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The interplay of prolactin and the glucocorticoids in the regulation of beta cell gene expression, fatty acid oxidation, and glucose-stimulated insulin secretion: implications for carbohydrate metabolism in pregnancy

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Abstract

Carbohydrate metabolism in pregnancy reflects the balance between counter-regulatory hormones, which induce insulin resistance, and lactogenic hormones, which stimulate beta cell proliferation and insulin production. Here we explored the interactions of prolactin (PRL) and glucocorticoids in the regulation of beta cell gene expression, fatty acid oxidation, and glucose-stimulated insulin secretion (GSIS). In rat insulinoma (INS-1) cells, rat PRL caused 30-50% ($p<0.001$) reductions in FoxO1, PGC1α, PPARα, and CPT-1 mRNAs and increased Glut-2 mRNA and GSIS; conversely, Dexamethasone (DEX) upregulated FoxO1, PGC1α, PPARα, CPT-1, and UCP-2 mRNAs in INS-1 cells and inhibited GSIS. Hydrocortisone had similar effects. The effects of DEX were attenuated by co-incubation of cells with PRL. In primary rat islets PRL reduced FoxO1, PPARα and CPT-1 mRNAs while DEX increased FoxO1, PGC1α, and UCP-2 mRNAs. The effects of PRL on gene expression were mimicked by constitutive over-expression of STAT5b. PRL induced STAT5 binding to a consensus sequence in the rat FoxO1 promoter, reduced nuclear FoxO1 protein levels, and induced its phosphorylation and cytoplasmic redistribution. DEX increased beta cell fatty acid oxidation (FAO) and reduced FA esterification; these effects were attenuated by PRL. Thus lactogens and glucocorticoids have opposing effects on a number of beta cell genes including FoxO1, PGC1α, PPARα, CPT-1, and UCP-2 and differentially regulate beta cell Glut-2 expression, fatty acid oxidation, and GSIS. These observations suggest new mechanisms by which lactogens may preserve beta cell mass and function and maternal glucose tolerance despite the doubling of maternal cortisol concentrations in late gestation.

Introduction

The pregnant mother in late gestation develops severe resistance to insulin action, with post-prandial free fatty acidemia and a 50% reduction in insulin-mediated glucose disposal (1-3). Maternal insulin resistance spares glucose, amino acids, essential fatty acids, and ketones for placental–fetal transport and is therefore obligatory for normal fetal development and growth. In uncomplicated pregnancies, the fall in insulin sensitivity in maternal tissues triggers a striking rise in pancreatic insulin production (1, 3), which derives from increases in maternal beta cell mass, insulin synthesis, and glucose-stimulated insulin secretion (GSIS). Pre-existing beta cell dysfunction and/or failure of the beta cell adaptive response lead to maternal glucose intolerance and, in more severe cases, gestational diabetes (3-5).

The insulin resistance and free fatty acidemia of pregnancy result from progressive increases in counter-regulatory hormones and inflammatory cytokines (2, 6-14) including placental GH (GH-V), cortisol, progesterone, and tumor necrosis factor alpha (TNFα). Placental lactogen (PL) and prolactin (PRL) may also play contributory roles. Interestingly, several of the factors that reduce insulin sensitivity in the mother have detrimental effects on beta cell mass and/or function; for example, glucocorticoids inhibit beta cell proliferation and reduce beta cell viability, insulin production, and GSIS (15-19). Likewise, TNFα and free fatty acids in chronic excess inhibit GSIS and may induce beta cell death (20, 21). Yet insulin production is sustained through late gestation in the majority of women, who thereby maintain plasma glucose concentrations within the normal range.

How is maternal insulin production sustained in late gestation in the face of a doubling of plasma cortisol concentrations (2) and a rise in free fatty acids (FFA) and inflammatory cytokines? The inhibitory effects of glucocorticoids on beta cell function are countered by progressive increases in the lactogenic hormones prolactin (PRL) and placental lactogen (PL). The lactogens and glucocorticoids have opposing effects on beta cell proliferation,

insulin production, glucokinase activity, and GSIS in insulinoma cells (2, 6, 18, 19, 22), and PRL blunts (but does not abolish) the apoptotic effect of Dexamethasone in rat islets (18).

The molecular mechanisms that govern the interactions of the glucocorticoids and lactogens are thus far unknown. The metabolic effects of the glucocorticoids in liver are mediated by induction of forkhead protein FoxO1, peroxisome proliferator activator receptor (PPAR) γ coactivator-1α (PGC1α), and PPARα, which promote hepatic gluconeogenesis, glycogenolysis, and fatty acid oxidation under conditions of fasting and stress (23-26). While their regulation by glucocorticoids in pancreatic islets has not been studied previously, the transcriptional regulators FoxO1, PGC1α, and PPARα play important roles in beta cell growth and function. Constitutive expression of FoxO1 in pancreatic islets suppresses beta cell proliferation, increases beta cell apoptosis, and reduces insulin production (27-30). Conversely, haploinsufficiency or phosphorylation and inactivation of FoxO1 by insulin/IGF signaling increases nuclear expression of PDX-1 and the D cyclins, thereby promoting beta cell proliferation (27-29, 31). Induction of islet PGC1α in beta cells *in vitro* is accompanied by increases in expression of peroxisome proliferator activator receptor α (PPARα), uncoupling protein 2 (UCP-2), and carnitine palmitoyltransferase 1 (CPT-1), the rate limiting enzyme in fatty acid oxidation (32-39). Activation of PPARα *in vivo* antagonizes the effects of high fat feeding and insulin resistance on islet insulin secretion (40, 41); *in vitro*, PPARα induces UCP-2 expression and, in some (but not all) studies suppresses GSIS (35). Conversely, deletion of PPARα and reductions in beta cell fatty acid oxidation increase GSIS in fasted mice (34), while over-expression of CPT-1 in islet beta cells inhibits GSIS (36-39, 42).

