-restricted activation of membrane progesterone receptors may represent a novel approach for stimulation of incretin hormone secretion and control of glucose homeostasis. *Diabetes* 62:283–290, 2013

Glucagon, a proglucagon-derived peptide (PGDP) secreted from islet α -cells, is a 29-amino acid peptide hormone that plays a key role in the control of blood glucose via regulation of hepatic glucose production (1). Glucagon excess in the setting of insulin deficiency or resistance contributes to the pathophysiology of diabetes mellitus, whereas defective glucagon secretion leads to an increased risk of hypoglycemia in diabetic subjects. Conversely, glucagon-like peptide-1 (GLP-1), a 30-amino acid PGDP secreted from gut endocrine cells regulates glucose homeostasis by augmenting β -cell and inhibiting α -cell function (2). Hence, understanding the control of PGDP biosynthesis and secretion in health and disease has direct relevance for the treatment of diabetes mellitus.

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Experimental approaches for studying enteroendocrine L cells have traditionally used primary intestinal cell cultures *in vitro* (3,4); however, L cells often represent <1% of the cells in the culture system. Hence, immortalized endocrine cell lines are commonly employed for analysis of intestinal proglucagon biosynthesis and gene transcription (4–7). More recently, targeting of reporter genes to murine enteroendocrine cells has facilitated isolation and analysis of purified populations of L cells and K cells, enabling more detailed molecular characterization of native enteroendocrine cell populations (4,8). Taken together, the use of primary cultures, cell lines, and purified endocrine cell populations examined *ex vivo* has greatly enhanced our knowledge of pathways regulating incretin hormone secretion.

Although islet α -cells and enteroendocrine L cells exhibit nutrient-sensitive regulation of proglucagon gene expression and PGDP secretion, the mechanisms controlling proglucagon gene transcription and PGDP secretion in these two cell types have diverged. Nutrients and insulin inhibit islet proglucagon gene expression and glucagon secretion (9) yet stimulate proglucagon gene expression and enhance PGDP secretion from gut endocrine cells in both animals and isolated cell cultures (10–13). To identify novel mechanisms regulating proglucagon biosynthesis and PGDP secretion, we carried out gene expression profiling of islet α TC-1 and enteroendocrine GLUTag cells (14,15) and identified a series of ion channels differentially expressed in GLUTag versus α TC-1 cells (15).

We demonstrate in this study that intestinal GLUTag cells express a functional progesterone receptor (PR). Progesterone increased levels of proglucagon mRNA transcripts and directly increased GLP-1 secretion from GLUTag cells *in vitro*. The progesterone-dependent stimulation of GLP-1 secretion was rapid, associated with extracellular signal-related kinase 1/2 (ERK1/2) activation, and insensitive to the classical progesterone antagonist RU486. Furthermore, a BSA-progesterone (P4) cell-impermeable conjugate retained the ability to activate ERK1/2 and stimulate GLP-1 secretion, and knockdown of membrane PRs in GLUTag cells eliminated the stimulatory effects of progestins on GLP-1 secretion. Furthermore, enteral but not parenteral P4 administration improved glucose tolerance and enhanced circulating levels of GLP-1 in mice. These findings identify novel intestinal targets for activating incretin hormone secretion, enabling development of ligands that target intestinal membrane PRs independent of absorption and systemic exposure.

RESEARCH DESIGN AND METHODS

Tissue-culture medium was from Hyclone (Logan, UT), and FCS was from Invitrogen Life Technologies (Burlington, Ontario, Canada). Antibiotics, chemicals, proteinase inhibitor cocktail, phosphatase inhibitors, BSA free of fatty acids

(BSA-FA), and Tri reagent were from Sigma Chemical Co. (St. Louis, MO). Forskolin, P4, and RU486 were from Sigma Chemical Co. R5020 and BSA-P4 were from Steraloids Inc. (Newport, RI). Kinase inhibitors were from Calbiochem (Billerica, MA). Insulin (Humulin R) was from Eli Lilly (Toronto, Ontario, Canada). Antibodies, unless specified otherwise, were from Cell Signaling Technology (Beverly, MA). Small interfering RNAs (siRNAs) were from Applied Biosystems (Carlsbad, CA). Ultrasensitive mouse insulin ELISA was from Alpco Diagnostics, (Salem, NH). Mouse/rat total GLP-1 assay kit was from Mesoscale Discovery (Gaithersburg, MD). Rat/mouse total gastrointestinal (gastric inhibitory) polypeptide glucose-dependent insulinotropic polypeptide (GIP) ELISA kit was from Millipore (Billerica, MA). An enzyme-linked immunosorbent assay kit from Neogen (Lexington, KY) was used to measure progesterone plasma levels.

Cell-culture and microarray experiments. Microarray experiments were carried out using RNA from islet α TC1 (16), enteroendocrine GLUTag (17), and simian virus (SV) 40-transformed mouse fibroblast SVT2 cell lines (18) using the Affymetrix MG_U74Av2 Gene Chip (Affymetrix). Microarray analysis and expression quantification was performed by guanine cytosine robust multi-array analysis from Bioconductor (<http://www.bioconductor.org>) as described previously (14,15). GLUTag cells were maintained, unless otherwise specified, in Dulbecco's modified Eagle's medium (DMEM; 4.5 g glucose/L) supplemented with 10% FCS.

RNA isolation and analysis. For RT-PCR, first-strand cDNA synthesis was generated from total RNA isolated from jejunum and colon of age-matched males and females C57BL/6 mice and from islet α TC1, GLUTag, and SVT2 cell lines using the SuperScript Preamplification System from Invitrogen. Target cDNA was amplified using specific oligonucleotide forward-reverse primer pairs, and PCR products transferred to membranes were probed with an internal 32 P-labeled oligonucleotide. Primers and probes sequences are provided in Supplementary Table 1. Quantitative PCR (qPCR) was performed using TaqMan assays on demand and ABI PRISM 7900HT (PE Applied Biosystems, Foster City, CA) for proglucagon (Mm00801712); PR (Mm01176082); progesterin and adipoQ receptor family member V (Paqr5, Mm01170057); and progesterin and adipoQ receptor family member VII (Paqr7, Mm00910958). For house-keeping genes, tubulin (Mm00846967), 18S (Hs99999901), and glyceraldehyde-3-phosphate dehydrogenase (Mm99999915) were analyzed.

Cell transfection. GLUTag cells were grown to 80% confluence in DMEM and transfected with murine mammary tumor virus (MMTV)-Luc (the MMTV Long Terminal Repeat promoter is a response to steroid hormones and used as a positive control for detection of functional PR activity) using Fugene (Roche Applied Science, Quebec, Canada) or Lipofectamine (Invitrogen), following the manufacturer's protocol. Twenty-four hours after transfection, the media was replaced with DMEM supplemented with 0.5% FCS, and 12 h later, cells were treated overnight with R5020 (20 nmol/L), P4 (20 nmol/L), BSA-P4 conjugate (BSA-P4; 20 nmol/L of P4), forskolin (20 μ mol/L), dexamethasone (10^{-7} mol), DMSO, or ethanol. Cells were then harvested for analysis of luciferase activity as described previously (19,20). Luciferase values were normalized to total protein content determined by a BCA protein assay kit (Pierce, Rockford, IL).