The effects of fasting and glucocorticoids, which inhibit beta cell growth and GSIS, contrast with those of the lactogenic hormones, which promote beta cell proliferation and GSIS. We therefore hypothesized that PRL would counteract the effects of nutrient deprivation and glucocorticoids on beta cell FoxO1, PGC1α, PPARα, UCP-2, and CPT-1 expression and thereby attenuate their effects on beta cell fatty acid oxidation and insulin

secretion. To test that hypothesis, we examined the interactions of PRL and Dexamethasone in the control of beta cell gene expression and function in rat insulinoma (INS-1) cells and primary rat islets.

Materials and Methods

Materials

Roswell Park Memorial Institute (RPMI) 1640, Dulbecco's modified Eagle's media (DMEM), L-glutamine, antibiotic/antimycotic solution, fetal bovine serum (FBS), and Trizol reagent were purchased from Life Technologies, Rockville, MD. Dexamethasone, hydrocortisone, and ovine PRL were from Sigma Corporation (St. Louis, MO). Rat PRL (lot AFP7545E) was purchased from Dr. Albert Parlow at the Hormone Distribution Program of the NIDDK. The Bradford protein reagents were from Sigma. Rat Insulin ELISA kits were purchased from LINCO Research., St Charles, MO. Insulin RIA DPC Coat-A-Count kits were purchased from DPC, Los Angeles, CA. The High Capacity cDNA Archive kits and SYBR Green PCR Master mixes were purchased from Applied Biosystems Inc., Foster City, CA. The NE-PER Nuclear and Cytoplasmic Extraction Reagents Kits and the Halt protease inhibitor cocktail kit were purchased from PIERCE Biotechnology (Rockford, IL). Rabbit polyclonal antibodies to FoxO1 and phosphorylated FoxO1 were purchased from Cell Signaling (Beverly, MA). A mouse monoclonal antibody to γ-tubulin was from Sigma. Rabbit polyclonal antibodies to STAT5 and to STAT5a were from Upstate Biotechnologies (Bedford, MA) and Santa Cruz Biotechnologies (Santa Cruz, CA), respectively.

Cell culture

Parental rat insulinoma (INS-1) cells (originally supplied by Dr. Claes Wollheim, Geneva) and an INS-1 cell line with high glucose responsivity (832/13 cells, from Dr. Christopher Newgard, Duke University) were used for these experiments. The two cell lines gave similar results in parallel experiments; however, we elected to use the 832/13 cells

specifically for studies of glucose-stimulated insulin secretion (GSIS). The INS-1 cells were grown in RPMI 1640 (11.1 mM glucose) supplemented with 10% fetal bovine serum (FBS), 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 10 mM HEPES, and 1% antibiotic/antimycotic solution in 5% CO_2 at 37°C. At approximately 80% confluence, the cells were washed and incubated with hormones or diluents for 20 hours (hrs) in 'basal medium' containing DMEM with 5.5 mM glucose, 0.1% human serum albumin, 10 μg/ml human transferrin, 50 μM ethanolamine, 0.1 nM tri-iodothyronine (T_3), 50 μM phosphoethanolamine, and 1% antibiotic/antimycotic solution. To study the effects of serum repletion on hormone action, we performed additional experiments in INS-1 cells incubated in medium containing 1% or 10% calf serum.

Primary rat islets were isolated from ~250g male Wistar rats by a previously described procedure (43). Use of the rats for this purpose was approved by the Duke University Institutional Animal care and Use Committee. The pre-incubation medium (used during the first 24 hrs after isolation) was RPMI 1640 containing 6.8 mM glucose, 10% FBS, 10 mM HEPES, and 1% antibiotic/antimycotic solution. After washing, the islets were incubated for 20 hrs with hormones or diluents in RPMI basal medium containing 6.8 mM glucose, 0.1% human serum albumin, 10 mM HEPES, and 1% antibiotic/antimycotic solution.

To assess cell viability in serum-free basal medium we grew INS-1 cells to confluence in RPMI containing 10% FBS. The cells were then washed and incubated for 20 hr in serum-free basal medium or basal medium containing 1% or 10% calf serum. Following trypsinization (0.05% trypsin/EDTA) the cells were washed and incubated with trypan blue (0.4%), counted by hemocytometry, and re-plated in RPMI/10% FBS. Under these conditions, there were no differences in cell viability among the three experimental groups (serum-free: cell counts 966,000±45,299 (mean±SE, n=4), trypan blue positive <0.5%, re-plating efficiency >99%; 1% calf serum: cell counts 885,000±16,523 (n=4), trypan blue

positive <0.5%, re-plating efficiency >99%; 10% calf serum: cell counts 873,000±21,564 (n=4), trypan blue positive <0.5%, re-plating efficiency > 99%).

Over-expression of Stat5 b in INS-1 cells

A constitutively active, murine STAT5b adenovirus was a gift from Dr. Nils Billestrup (Novo) and has been described previously (44). A recombinant adenovirus containing green fluorescent protein served as a control. INS-1 cells were transduced with adenovirus for 24 hrs and cultured for an additional 24 hrs. The transfected cells were subsequently incubated in serum-free basal medium for an additional 24 hrs with hormones or diluents.

Basal and glucose-stimulated insulin secretion (GSIS)

Basal insulin secretion and GSIS were assessed using INS-1 832/13 cells. To assess the effects of serum deprivation on basal and GSIS the cells were grown to 80% confluence and washed with basal media. Half the cells were then incubated for 16 hrs in basal media containing 5.5 mM glucose; the remaining cells were re-incubated for 16 hrs in growth medium containing 10% FBS. All the cells were then washed and incubated for 2 hr in basal medium containing 2.8 mM glucose. After collection of the conditioned media the cells were incubated for an additional 2 hr in basal medium containing 15 mM glucose. The conditioned media samples were then analyzed for insulin content.