For knockdown of RNA transcripts, GLUTag cells were grown to 50% cell density and siRNA was transfected for 48 h using lipofectamine RNAiMAX (Invitrogen) following the manufacturer's protocol. siRNAs included the nuclear PR (si-PR) (s71548 plus s71549), the progesterin and adipoQ receptor family member V (si-Paqr5) (s92240), the progesterin and adipoQ receptor family member VII (163230) (silencer select pre-designed siRNA, Invitrogen-Applied Biosystems) and the negative control (si-Neg. Ctrl.). Efficiency of RNA knockdown was assessed by qPCR. Concomitant quantification of glyceraldehyde-3-phosphate dehydrogenase mRNA transcripts was used to normalize mRNA transcript levels.

Signal transduction studies. GLUTag cells were incubated in DMEM high glucose supplemented with 10% FCS and 12 h before treatment, cells were washed twice with PBS and media was replaced with DMEM high glucose supplemented with 0.5% FCS. The day of the experiment, cells were washed twice with PBS and incubated in DMEM high glucose without FCS for 2 h followed by treatment with the progesterin R5020 (20 nmol/L), progesterone (P4) (20 nmol/L), BSA-P4 (20 nmol/L of progesterone), forskolin (20 μ mol/L) or ethanol (vehicle control). Whole cell extracts were prepared in radioimmunoprecipitation assay buffer (RIPA) (Tris 50 mmol/L, NaCl 150 mmol/L, SDS 0.1%, Na-Deoxycholate 0.5%, Triton \times 100 1%) containing proteinase inhibitor cocktail and phosphatase inhibitor. Protein content was measured by BCA protein assay kit (Pierce) and 40 μ g of total protein was separated by electrophoresis in a 10% SDS-PAGE gel. Following transfer to a nylon membrane, blots were probed with antisera directed against P-Creb (Ser133) or Phospho-P44/42 mitogen-activated protein kinase (MAPK). Anti-heat shock protein 90 (HSP90) was used to monitor loading and transfer conditions. In separate experiments, cells were pretreated for 30 min with U0126 (10 μ mol) or for 15 min with the classical nuclear PR antagonist RU486 (1 μ mol) followed by R5020 (20 nmol/L), P4 (20 nmol/L), epidermal growth factor (25 ng/mL), or vehicle.

cAMP was measured in dried aliquots of ethanol cell extracts from GLUTag cells incubated as described above and treated for 30 or 60 min with R5020 (20 nmol/L) or forskolin (100 μ mol) using a cAMP radioimmunoassay kit (Biomedical Technologies, Stoughton, MA). Densitometry analysis was done with a Kodak image station 4000 MM PRO (Kodak), and values were normalized by HSP90.

GLP-1 secretion from GLUTag cells. GLUTag cells grown to 80% confluence in DMEM with 10% FCS were washed with PBS and preincubated for 2 h in DMEM without FCS. Media was replaced with fresh DMEM high glucose (4.5 g glucose/L) without FCS supplemented with vehicle (ethanol), R5020 (20 nmol/L), P4 (20 nmol/L), or BSA-P4 (20 nmol/L of P4 equivalent) for 2 h. Media and cell extracts were collected in the presence of proteinase inhibitor cocktail, and GLP-1 levels were measured using the total GLP-1 radioimmunoassay (Millipore) or the total GLP-1 assay kit (Mesoscale Discovery, Gaithersburg, MD). Total GLP-1 secreted into the media was normalized to total protein content. **Animal experiments.** Mouse experiments were carried out according to protocols approved by Mt. Sinai Hospital and the Toronto Centre for Phenogenomics Animal Care Committees.

Glucose tolerance tests. Age-matched C57BL/6, *Glpl1r^{-/-}*, and double incretin receptor knockout (DIRKO) male mice (8–10 weeks old) were fasted for 4 h. On day 1 mice received a single oral dose of vehicle (100 μ l of 80% PEG400, 10% Tween 80, 10% ethanol) 15 min prior to oral glucose tolerance testing (OGTT) (1.5 g/kg body weight). Four days later, the same mice received a single oral dose of P4 (100 μ g in 100 μ L/mouse) 15 min prior to OGTT. In a separate experiment, mice were given a single oral dose of either vehicle (100 μ L of 80% PEG400, 10% Tween 80, and 10% ethanol) or the classical nuclear progesterone receptor antagonist RU486 (100 mg/kg administered in \sim 90–110 μ L) as described (21). Sixty minutes later, mice received a single oral dose of vehicle (80% PEG400, 10% Tween 80, and 10% ethanol) (day 1) or P4 (100 μ g/mouse) (day 2) followed by an oral glucose load (1.5 g/kg body weight) 15 min later. Blood glucose levels were measured by sampling from the tail vein of gently held conscious mice from 5–90 min after glucose administration. In all experiments, at the 5-min time point following glucose administration, a blood sample (150 μ L) was collected and immediately mixed with 15 μ L of a chilled solution containing 5000 kIU/mL Trasylol (Bayer, Toronto, Ontario, Canada), 32 mmol/L EDTA, and 0.01 mmol/L Diprotin A (Sigma Chemical Co.) for measurement of insulin, total GLP-1, and total GIP levels. Using a separate set of DIRKO mice (22), plasma insulin levels were assessed in blood samples collected 10, 15, and 20 min following oral glucose administration. For analysis of intraperitoneal progesterone action, mice were fasted for 4 h and were given a single intraperitoneal dose of either vehicle (corn oil) (day 1) or P4 (100 μ g, day 2) 15 min prior to oral glucose load.

Intestinal proglucagon expression. Ten-week-old C57BL/6 male mice were given regular chow supplemented with P4 or vehicle (corn oil) for 48 h. Briefly, P4 was dissolved in corn oil, and the chow was then mixed with corn oil/P4 to obtain a diet of uniform consistency. The daily estimated dose of P4 was 10 mg/day/mouse. Mice were then killed, and jejunum and colon were cleaned with cold PBS and collected for RNA preparation. Real-time PCR was performed as described above using the assay on demand for proglucagon (Mm00801712; Applied Biosystems). Blood was collected by cardiac puncture and immediately mixed with a chilled solution containing 5000 kIU/mL Trasylol (Bayer, Toronto, Ontario, Canada), 32 mmol/L EDTA, and 0.01 mmol/L Diprotin A (Sigma Chemical Co.). Plasma was obtained by centrifugation at 4°C and stored at -80° C until determination of total GLP-1 and progesterone levels.

Gastric emptying. Liquid-phase gastric emptying was assessed using the acetaminophen absorption test (23,24). C57BL/6 and DIRKO male mice, 10–12 weeks of age, were fasted for 4 h and given a single dose of either vehicle (V; 80% PEG400, 10% Tween 80, and 10% ethanol; day 1) or P4 (100 μ g/mouse; day 2) 15 min before oral administration of a solution of glucose 15% and acetaminophen 1% (Sigma Chemical Co.) at a dose of 1.5 g/kg glucose to 0.1 g/kg acetaminophen. Tail vein blood (50 μ L) was collected into heparinized tubes at 15 and 30 min after glucose/acetaminophen administration. Plasma was separated by centrifugation at 4°C and stored at -20° C until measurement of acetaminophen levels using an enzymatic-spectrophotometric assay (Diagnostic Chemicals Ltd., Oxford, CT).