To assess the effects of PRL and DEX on basal and GSIS the cells were grown to 80% confluence, washed, and incubated in basal medium (5.5 mM glucose) containing rat PRL (20 nM), DEX (0.1 or 1 μM, which gave similar results), a combination of the two, or diluent(s) for 16 hrs. The cells were then washed with a secretion buffer (SAB) (114 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.16 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 20 mM HEPES, and 1% fatty acid-free BSA, pH 7.4) and incubated for 2 hr in SAB containing 2.8 mM glucose in the presence or absence of the various hormones. The cells were then

washed and incubated for 2 hr in fresh SAB containing 2.8 mM or 15mM glucose; the media was then analyzed for basal and glucose stimulated insulin secretion. Insulin was measured with an Insulin ELISA kit or with the DPC Coat-A-Count kit and expressed per mg of cell protein.

Quantification of mRNA expression

INS-1 cells and rat islets were incubated with hormones or diluents for 16-20 hrs as described above. Total RNA was extracted using Trizol reagent according to the manufacturer's protocol. cDNA was synthesized from 2.0 μg RNA using the High Capacity cDNA Archive kit (ABI) according to the manufacturer's protocol. Quantitative real-time PCR was performed using an ABI 7300 Real Time PCR System. Oligonucleotide primers were designed using the Primer Express program from ABI. Amplicon lengths ranged from 90-150 bp; all primer pairs spanned introns. Negative controls were processed without reverse transcriptase. All samples from a single experiment were run using a single PCR mixture. Expression levels were normalized against the levels of acidic ribosomal phoshoprotein PO (riboprotein), a housekeeping gene that shows little change during cellular growth or differentiation (45).

The levels of mRNA were quantified using the comparative threshold cycle (C_T) method. **Table 1** shows the oligonucleotide primer pairs, all of which encode rat genes, and C_T values obtained in cells at 80% confluence (prior to the change to basal medium).

FoxO1 protein content and cellular distribution

INS-1 832/13 cells at 80% confluence were washed and pre-incubated for 4 hrs in serum-free basal medium. The cells were then incubated with hormones or diluent for 15 minutes or for 16 hrs to assess changes in FoxO1 phosphorylation and in the cellular distribution of FoxO1 protein. The cells were collected and centrifuged at 200 x g for 5 minutes. The resulting cell pellets were then either frozen at -80^{o}C or used immediately for

cytoplasmic and nuclear extraction in preparation for Western blotting. Cytoplasmic and nuclear extracts were prepared on ice using the NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (PIERCE biotechnology, Rockford, IL) with the addition of protease inhibitors (Halt protease inhibitor cocktail kit, PIERCE biotechnology, Rockford, IL).

The distribution and levels of FoxO1 protein were estimated by Western blot. 35 µg of each extract protein was separated on a 4-12% Bis-Tris SDS-PAGE gel (Invitrogen, Carlsbad, CA) and transferred by electrophoresis to a polyvinylidine floride membrane. The membrane was washed in Tris-buffered saline (TBS) and then blocked in 3% milk buffer (Chemicon, Temicula, CA) for 15 min followed by quick washes in TBS. Membranes were probed with primary antibodies (rabbit polyclonal anti-FoxO1 or anti-phospho-FoxO1) according to manufacturer's guidelines. The membranes were exposed to chemiluminescent substrate (ECL Advance™ Western blotting detection kit, GE Health Care, Piscataway, NJ) and imaged using the VersaDoc imaging system (BioRad, Los Angeles, CA). Mouse monoclonal anti-tubulin antibody was used as internal control. Proteins were quantified by densitometric analysis of the blots using Image J software (rsb.info.nih.gov/ij).

Electrophoretic mobility gel shift assays

To determine if PRL induces binding of STAT5 to consensus sequences in the rat FoxO1 promoter we incubated INS-1 cells in RPMI containing 10% FBS until 60-70% confluence. The cells were then washed, incubated for 4 hr in serum-free basal medium, and treated with PRL (20 nM) or diluent for 30 minutes. Nuclear extracts, prepared as described previously (46, 47), were incubated for 30 min at room temperature with radiolabeled double stranded oligonucleotide probes encoding STAT5 consensus sequences in the rat FoxO1 promoter (5'-ccggttTTCTTGGAAgcctca-3', bp -1241 to -1221) or the rat beta casein promoter (5'-agatTTCTAGGAAttcaatcc-3'). Parallel incubations included a rabbit polyclonal antibody to STAT5a or a 200-fold excess of non-radiolabeled ("cold competitor") double-stranded oligonucleotide primers encoding the STAT5 consensus sequence in the rat

beta casein promoter. The complexes were resolved by PAGE as described previously (46, 47).

$^{1\text{-}14}$C-Palmitate Oxidation

Palmitate oxidation was assessed by measuring the production of $^{1\text{-}14}$C-labeled acid-soluble metabolites (ASM), tricarboxylic acid (TCA) intermediates, and acetyl esters (incomplete oxidation), and [^{14}C] CO_2 (complete oxidation), by use of a modified 48-well microtiter plate described by Kim et al. (48, 49). $^{1\text{-}14}$C-palmitate was dried under nitrogen and incubated in media for 15 minutes at 37°C; under these conditions the $^{1\text{-}14}$C -palmitate complexes with media protein. INS-1 cells were grown and treated with PRL (20 nM), DEX (1 μM), or a combination of the two as described previously. After 20 hrs, the media was aspirated; the cells were then incubated for 2 hr at 37°C in media containing $^{1\text{-}14}$C-palmitate (1 μCi/ml). Aliquots of conditioned media were added to odd-numbered rows of the modified microtitre plate (Costar, Cambridge MA). 200 μl of 1 N NaOH was added to adjoining even-numbered rows. The plate was sealed with a rubber gasket and 100 μl of 70% perchloric acid was added to the conditioned media in the odd-numbered rows; this produces [^{14}C]-CO_2 which then diffuses into the adjoining even-numbered well containing NaOH. This reacts with the CO_2 to form radiolabeled $NaHCO_3$ during incubation at room temperature for 75 min on a shaker set at 125 rpm. At the end of the incubation, 150 μl of the labeled $NaHCO_3$ solution was transferred to scintillation vials and counted using high salt scintillation fluid (complete oxidation).

To quantify acid soluble metabolites (incomplete oxidation), the acidified media samples were collected from the plate, incubated at 4°C overnight, and then centrifuged at 14,000 x g for 10 minutes at 4°C. The supernatants were transferred and centrifuged at 14,000 x g for 10 minutes at 4°C; 300 μl of the final supernatant was assayed for radioactivity by liquid scintillation counting.

The total fatty acid oxidation is derived from the sum of radioactivity obtained from CO_2 absorption (complete oxidation) and acid-soluble metabolites (incomplete oxidation). The results were expressed as nmol/mg protein/hr. Protein was determined by the BCA assay (50) using bovine serum albumin as standard.

$^{1\text{-}14}$C-Palmitate esterification

The incorporation of $^{1\text{-}14}$C-palmitate into cellular lipid was assessed using procedures described previously (48, 49, 51). INS-1 cells were grown and treated with PRL, DEX or a combination of the two as described previously. After 24 hrs, the media was aspirated; the cells were then incubated for 2 hr at 37°C with $^{1\text{-}14}$C-palmitate (1 µCi/ml). The resulting conditioned media was collected for measurements of fatty acid oxidation (see above); the remaining media was aspirated and the cells were washed with PBS, extracted with methanol:PBS (2:3), collected, and centrifuged at 700 x g for 5 minutes. The supernatant was aspirated and the pellet was washed with 1 ml of PBS and re-centrifuged at 700 x g for 5 minutes. The resulting supernatant was removed and 200 µl of 0.2 M NaCl was added to each pellet. The cell pellet was frozen in a dry ice/ethanol bath, thawed at room temperature, and extracted by vortexing in 750 µl $CHCl_3$:MeOH (2:1) and 50 µl 0.1 N KOH. Each sample was then centrifuged at 2000 x g for 20 minutes; the top aqueous layer and the white protein precipitate were removed. 200 µl of MeOH:H_2O:$CHCl_3$ (48:47:3) was added to the bottom layer; after vortexing, the tubes were centrifuged at 2000 x g for 10 minutes. 200 µl of top aqueous phase was assayed for analysis of residual glycerol products and 200 µl of the bottom phase containing lipid was counted by liquid scintillation. The results were expressed as nmol/mg protein/hr, calculated using the following formula = $[(dpm_{sample}–dpm_{blank})/volume]/(dpm/nmol)]/mg\ protein]/2\ h$

Statistical analysis

All assays were performed in triplicate or quadruplicate unless otherwise noted. Data are expressed as mean±SEM of all values obtained in 2-5 independent experiments. Differences among sample means were tested by ANOVA, followed by the Neuman-Keuls or Bonferroni tests of multiple comparisons. A p value <0.05 was considered statistically significant.

Results

1. **Effects of serum deprivation on beta cell gene expression and insulin secretion**

Beta cell growth and function are regulated by glucose availability and nutritional status. To assess the effects of nutrient deprivation on beta cell gene expression and insulin production we compared the levels of FoxO1, PGC1α, PPARα, UCP-2, and CPT-1 mRNAs and the rates of basal and GSIS in INS-1 cells subjected to serum starvation for 20 hr with those in cells growing in serum-replete medium. The concentration of glucose (5.5 mM) in serum-free basal medium was lower than the concentration of glucose in serum-containing growth medium (11.1 mM). As noted previously (Materials and Methods), the viability of cells incubated for 20 hr in serum-free basal medium was comparable to that of cells incubated for 20 hr in basal medium containing 1% or 10% calf serum.

As shown in **Figures 1a and 1b**, deprivation of serum and reduction in ambient glucose concentrations (from 11.1 to 5.5 mM glucose) stimulated a 50% increase in FoxO1 mRNA, 4-5 fold increases in PGC1α and PPARα mRNAs, a smaller increase in PPARγ mRNA, and a 2-fold increase in the ratio of PPARα to PPARγ (all $p<0.01$). In contrast, glucose transporter 2 (Glut-2) mRNA declined 70% ($p<0.01$), and there were small but statistically significant reductions ($p<0.05$) in UCP-2 and CPT-1 mRNAs.

These changes in gene expression were accompanied by sharp reductions in basal and glucose-stimulated insulin secretion (**Figure 2**). Serum deprivation did not abolish the

insulin secretory response to glucose: under serum-free conditions, media insulin concentrations were 4.5-fold higher in cells treated with 15 mM glucose than with 2.8 mM glucose. However, the absolute basal and glucose-stimulated insulin concentrations were 80% lower ($p<0.01$) in cells pre-incubated in serum-free medium than in cells pre-incubated in serum-replete medium. Indeed, glucose (15 mM)-stimulated insulin concentrations in cells pre-incubated in serum-free medium were 40% lower than insulin concentrations in cells pre-incubated in serum and then treated with 2.8 mM glucose.

2. Effects of PRL and glucocorticoids on beta cell gene expression

The effects of PRL on beta cell gene expression were studied in INS-1 cells and primary islets incubated in serum-free basal medium. Preliminary experiments (**Figure 3**) showed that rat PRL (10-500 ng/ml, 0.4-20 nM) caused dose-dependent reductions in the levels of FoxO1, PGC1α, and PPARα mRNAs in INS-1 cells; the half-maximal concentration of PRL approximated 25 ng/ml. Ovine PRL had similar effects (data not shown). Subsequent experiments used PRL at a concentration of 20 nM.