Insulin tolerance test. Ten-week-old age-matched C57BL/6 and DIRKO male mice were fasted for 5 h. A single oral dose of vehicle (V; 80% PEG400, 10% Tween 80, 10% ethanol) (day 1) and P4 (100 μ g/mouse) (day 2) was given 15 min prior to the administration of 1.2 U/kg of insulin (Humulin R; Eli Lilly, Ontario, Canada). Blood glucose was determined at 0, 15, 30, 60, 120, and 180 min.

Statistical analysis. Statistical significance was assessed by one-way ANOVA using Bonferroni multiple comparison post hoc test and, where appropriate, by paired Student *t* test using GraphPad Prism 4 (GraphPad Software, San Diego, CA). A *P* value <0.05 was considered to be statistically significant.

RESULTS

Differential gene expression in glucagon-producing cell lines. To identify novel pathways in enteroendocrine cells coupled to control of GLP-1 secretion, we searched for genes with functional activity potentially coupled to hormone synthesis or secretion differentially expressed in enteroendocrine cells. We identified several mRNA transcripts preferentially expressed in GLUTag versus α TC1 cells (Fig. 1A). For example, the transcription factors *Cdx2*, *Uncx4.1*, and *Tal1*, mRNA transcripts for the ion channel *Kcnd2*, the glutamate receptor *Grik1*, the bombesin-like receptor *Brs3*, and the protein tyrosine phosphatase *Ptpn5* were preferentially expressed in RNA from GLUTag cells. Unexpectedly, we detected robust expression of the PR in GLUTag but not α TC1 cells (Fig. 1A).

As sex steroids have not previously been implicated in the control of GLP-1 secretion (25), we assessed whether progesterone activated proglucagon gene expression or GLP-1 secretion in GLUTag cells. To first ascertain whether the GLUTag PR mRNA transcript encodes a transcriptionally competent PR, cells were transfected with the MMTV-Luc reporter plasmid and treated with the synthetic progestin R5020 or the glucocorticoid dexamethasone. R5020 and, to a lesser extent, dexamethasone significantly increased MMTV-Luc activity in GLUTag cells (Fig. 1B). In contrast, R5020 had no effect on MMTV-Luc activity in

α TC1 cells; however, dexamethasone robustly induced MMTV-Luc activity in the same experiments (Fig. 1C). R5020 also increased levels of proglucagon mRNA transcripts in GLUTag cells (Fig. 1D); however, enteral P4 administration for 2 days had no effect on intestinal proglucagon mRNA transcripts in the mouse jejunum or colon (Fig. 1E). Plasma P4 levels were significantly increased, and levels of total circulating GLP-1 trended higher after chronic enteral P4 administration (Fig. 1F).

As P4 may regulate hormone secretion independent of changes in gene expression, we examined GLP-1 secretion in enteroendocrine cells. R5020 significantly stimulated GLP-1 secretion from GLUTag cells (Fig. 2A). Forskolin, a potent activator of adenylate cyclase and classical activator of GLP-1 secretion in L cells (3,6), increased both GLP-1 secretion (Fig. 2A) and produced a sustained activation of cAMP-responsive element-binding protein (CREB) phosphorylation (Fig. 2B). In contrast, R5020 rapidly but transiently increased levels of phospho-CREB at 5 min, followed by a secondary increase at 30–60 min (Fig. 2B). R5020 also increased ERK1/2 (Fig. 2C) but not AKT phosphorylation (data not shown). Furthermore, the ERK1/2 inhibitor UO126 abolished the stimulatory effect of R5020 on GLP-1 secretion (Fig. 2D). Unlike forskolin, R5020 had no effect on cyclic AMP accumulation in GLUTag cells (Fig. 2E).