In INS-1 cells (**Figure 4a and 5a)**, rat PRL reduced by 30-50% the expression of FoxO1, PGC1α, PPARα, and CPT-1 (all $p<0.001$); PRL also reduced by 12-25% the levels of UCP-2 mRNA ($p<0.05$). In contrast, PRL stimulated a 65% increase in Glut-2 mRNA ($p<0.01$). In primary rat islets, (**Figure 4b**), PRL caused a 30% reduction in FoxO1 mRNA ($p<0.05$), a 48% reduction in PPARα mRNA ($p<0.01$) and a 40% reduction in CPT-1 mRNA ($P<0.01$). In contrast, PRL stimulated a 35% increase in Glut-2 mRNA ($p<0.05$) but had no significant effect on PGC1α (-18%, $p=0.1$) or UCP-2 mRNAs.

To assess the effects of glucocorticoids on beta cell gene expression we conducted preliminary studies using Dexamethasone (DEX) and hydrocortisone. As shown in **Figure 3**, DEX (0.02-1 μM, 20-1000nM) stimulated dose-dependent increases in FoxO1, PGC1α, and PPARα mRNAs; the effect of DEX was mimicked by hydrocortisone at concentrations (15 μg/dl, 410 nM) within the range of maternal cortisol concentrations during pregnancy (10-

55 μg/dl, 280-1520 nM, reference 52). Subsequent experiments used DEX at concentrations of 0.1 or 1 μM, which gave similar results.

In INS-1 cells, DEX stimulated 2-5 fold increases in the expression of FoxO1, PGC1α, PPARα, and CPT-1 mRNAs (all $p<0.001$) and increased by 18% ($p<0.05$) the levels of UCP-2 mRNA (**Figure 5a**). The effects of DEX were attenuated by co-incubation of cells with PRL; in all cases, the effects of PRL plus DEX on gene expression differed significantly from those of DEX alone. Neither PRL nor DEX had significant effects on levels of mRNA encoding rat adipose differentiation and development 1 (ADD-1, also called srEBPc, data not shown). In primary islets DEX stimulated a 73% increase in FoxO1 mRNA ($p<0.01$), a 79% increase in PGC1α mRNA ($p<0.01$), and a 40% increase in UCP-2 mRNA ($p<0.05$, figure not shown) but had no significant effect on Glut-2 mRNA (-15%, $p=0.21$), PPARα, or CPT-1 mRNAs. The combination of PRL and DEX yielded a 38% increase in FoxO1 mRNA ($p=0.07$ vs DEX alone) and a 26% increase in UCP-2 mRNA ($p=0.18$ vs DEX alone).

3. Effects of serum repletion on hormone action

To assess the effects of serum repletion on hormone action we compared the effects of PRL and DEX on gene expression in cells incubated in 1% (**Figure 5b**) or 10% (**Figure 5c**) calf serum to their effects on gene expression in cells incubated in serum-free medium (**Figure 5a**). The effects of hormones in 1% serum were similar to those in serum-free medium. However in 1% serum PRL had no significant effect on PGC1α mRNA and had lesser (and statistically insignificant) effects on UCP-2 mRNA and on the ratio of PPARα to PPARγ. In cells incubated in 10% serum, the induction of FoxO1, PGC1α, and PPARα by DEX was maintained. However, the effect of DEX on CPT-1 was blunted and its induction of UCP-2 was abolished. PRL alone had no significant effects on gene expression in the presence of 10% calf serum but attenuated the effect of DEX on FoxO1 expression.

4. Effects of PRL and DEX on FoxO1 protein content, phosphorylation, and cellular distribution

FoxO1 activity can be regulated by changes in total cellular FoxO1 content or in the phosphorylation and cellular distribution of the protein (27-29, 31). Dephosphorylation and nuclear localization of FoxO1 increases FoxO1 activity, while phosphorylation and cytoplasmic redistribution of FoxO1 suppresses FoxO1 action. To examine the effects of PRL and DEX on FoxO1 content, phosphorylation, and cellular distribution we incubated INS-1 cells in RPMI containing 10% FBS until they were 70-80% confluent. The cells were then washed and incubated in serum-replete or serum-free basal medium with PRL (40 nM), DEX (1 μM), PRL+DEX (P+D), Insulin (INS, 1 μM), or diluent for 15min or for 16 hrs to assess effects on levels of phosphorylated (cyto phos) and total FoxO1 in cytoplasmic and nuclear compartments. Cell extracts were analyzed by Western blot (**Figure 6**).

Serum depletion acutely increased nuclear FoxO1 and decreased cytoplasmic, phosphorylated FoxO1 while insulin reduced nuclear FoxO1 and increased cytoplasmic, phosphorylated FoxO1 (**Figure 6**). PRL reduced by 35-40% ($p<0.05$) the effect of serum deprivation on appearance of FoxO1 in the nucleus. At 15 minutes and at 16 hours of incubation, PRL increased cytoplasmic phosphorylated FoxO1 levels relative to basal controls and thereby increased by 52% ($p<0.05$) the ratio of cytoplasmic phosphorylated FoxO1 to nuclear FoxO1.

5. Induction of STAT5 binding to a consensus sequence in the rat FoxO1 promoter

Many of the biological actions of PRL in target tissues are mediated through activation and induction of binding of STAT5 to consensus sequences in the promoters of target genes. An analysis of the promoters of the various genes studied here identified a potential STAT5 consensus sequence 5'-TTCTTGGAA-3' in the promoter (bp -1235 to -1227) of the rat FoxO1 gene. To determine if that sequence could bind activated STAT5 we performed an

electrophoretic mobility shift assay using double stranded oligonucleotide primers encoding bp -1241 to -1221 of the rat FoxO1 promoter. A consensus STAT5 sequence in the rat beta casein promoter served as a positive control. As shown in **Figure 7a**, PRL stimulated the binding of STAT5 to its consensus sequence in the rat FoxO1 promoter; the radiolabeled complex was abolished by the addition of non-radiolabeled primers encoding the consensus STAT5 sequence in the beta casein promoter and supershifted by addition of anti-STAT5a antisera to the pre-electrophoresis incubation mix (**Figure 7b**). These findings suggest that PRL might regulate the expression of FoxO1 through induction of STAT5.