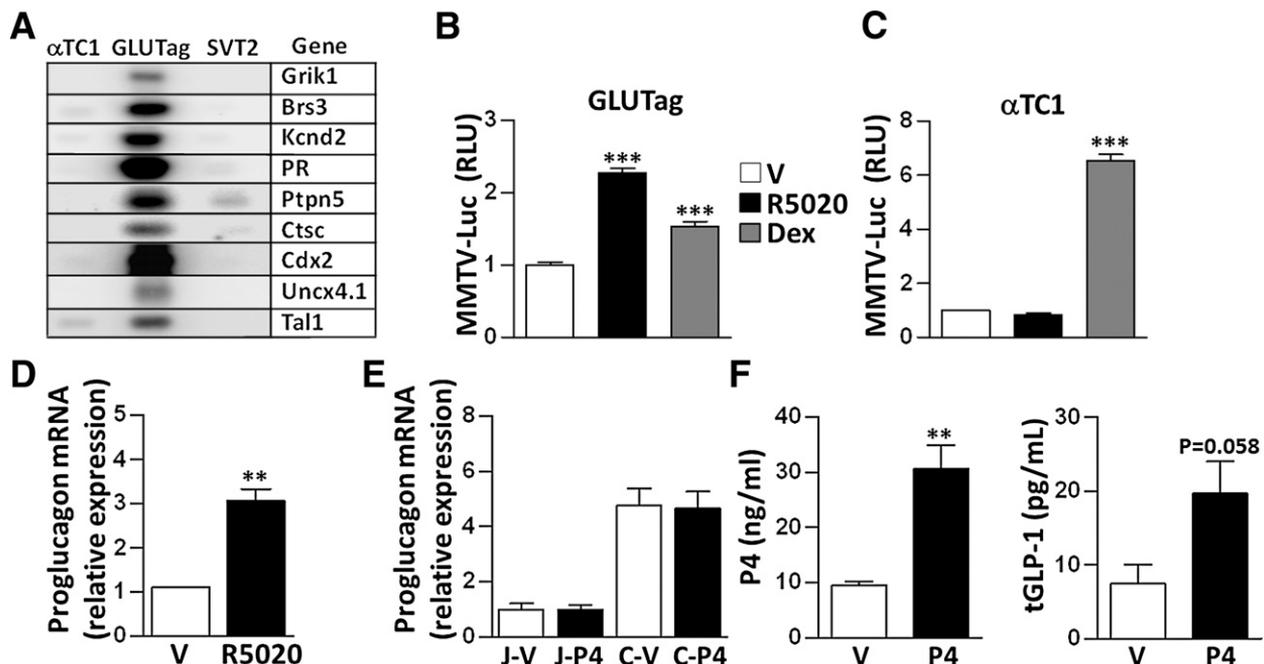


FIG. 1. A functional PR is expressed in enteroendocrine cells and the gastrointestinal tract of mice. **A:** Examples of genes differentially expressed in enteroendocrine GLUTag cells compared with islet α TC1 cells by RT-PCR. SVT2 cells were used as a control SV40 T antigen-transformed nonendocrine cell line. PCR products were probed with an internal 32 P-labeled oligonucleotide. Glutamate receptor, ionotropic, kainate 1 (*Grik1*), bombesin-like receptor 3 (*Brs3*), potassium voltage-gated channel, Shal-related family, member 2 (*Kcnd2*), PR, protein tyrosine phosphatase, nonreceptor type 5 (*Ptpn5*), cathepsin C (*Ctsc*), caudal type homeobox 2 (*Cdx2*), homeobox protein *Uncx4.1* (*Uncx4.1*), and T-cell acute lymphocytic leukemia 1 (*Tal1*) were differentially expressed in GLUTag cells. GLUTag (**B**) and α TC1 (**C**) cells transfected with MMTV-Luc were treated with R5020 (20 nmol/L) or vehicle (V [ethanol]) as described in RESEARCH DESIGN AND METHODS. Luciferase activity was assessed 12 h after treatment. Dexamethasone (Dex; 10^{-7} mol) was used as a positive control. Results depict mean \pm SD from two independent experiments each done in quadruplicate and are expressed relative to the luciferase activity measured for V-treated cells. *** $P < 0.001$ vs. V-treated cells. **D:** The progestin R5020 (20 nmol/L) increases levels of proglucagon mRNA transcripts in GLUTag cells. Proglucagon mRNA levels were assessed by qPCR following incubation of the cells with R5020 (20 nmol/L) or V for 48 h in the presence of 0.1% FCS. Data are mean \pm SD of two independent experiments and are expressed relative to V-treated cells. ** $P < 0.01$ vs. V-treated cells. **E:** Enteral P4 does not increase intestinal proglucagon mRNA levels in vivo. Male mice ($n = 4$ /group) were fed a regular chow diet supplemented with P4 (daily estimated dose of P4: 10 mg/day) or V (corn oil) for 48 h. Jejunum (J) and colon (C) were collected for mRNA analysis by qPCR. Results are expressed relative to the values for J-V. **F:** Blood was collected from mice studied in **E** for the assessment of postprandial circulating P4 and total GLP-1 (tGLP-1). ** $P < 0.01$ vs. V-treated mice.

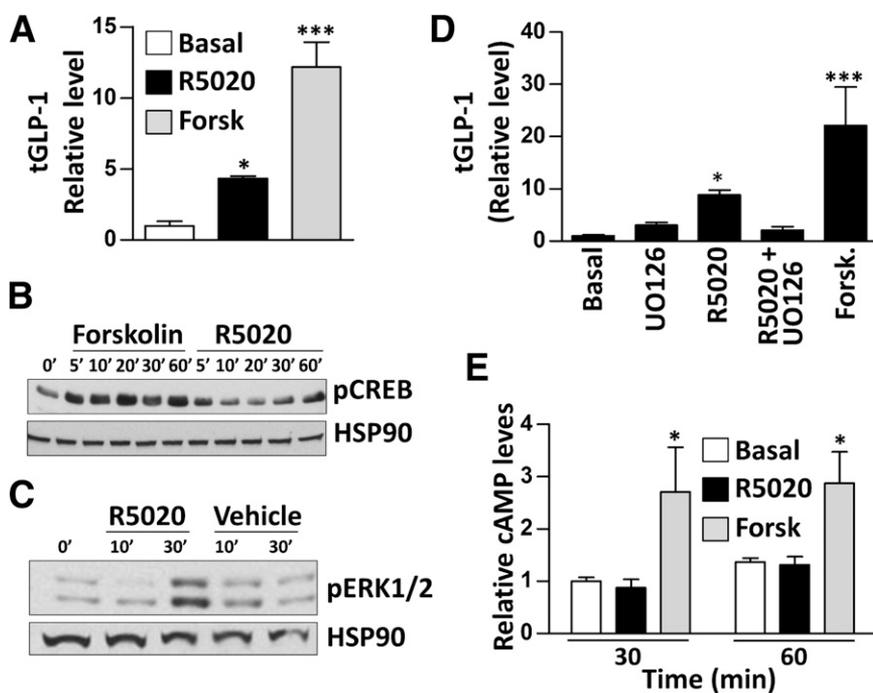


FIG. 2. P4 stimulates GLP-1 secretion in an MAPK-dependent manner in GLUTag cells. **A:** GLP-1 secretion in GLUTag cells treated with R5020. Total GLP-1 (tGLP-1) secreted in the media (pg/mL) over 2 h was normalized to total cell protein content (μg). Results depict mean \pm SD from two independent experiments performed in triplicate and are expressed relative to the levels of GLP-1 secreted under basal conditions (V-treated cells). Western blot analysis of CREB phosphorylation (**B**) and ERK1/2 phosphorylation (**C**) in GLUTag cells treated with R5020 (20 nmol/L). Anti-HSP90 was used to monitor loading and transfer conditions. **D:** R5020 (20 nmol/L) stimulates GLP-1 secretion in a U0126-sensitive manner in GLUTag cells. GLUTag cells were pretreated for 30 min with U0126 (10 $\mu\text{mol/L}$) or V (DMSO) followed by treatment with R5020 (20 nmol) or V (ethanol). Total GLP-1 secreted in the media (pg/mL) over 2 h was normalized by protein cell content (μg) and expressed relative to basal conditions (V-treated cells). **E:** R5020 (20 nmol/L) does not increase levels of cAMP. GLUTag cells were incubated as described in RESEARCH DESIGN AND METHODS and treated for 30 or 60 min with R5020 (20 nmol/L) or forskolin (Forsk) (100 $\mu\text{mol/L}$). cAMP was measured in dried aliquots of ethanol cell extracts. Results shown are the mean \pm SD of a representative experiment done in quadruplicate. * $P < 0.05$, *** $P < 0.001$ vs. V-treated cells.

The induction of ERK1/2 phosphorylation by R5020 and the inhibition of R5020-stimulated GLP-1 secretion by U0126 raised the possibility of a nongenomic mechanism of action. Consistent with this possibility, both R5020 and P4 rapidly increased ERK1/2 phosphorylation in GLUTag cells in the presence of the classical PR antagonist RU486 (Fig. 3A). Furthermore, we detected expression of mRNA transcripts for membrane PRs, specifically the progesterin and adipoQ receptor family member V (*Paqr5*) and the progesterin and adipoQ receptor family member VII (*Paqr7*) in RNA from murine jejunum and colon, as well as in GLUTag cells (Supplementary Fig. 1). To examine the importance of membrane-localized progesterone in GLUTag cells, we used BSA-P4, a P4-albumin conjugate (26). BSA-P4 rapidly increased ERK1/2 phosphorylation (Fig. 3B) and GLP-1 secretion (Fig. 3C) from GLUTag cells to levels comparable to or modestly greater than that seen with R5020 or P4. In contrast, BSA-P4, unlike R5020, had no effect on the activity of MMTV-Luc in transfected GLUTag cells (Fig. 3D).

To identify receptors required for the progesterone-regulated control of enteroendocrine GLP-1 secretion, we used siRNA to reduce expression of the PR, *Paqr5*, or *Paqr7* in GLUTag cells (Fig. 3E). A reduction of PR mRNA by >50% unexpectedly enhanced the stimulatory effects of P4 on GLP-1 secretion (Fig. 3F). In contrast, reduction in levels of either *Paqr5* or *Paqr7* abrogated the ability of P4 to stimulate GLP-1 secretion (Fig. 3F). Hence, P4 stimulates GLP-1 secretion via activation of both *Paqr5* and *Paqr7*, likely in a cooperative manner in GLUTag cells.

We next examined whether P4 acutely lowers glycemia and increases plasma levels of GLP-1 in mice. A single dose of oral P4 significantly improved oral glucose tolerance, in association with increased plasma levels of insulin and GLP-1 (Fig. 4A–C). Consistent with the RU486-independent actions of progesterone on ERK1/2 phosphorylation in GLUTag cells (Fig. 3A), the P4-mediated reduction in glycemic excursion and increase in plasma levels of GLP-1 was not attenuated by concomitant RU486 administration (Fig. 4D–F). In contrast to the glucoregulatory effects of enteral P4, acute intraperitoneal P4 administration failed to improve glucose tolerance in wild-type (WT) mice (Supplementary Fig. 3) and did not increase circulating levels of plasma GLP-1 (data not shown).

As P4 stimulated GLP-1 secretion from GLUTag cells and increased plasma levels of GLP-1 following enteral administration in mice, we examined the importance of the GLP-1 receptor for the glucoregulatory actions of P4. Unexpectedly, P4 improved oral glucose tolerance and significantly increased insulin levels in both *Glp1r*^{+/+} and *Glp1r*^{-/-} mice (Fig. 5A and B). *Glp1r*^{-/-} mice exhibit compensatory changes in the incretin axis including upregulation of GIP secretion and enhanced GIP sensitivity (27,28); hence, we examined GIP levels following oral P4. P4 significantly increased plasma levels of GIP in WT and DIRKO mice and GIP levels also trended higher in *Glp1r*^{-/-} mice (Fig. 5C). Accordingly, we assessed the importance of the GIPR and the GLP-1 receptor as targets for P4 action in *Glp1r*^{-/-}:*Gipr*^{-/-} (DIRKO) mice. Surprisingly, P4 significantly improved glucose tolerance in DIRKO mice

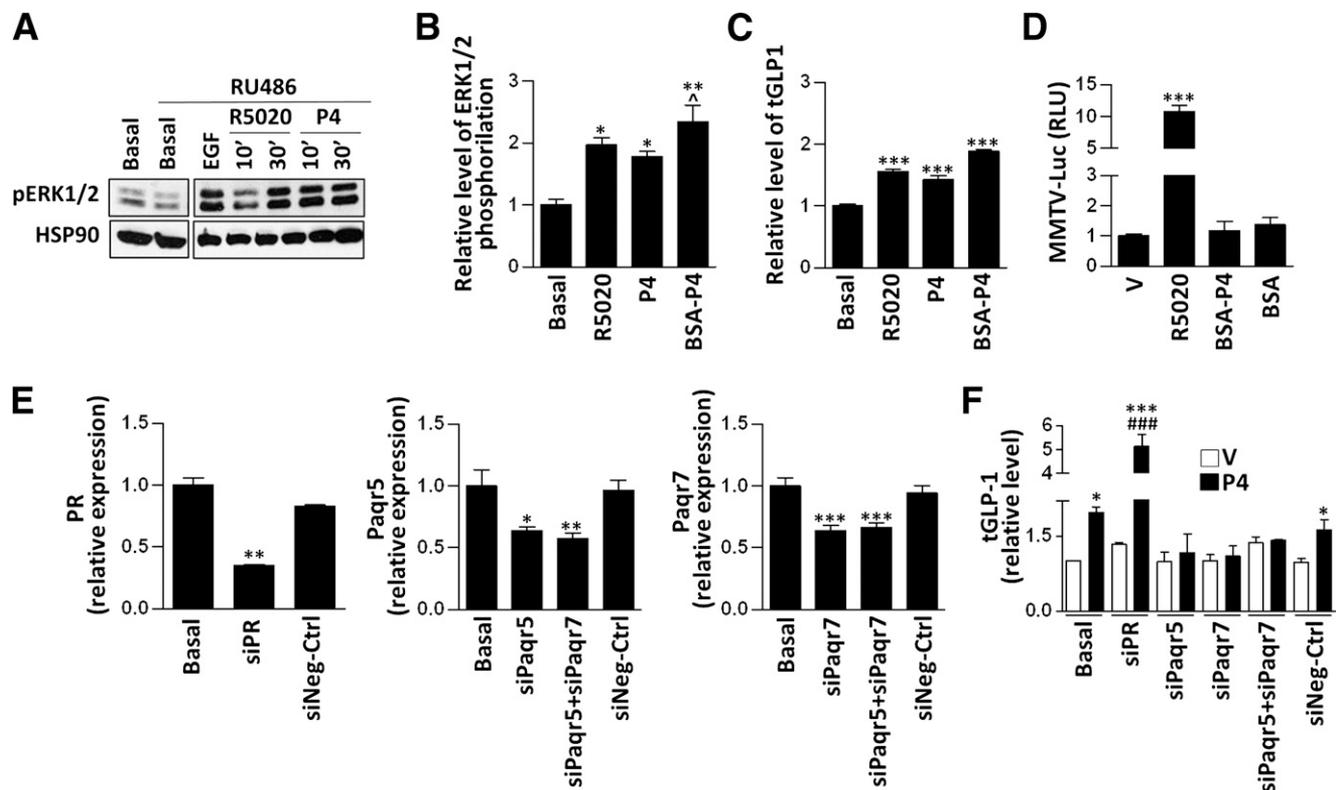


FIG. 3. P4 stimulates ERK1/2 phosphorylation and GLP-1 secretion independent of the classical PR in GLUTag cells. **A:** P4-stimulated ERK1/2 phosphorylation is not inhibited by RU486 in GLUTag cells. Western blot analysis of ERK1/2 phosphorylation in cells pretreated for 15 min with the PR antagonist RU486 (1 μ mol) followed by treatment for 10 or 30 min with R5020 (20 nmol), P4 (20 nmol), and epidermal growth factor (EGF; 25 ng/mL, positive control for ERK1/2 phosphorylation). Anti-HSP90 was used to monitor loading and transfer conditions. **B:** Covalently bound BSA-P4 increases ERK1/2 phosphorylation. GLUTag cells were treated for 10 min with the progestin R5020 (20 nmol), P4 (20 nmol), or covalently bound BSA-P4 (20 nmol P4). Cells treated with V (ethanol) plus BSA-FA (20 nmol/L) were used as the basal control. Figure shows the densitometric analysis of ERK1/2 phosphorylation normalized to HSP90 in the same experiment and expressed relative to basal conditions. Results depict mean \pm SD of two independent experiments each done in triplicate. **C:** BSA-P4 (20 nmol/L P4 equivalent) stimulates GLP-1 secretion in GLUTag cells. Cells treated with V (ethanol) plus BSA-FA (20 nmol/L) were used as the basal control. Total GLP-1 secreted in the media (2 h incubation) (pg/mL) was normalized to total cell/protein content (μ g). Results depict mean \pm SD of two independent experiments, each performed in quadruplicate and expressed relative to levels of GLP-1 secreted under basal conditions. **D:** BSA-P4 (20 nmol/L P4) does not transactivate the MMTV-Luc promoter. GLUTag cells transfected with MMTV-Luc were treated for 12 h with R5020 (20 nmol/L), BSA-P4 (20 nmol/L of P4), V, or BSA-FA (20 nmol/L). Results are expressed relative to the luciferase activity in V-treated cells and represent the mean \pm SD of a representative experiment done in triplicate. **E:** siRNA knockdown reduces levels of PR, Paqr5, and Paqr7 mRNA transcripts in GLUTag cells. siRNA was transfected as described in RESEARCH DESIGN AND METHODS, and the efficiency of RNA knockdown was assessed by qPCR. Levels of mRNA transcripts after knockdown are expressed relative to the levels detected under basal conditions. **F:** Relative GLP-1 secretion in cells transfected with the indicated siRNAs and treated for 2 h with R5020 (20 nmol/L), P4 (20 nmol/L), or V (ethanol). Total GLP-1 secreted in the media (pg/mL) was normalized to total cell protein content (μ g). Results are expressed relative to levels of GLP-1 in cells treated with V and depict mean \pm SD from three independent experiments each done in triplicate. $^*P < 0.05$, $^{***}P < 0.001$ vs. basal, $^{###}P < 0.001$, small interfering PR (siPR) + P4 vs. basal + P4.