6. Effects of over-expression of STAT5b on beta cell gene expression

To assess further the role of STAT5 activation in PRL action we examined the effects of over-expression of STAT5 on beta cell gene expression. INS-1 cells were transfected transiently with an adenoviral vector encoding a constitutively activated STAT5b or a control vector expressing green fluorescent protein. Transfection of INS-1 cells with activated STAT5b increases STAT5b mRNA and protein levels ~5-fold (**Figure 8a**). The cells were analyzed after incubation for 20 hrs in the presence or absence of PRL or diluent.

As shown in **Figure 8b**, STAT5b over-expression alone reduced expression of FoxO1, PGC1α, PPARα, UCP-2 and CPT-1, mimicking the effects of PRL. The effects of STAT5b were potentiated by concurrent treatment with PRL; the effects of PRL plus STAT5b on gene expression exceeded ($p<0.05$) the effects of STAT5b or PRL alone. These findings suggest that the effects of PRL on beta cell gene expression might be mediated, at least in part, by activation of STAT5.

7. Effects of PRL and DEX on fatty acid oxidation and esterification

CPT-1 is the rate limiting step in beta cell fatty acid oxidation. To assess the effects of PRL and DEX on fatty acid oxidation and esterification we incubated INS-1 cells in basal medium (5.5 mM glucose) with hormones or diluent for 20 hrs and then exposed the cells for 2 hr to

$^{1\text{-}14}$C-palmitate. As shown in **Figure 9**, PRL alone had no significant effect on cellular fatty acid oxidation but attenuated ($p<0.01$) the effect of DEX, which stimulated a 56% increase ($p<0.001$) in [^{14}C]-CO_2 production. DEX also reduced fatty acid esterification (the incorporation of palmitate into total cellular lipid) by 50% ($p<0.01$); the effect of DEX was attenuated ($p<0.001$) by co-incubation of cells with PRL.

8. Effects of PRL and Dexamethasone (DEX) on GSIS

In parallel experiments we examined the effects of PRL and DEX on GSIS in INS-1 832/13 cells. As shown in **Figure 10**, PRL stimulated a 48% increase in GSIS ($p<0.05$) during a 24 hour incubation in serum-free medium. In contrast, DEX reduced GSIS by 50% ($p<0.01$). The effects of DEX were reversed by PRL ($p<0.01$ PRL+DEX vs DEX alone).

Discussion

Maintenance of glucose tolerance in mid-late gestation requires a 2-2.5-fold increase in maternal insulin production (1, 3-5, 52) to counter the insulin resistance induced by counter-regulatory hormones, cortisol, and TNFα (2, 6-14). That the lactogenic hormones subserve a critical role in the beta cell adaptive response is suggested by several lines of evidence. First, the increase in maternal insulin secretion during mid-late gestation parallels the rise in maternal PL and PRL concentrations (2) and the increased expression of islet PRL receptors (53), which bind PL as well as PRL. Second, the lactogens stimulate beta cell proliferation, insulin gene expression, and GSIS in human and rodent islets and insulinoma cells and inhibit beta cell apoptosis (6,18,19,22,54-61). Third, exogenous administration or constitutive expression of PRL or PL protects against the development of diabetes in streptozotocin-treated mice (57, 60, 62-64). Finally, insulin production is increased in men and women with hyperprolactinemia (65, 66), while targeted deletion of the PRL receptor in mice causes 30–40% reductions in beta cell mass, pancreatic insulin mRNA, and GSIS (67).

In this study we explored the interactions of lactogens and glucocorticoids in the regulation of beta cell gene expression and function. Our initial studies were conducted using rat insulinoma (INS-1) cells. We demonstrated that serum deprivation and reduction in ambient glucose concentrations stimulated increases in beta cell expression of key transcriptional determinants of energy metabolism including FoxO1, PGC1α, and PPARα and increased the ratio of PPARα to PPARγ. These changes in gene expression were accompanied by striking reductions in beta cell Glut-2 expression and insulin secretion. We then showed that PRL inhibits expression of FoxO1, PGC1α, PPARα, CPT-1, and UCP-2 in serum-free medium and blunts or reverses their induction by Dexamethasone (DEX). The effects of PRL on beta cell gene expression were observed at concentrations (half-maximal dose ~25 ng/ml) comparable to or less than (reference 2) those observed in non-pregnant adults and children (3-20 ng/ml), pregnant women (50-180 ng/ml), and late fetal and newborn boys and girls (50-400 ng/ml). Repletion of serum, which contains a panoply of beta cell growth factors and insulinotropic hormones, obscured most of the effects of PRL on gene expression. In accordance with its suppression of CPT-1 and induction of Glut-2 mRNAs, PRL blunted the effects of DEX on beta cell fatty acid oxidation and increased GSIS. It should be noted that the effect of PRL on cellular fatty acid oxidation was less striking in magnitude than the effect of the hormone on CPT-1 expression or insulin production, possibly because the absolute level of fatty acid oxidation in beta cells is relatively low.

The effects of PRL and DEX on gene expression in primary rat islets differed in certain respects from the effects of the hormones in INS-1 cells. In primary islets as in INS-1 cells, PRL reduced expression of FoxO1, PPARα, and CPT-1 mRNAs and stimulated an increase in Glut-2 mRNA. However, PRL had no effect on PGC1α or UCP-2 mRNAs in primary islets. DEX stimulated increases in FoxO1, PGC1α, and UCP-2 mRNAs in primary islets as well as INS-1 cells but had no effect on islet Glut-2, PPARα, or CPT-1 expression. Differential effects of the hormones in INS-1 cells and islets may reflect differences in cell

composition (clonal beta cells vs mixed islet cells), glucose sensitivity (higher in islets than in parental INS-1 cells), proliferation rates (high in INS-1 cells, low in primary adult islets), and sensitivity to apoptosis/necrosis (higher in INS-1 cells, low in islets).