(Fig. 5D). Plasma insulin levels were significantly increased after P4 administration in WT and *Glp1r*^{-/-} mice 5 min after glucose challenge (Fig. 5E) and at later time points in DIRKO mice (Fig. 5F). Enteral P4 had no effect on the rate of gastric emptying in WT or DIRKO mice (Supplementary Fig. 2A) and produced a modest impairment in insulin sensitivity in both WT and DIRKO mice (Supplementary Fig. 2B and C).

DISCUSSION

To identify molecular mechanisms important for PGDP synthesis and secretion in gut endocrine cells, we and others have studied the genes and proteins expressed in murine GLUTag cells (6), which retain many of the properties associated with differentiated gut endocrine cells, including cAMP-dependent and nutrient-sensitive regulation of PGDP synthesis and secretion (6,29,30). The current

studies demonstrate that the murine PR is expressed in cultured enteroendocrine cells and within different regions of the mouse gastrointestinal tract. Nevertheless, both P4 and membrane-restricted BSA-P4 activate ERK1/2 and GLP-1 secretion in GLUTag enteroendocrine cells, whereas the nuclear receptor antagonist RU486 does not block the stimulatory actions of progesterone on GLP-1 secretion in mice. Moreover, enteral but not parenteral progestin administration acutely increased GLP-1 levels and improved oral glucose tolerance in mice. Together, these findings establish a role for enteral progesterone in the pharmacological control of incretin secretion and glucose homeostasis.

Despite expression of a functional classical PR in GLUTag cells, the actions of R5020 and P4 to stimulate GLP-1 secretion were not diminished by the PR antagonist RU486 in mice. However, the MAPK inhibitor UO126 completely eliminated the stimulatory effect of progesterone on

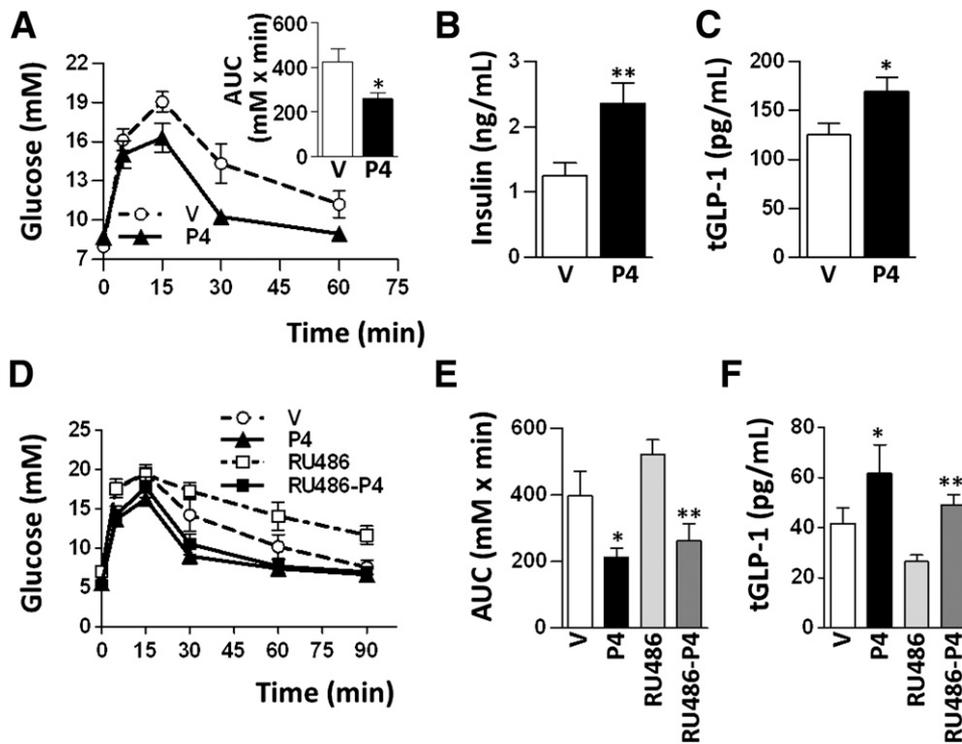


FIG. 4. P4 improves oral glucose tolerance and increases plasma GLP-1 levels in mice via RU486-insensitive mechanisms. **A:** Plasma glucose excursion during an OGTT and area under the curve (AUC) (inset) in mice treated orally with P4 (100 μ g). P4, solid triangle and solid line; V (vehicle), open circle and dashed line. **B:** Glucose-stimulated insulin levels in plasma obtained at the 5-min time point of the OGTT shown in **A**. **C:** Plasma total GLP-1 (tGLP-1) levels at the 5-min time point from the OGTT shown in **A**. C57BL/6 mice were fasted and given vehicle or P4 (100 μ g) 15 min before oral glucose administration, followed by tail bleed, 5 min after glucose loading for the measurement of insulin and tGLP-1 ($n = 9$ mice). * $P < 0.05$, ** $P < 0.01$ vs. vehicle-treated mice. **D:** Glucose excursion during an OGTT in mice pretreated with the PR antagonist RU486 (100 mg/kg) in the presence or absence of enteral P4 (100 μ g) administration. P4, solid triangle and solid line; RU486, open square and dashed line; RU486-P4, solid square and solid line; V, open circle and dashed line. **E:** AUC for glucose levels of OGTT shown in **D**. **F:** tGLP-1 levels at the 5-min time point from the OGTT shown in **D**. Mice were fasted and given orally vehicle or RU486 (100 mg/kg) 60 min before oral P4 (100 μ g) or V administration followed by OGTT 15 min later. Mice were then bled 5 min after oral glucose administration for the measurement of tGLP-1 ($n = 7$ mice/group). * $P < 0.05$, P4 vs. V-treated mice; ** $P < 0.01$, RU486-P4- vs. RU486-treated mice.

ERK1/2 phosphorylation and GLP-1 secretion in GLUTag cells. Together, these findings strongly suggested that P4 stimulates GLP-1 secretion through nongenomic mechanisms linked to ERK1/2 signal transduction. Consistent with this possibility, we observed robust activation of both ERK1/2 phosphorylation and GLP-1 secretion in GLUTag cells using BSA-P4, a hybrid molecule that restricts passage of P4 across the cell membrane. In contrast, BSA-P4 had no effect on the transcriptional activity of MMTV-Luc in GLUTag cells, whereas R5020 robustly increased MMTV-Luc activity in the same experiments.

Limited information is available on whether and how P4 controls glucose homeostasis or islet function. A previous study demonstrated that female but not male *PR*^{-/-} mice exhibited lower fasting glucose and higher insulin levels; however, the precise mechanisms accounting for this sexual dimorphism and phenotype were not clearly elucidated (31). Our data, using both the nuclear PR antagonist RU486 in mice and BSA-P4, together with siRNA knockdown studies in GLUTag cells, clearly implicates a potential role for membrane PRs in the control of GLP-1 secretion. The membrane-restricted actions of P4 are thought to be linked to the activity of at least three G protein-coupled receptors, principally Paqr5, Paqr7, and Paqr8 (32,33). Although actions for these receptors have been described in several tissues, principally reproductive organs and the immune system (34), no previous studies have linked membrane PRs to the control of gut hormone secretion or glucose homeostasis.

Furthermore, experiments designed to assess the putative physiological importance of endogenous membrane PRs in the control of incretin secretion and glucose homeostasis are limited by the lack of suitable antagonists, highly specific antisera, or knockout mice.

Acute enteral progestin administration rapidly increased plasma levels of both GIP and GLP-1. Although the majority of enteroendocrine L cells are localized to the distal gut, a substantial proportion of enteroendocrine cells within the proximal gut also exhibit the capacity for GLP-1 synthesis and secretion (35–37). Hence, the precise site of action for P4 within the gut requires further investigation. The gluoregulatory actions of enteral P4 were unexpectedly preserved in *Glp1r*^{-/-} and DIRKO mice, indicating that the actions of P4 to improve glucose tolerance may be also be observed independent of classical incretin receptor signaling. In contrast to the glucose lowering observed following enteral P4, intraperitoneal administration of P4 did not stimulate GLP-1 secretion or improve oral glucose tolerance. Taken together, these findings are consistent with a mechanism whereby exposure of the gastrointestinal epithelium to enteral P4 triggers one or more gut-derived mechanisms promoting insulin secretion and glucose homeostasis. The preservation of the gluoregulatory actions of enteral P4 in DIRKO mice strongly suggests the possibility of P4-sensitive incretin-like compensatory mechanisms. Indeed, we recently described upregulation of multiple incretin-like compensatory

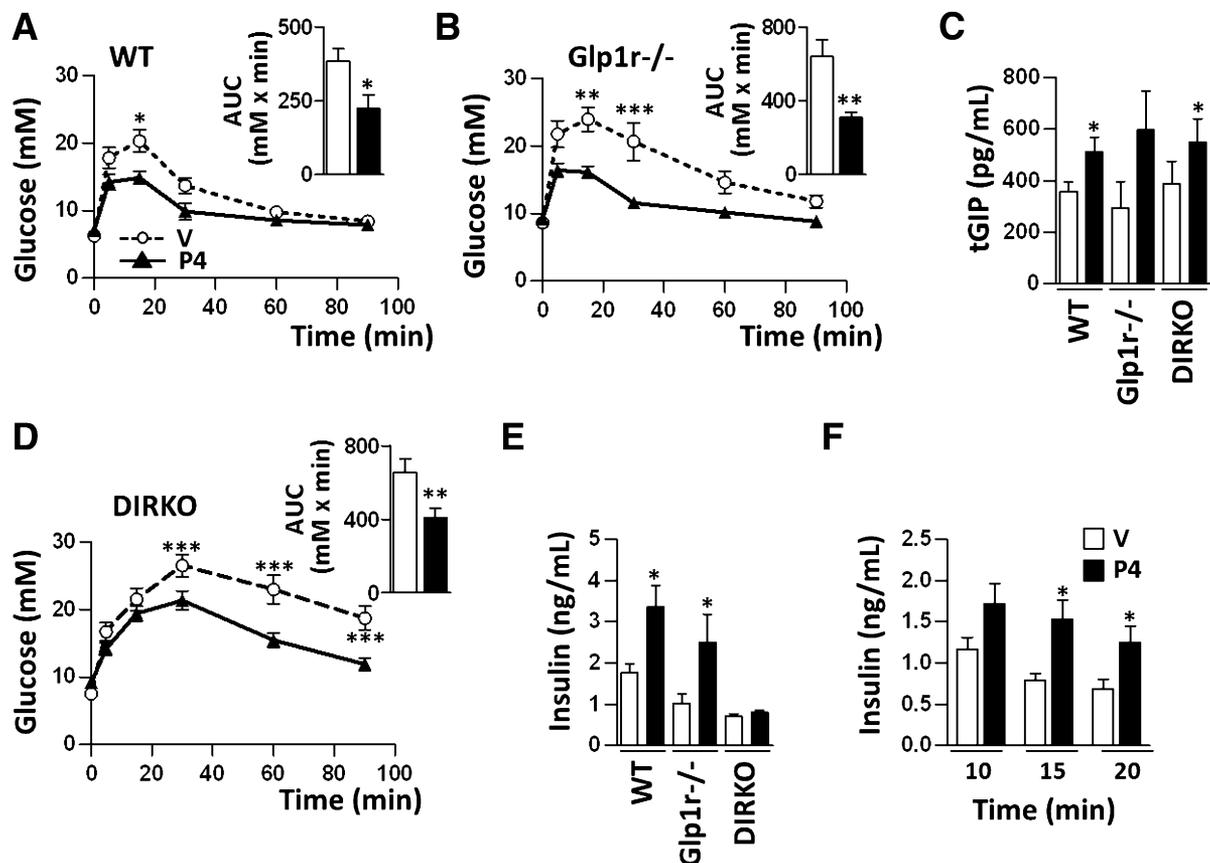


FIG. 5. P4 improves oral glucose tolerance independent of incretin receptors. Glucose excursions and area under the curve (AUC, inset) during OGTT for WT ($n = 14$ mice) (A), $Glp1r^{-/-}$ ($n = 8$ mice) (B), and DIRKO mice ($n = 17$ mice) (D). Age-matched WT, $Glp1r^{-/-}$, and DIRKO mice were treated with a single oral dose of P4 (20 nmol/L; solid line) or V (vehicle) (dashed line) 15 min before an oral glucose load. For C and E, a blood sample was collected 5 min after oral glucose administration for the assessment of glucose-stimulated total GIP (tGIP) and insulin plasma levels. For F, an independent group of DIRKO mice ($n = 10$ mice) was treated as described above and bled at the indicated time points following glucose administration for the assessment of plasma insulin levels during an OGTT. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. vehicle-treated mice.

mechanisms in DIRKO mice treated with antisense oligonucleotides against the *Gcgr* (28), revealing considerable redundancy in how the gastrointestinal tract and islet β -cells maintain glucose homeostasis in response to enteral glucose administration.