The effects of PRL on FoxO1, PPARα and CPT-1 mRNAs and beta cell fatty acid oxidation resemble in some ways those of glucose (38, 42, 68). Glucose uptake and utilization likely play roles in PRL action because the hormone increases Glut-2 mRNA and glucokinase activity in pancreatic beta cells (this study and (19, 56, 69). However unlike glucose, PRL is not an acute insulin secretagogue (18,19,22). Moreover, PRL and glucose have synergistic effects on beta cell replication (70), rat insulin gene expression (47, 56), and GSIS (18, 19). These observations suggest that PRL and glucose exert insulinotropic effects through overlapping mechanisms of action.

At least two mechanisms appear to mediate the effect of PRL on FoxO1 expression. PRL reduced FoxO1 mRNA during a 16-20 hour incubation and increased the ratio of cytoplasmic, phosphorylated (inactivated) FoxO1 to nuclear (active) FoxO1. The effects of PRL on FoxO1 mRNA might be exerted at the level of transcription, because the hormone induced binding of STAT5 to a consensus sequence in the rat FoxO1 promoter, and over-expression of STAT5 reduced FoxO1 mRNA and potentiated the (suppressive) effect of PRL. However, additional experiments will be required to define the role of Stat5 in this action of PRL. The mechanism by which PRL induces cytoplasmic re-distribution of phosphorylated (inactive) FoxO1 is currently unknown, but similar effects are achieved through induction of Akt by other hormones that induce beta cell proliferation including insulin, insulin-like growth factor-2 (IGF-2), glucagon-like peptide 1 (GLP-1), and glucose-dependent insulinotropic polypeptide (GIP) (29, 30, 68, 71). Glucose also induces FoxO1 phosphorylation; this effect appears to be mediated by induction of insulin secretion (68,71). A role for glucose and/or insulin action in PRL induction of FoxO1 phosphorylation is possible given that PRL increases beta cell Glut-2 expression and GSIS.

Activation and/or over-expression of nuclear FoxO1 inhibits beta cell proliferation and induces beta cell apoptosis (27-29, 31). In contrast, haploinsufficiency of FoxO1 promotes an increase in beta cell mass (27). In our studies PRL, which induces beta cell replication and inhibits beta cell apoptosis (54, 60, 70), reduced FoxO1 expression. Conversely DEX, which reduces beta cell replication and induces beta cell apoptosis (16, 18), increased FoxO1 mRNA levels. Thus, the PRL-dependent reduction of FoxO1 expression provides a new mechanism by which the lactogens may promote beta cell growth and increase beta cell mass.

FoxO1 suppression might also explain in part the effects of PRL on other beta cell genes. Over-expression of FoxO1 increases PGC1α mRNA in skeletal muscle and liver (72, 73), while deletion of FoxO1 reduces hepatic PGC1α mRNA levels (26). In beta cells, induction of PGC1α induces expression of PPARα and CPT-1 and up-regulates expression of UCP-2 (34-39, 42, 73). Thus, inhibition of FoxO1 by PRL might initiate a cascade that reduces expression of PGC1α and PPARα and thereby inhibits beta cell expression of UCP-2 and CPT-1.

Together with induction of glucose uptake and utilization, these changes in gene expression may facilitate the effects of PRL on beta cell insulin production. This is because over-expression of FoxO1, PGC1α, UCP-2, or CPT-1 inhibits GSIS (30, 32, 74-78). UCP-2 reduces insulin secretion by limiting cellular ATP production from glucose utilization, while up-regulation of CPT-1 reduces insulin production by increasing rates of fatty acid oxidation. Induction of beta cell fatty acid oxidation does not alter glucose oxidation or ATP production and plays no role in the K_{ATP} channel-dependent insulin secretory response to glucose (38, 42). Rather, induction of fatty acid oxidation may reduce the levels of critical lipid mediators that regulate protein kinases and thereby amplify insulin secretion through a K_{ATP}-independent pathway (38, 42). In our studies, PRL suppressed CPT-1 expression and blunted the induction of beta cell fatty acid oxidation by DEX; concurrently, PRL stimulated an increase in GSIS and blocked the inhibitory effect of DEX. Although the magnitude of the

effect of PRL on insulin production exceeded the magnitude of its effect on fatty acid oxidation, our findings are consistent with previous investigations that showed an inverse relationship between fatty acid oxidation and GSIS (38, 74-80). Other studies, however, report that activation of PPARα and induction of fatty acid oxidation may increase beta cell insulin secretion (74); moreover, attenuation of the effect of glucose on fatty acid oxidation (81) and inhibition of fatty acid synthase in INS-1 832/13 cells or primary rat islets are reported to have no effect on GSIS (82, 83). Thus the roles of fatty acid oxidation in beta cell insulin secretion and PRL action remain unclear.

In summary, the lactogens and glucocorticoids have opposing effects on a number of beta cell genes including FoxO1, PGC1α, PPARα, CPT-1 and UCP2 and differentially regulate beta cell glut-2 expression, insulin production, and fatty acid oxidation. These observations suggest new mechanisms by which lactogens may preserve beta cell mass and function and maternal glucose tolerance despite the doubling of maternal cortisol concentrations in late gestation.

It should be noted that PRL only partially antagonized the effect of DEX on gene expression and fatty acid metabolism in INS-1 cells and primary rat islets. There are a number of possible explanations for this finding; most important, the *in vitro* cell culture model clearly does not mimic perfectly the metabolic milieu of pregnancy. For example: (a) the timing, duration and level of exposure to DEX or hydrocortisone *in vitro* do not replicate perfectly the timing, duration and level of exposure to hydrocortisone *in vivo*; and (b) PRL is only one of a number of pregnancy serum factors (e.g. placental GH, progesterone, menin, insulin, insulin-like growth factors, incretins, etc) that may regulate maternal beta cell mass and GSIS.