Taken together, the observations that BSA-P4 stimulates GLP-1 secretion from GLUTag cells, coupled with the loss of progestin action on GLP-1 secretion following knockdown of membrane PRs strongly suggests that membrane, rather than nuclear PRs, activate GLP-1 secretion in response to P4. Furthermore, these findings are consistent with the inability of RU486, a classical nuclear PR antagonist, to diminish the GLP-1-stimulation and glucoregulatory effects observed following enteral P4 administration in mice. Our data raise interesting questions about the potential for enteral P4 or membrane PR agonists to augment incretin secretion and control glucose homeostasis under different physiological and pathophysiological situations, including type 2 diabetes. The observation that BSA-P4 enhances GLP-1 secretion in GLUTag cells, coupled with findings that enteral P4 promotes GLP-1 secretion and enhances glucose homeostasis *in vivo*, suggest that the enteroendocrine membrane PR system may be a potential intestinal target for selectively enhancing incretin secretion, independent of systemic progesterone exposure, for the treatment of metabolic disorders.

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G.B.F. and X.C. carried out experiments and wrote and reviewed the manuscript. M.M. analyzed microarray data and reviewed the data and manuscript. D.J.D. planned experiments, reviewed data, and wrote the manuscript. D.J.D. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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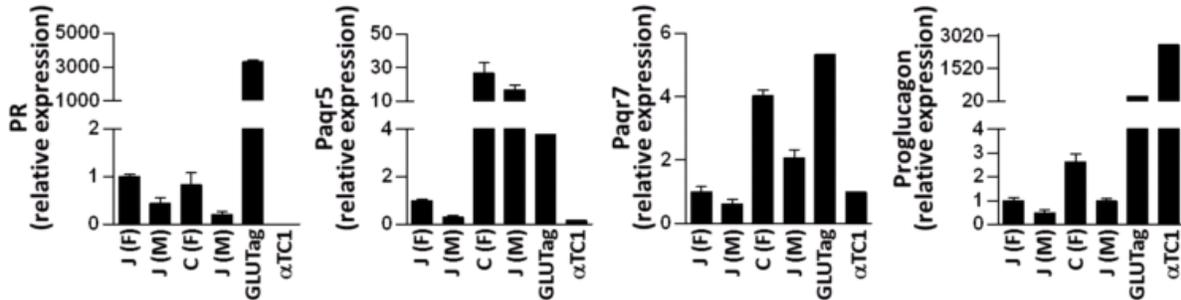
SUPPLEMENTARY DATA

Supplementary Table 1. Primers and internal probe sequences used to validate microarray results obtained from GLUTag vs. α TC1 cell lines.

Gene	Symbol	Forward 5'-3'	Reverse 5'-3'	Probe
Glutamate receptor, ionotropic, kainate 1	Grik1	CTCTCATGCGGCAAGGATC	GGCTTTCTGTTTTGCTCCC	CGTCCTTTCTGTGTTTGTAGC
Bombesin-like receptor 3	Brs3	GAATCCCGGAAGAGAATTGC	CCTTCCTGGCACTACTGCC	GTAATTCCTGCGTGAACCCC
Potassium voltage-gated channel,	Kcnd2	GACTCTGTGGCCCTTTGAC	CCCCATGAGAAACACTGTG	GTGTGGACTGAAGGAAACCA
Progesterone receptor	PR	GGTCGTACAAGCATGTCAG	GGATCTTGGGCAACTGGG	CTTACCATGTGGCAAATCCC
Protein tyrosine phosphatase, non-receptor type 5	Ptpn5	CCCCATCTATTGTCCTGGC	GGTCTCTGCCATCCACATC	GCCAGGAAAGGAGCACTGAA
Cathepsin C	Ctsc	CCTTTCAACCCCTTCGAGC	GACAACCTGAACCACTGCTC	CGGGTCTTTATCACTCACAG
Caudal type homeo box 2	Cdx2	CAGAACC GCAGAGCCAAG	CTTCGTTTGTGTTGCTGG	CTGAGCCATGAGGAGTATGG
Unc4.1 homeobox (C. elegans)	Uncx4.1	CTTTCCGGGACCTAACAGC	CCATCGACACAGCGTTTTTC	GATGCGCTTAGCCAGTCGAG
T-cell acute lymphocytic leukemia 1	Tal1	CCAAGGGCACAGCAACTAG	GTACAAAAGTCCAGGCCCC	CATAGCACGCCATGTCTGTG

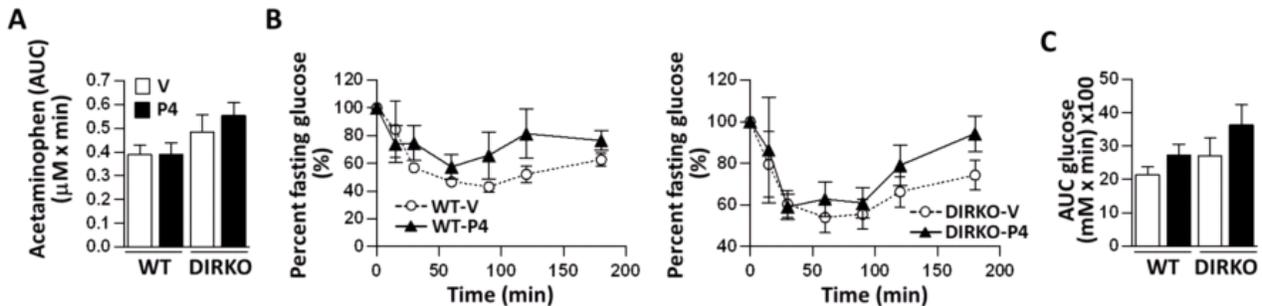
SUPPLEMENTARY DATA

Supplementary Figure 1. PR, Paqr5 and Paqr7 mRNA transcripts are present in the murine intestine. Expression of PR, Paqr5, Paqr7 and proglucagon (*Gcg*) mRNA transcripts in the jejunum (J) and colon (C) of female (F) and male (M) C57Bl/6 mice (n=5 per group) as assessed by qPCR. Levels of mRNA transcripts are normalized to the expression in the jejunum of female mice.



Supplementary Figure 2. Acute enteral progesterone administration does not modulate gastric emptying and or insulin sensitivity in mice.

(A) AUC for plasma acetaminophen levels in gastric emptying studies with vehicle (V) or progesterone (P4) in WT and DIRKO mice. Liquid-phase gastric emptying was assessed using the acetaminophen absorption test as described in Methods. (B) Relative decline in glucose levels during an ITT for WT (n = 8) and DIRKO (n = 10) mice pre-treated with oral vehicle (V) or progesterone (P4) (100 μ g) 15 min before administration of a single dose of insulin (1.2 U/kg). Values are expressed as a percentage relative to fasting blood glucose levels. (C) AUC for glucose during ITT in (B).



Supplementary Figure 3. Progesterone administered via intraperitoneal injection fails to improve glucose homeostasis during an OGTT in mice.

Glucose excursions during OGTT. Mice (n = 6 mice) were fasted and given a single ip dose of vehicle (dashed line) or (P4) (100 μ g) (solid line), 15 min before an OGTT.