It is, also, clear that the insulinotropic effects of PRL are not mediated solely by suppression of FoxO1, PPARα or CPT-1 or by changes in beta cell lipid metabolism. In addition to up-regulating glucose uptake and utilization, PRL stimulates beta cell proliferation and inhibits beta cell apoptosis through induction of cyclin D expression (44)

and Bcl-X_L (61) and suppression of menin (84). PRL also promotes insulin gene transcription (47, 56) and enhances the expression of SNARE proteins involved in insulin secretion (85). Thus PRL is a multifunctional beta cell tropin with complex effects on growth and function.

Our studies of the interactions between lactogens and glucocorticoids may have implications for the physiology of insulin production in states other than pregnancy. For example, beta cell mass and insulin production increase markedly in the late gestational fetus and newborn infant despite a surge in cortisol secretion (86); the high levels of lactogens in fetal (PL and PRL) and neonatal (PRL) blood ((55, 67, 87) may promote or sustain beta cell proliferation and insulin production at this stage of development. The ability of PRL to maintain insulin secretion during nutrient deprivation or in the presence of Dexamethasone (this study) may also implicate a role for lactogens in the preservation of beta cell function during fasting, stress, or states of glucocorticoid excess. Finally, since FoxO1, PGC1α, and UCP-2 are over-expressed in islets of diabetic humans and rodents (29, 32, 33, 88, 89), our findings may suggest novel pathways by which lactogens might prevent or reverse beta cell failure in types 1 and 2 diabetes.

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Figure Legends

Figure 1. Effects of serum deprivation and reduction in ambient glucose concentrations on beta cell gene expression.

INS-1 cells were grown in RPMI 1640 containing 10% FCS and 11.1 mM glucose (growth medium). At 80% confluence the cells were washed and incubated for 24 hrs in growth medium or in serum-free DMEM (5.5 mM glucose, 0.1% human serum albumin, HSA). RNA levels were measured by Q-PCR. Values for cells incubated in growth medium (Serum) were adjusted so that the mean equaled 1.0; values for cells in serum-free medium were calculated as a function of the mean of serum-replete values. The figures show the means±SEM of all data from three independent experiments, each of which contained 4 flasks per treatment group. Statistically significant differences between the experimental groups are indicated by asterisks *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Figure 2. Effects of serum deprivation on basal and glucose-stimulated insulin secretion. INS-1 832/13 cells were grown in RPMI 1640 (11.1 mM glucose) supplemented with 10% FBS (growth medium); at 80% confluence the cells were washed and incubated in growth medium or in basal serum-free medium for 16 hrs. Insulin secretion was measured as described in Methods. Values represent the means±SEM of 6 samples in a representative experiment. The figures show the means±SEM of all data from two independent experiments, each of which contained 6 wells per treatment group. Statistically significant differences between the experimental groups are indicated by asterisks *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Figure 3. Dose-dependent effects of PRL and glucocorticoids on gene expression in INS-1 cells. INS-1 cells were incubated for 20 h in serum-free basal medium in the presence or absence of rat PRL (10-500 ng/ml, 0.4-20 nM), DEX (0.02-1 μM, 20-1000 nM), hydrocortisone (HC, 15-60 μg/dL, 410-1640 nM), or diluent. mRNA levels were measured by Q-PCR. Values in diluent-treated control cells were adjusted so that the mean equaled 1.0; values for hormone-treated cells were calculated as a function of the mean of control values. The figure shows the mean±SEM of 4 flasks per treatment group. PRL had statistically significant effects ($p<0.01$) on gene expression at concentrations exceeding 10 ng/ml (0.4 nM). DEX and HC had statistically significant effects ($p<0.001$) on gene expression at all concentrations tested.

Figure 4. Effects of PRL on beta cell gene expression in INS-1 cells and primary rat islets. INS-1 cells **(a)** or primary rat islets **(b)** were incubated for 20 hrs in serum-free DMEM (5.5 mM glucose, 0.1% HSA for INS-1 cells) or serum-free RPMI (6.8 mM glucose, 0.1% HSA for rat islets) in the presence or absence of rat PRL (20 nM). mRNA levels were measured by Q-PCR. Values in diluent-treated control cells (-) were adjusted so that the mean equaled 1.0; values for hormone-treated cells were calculated as a function of the mean of control values. The figures show the means±SEM of all data from 2 (islets) – 5 (INS-1 cells) independent experiments, each of which contained 4 flasks per treatment group. Statistically significant differences between the PRL and control (-) groups are indicated by asterisks *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Figure 5. Effects of PRL and DEX on gene expression in INS-1 cells. INS-1 cells were incubated for 20 h in serum-free basal medium **(a)** or in basal medium containing 1% **(b)** or 10% **(c)** calf serum in the presence or absence of rat PRL (20 nM), DEX (0.1 μM) a combination of the two, or diluent. mRNA levels were measured by Q-PCR. Values in

diluent-treated control cells were adjusted so that the mean equaled 1.0; values for hormone-treated cells were calculated as a function of the mean of control values. The figures show the means±SEM of 4 flasks per treatment group. Statistically significant differences between the experimental (hormone-treated) and control groups are indicated by asterisks *p<0.05, **p<0.01, ***p<0.001. Statistical differences between the DEX group and the PRL + DEX group are indicated by the p values above the horizontal connecting lines.

Figure 6. Effects of PRL and DEX on FoxO1 content, phosphorylation and cellular distribution. INS-1 832/13 cells were grown in serum-containing medium and then incubated in serum-free in the presence of rat PRL (40 nM), DEX (1 μM), a combination of the two, or diluent in basal medium for 15 minutes or 16 hrs. Phoshorylated FoxO1 (cyto phos), nuclear FoxO1, and tubulin were detected by Western blot, as described in Methods. Similar results were obtained in 3 experiments.

Figure 7. PRL induces STAT5 binding to a consensus sequence on the rat FoxO1 promoter. Nuclear proteins were prepared from INS-1 cells treated with PRL (20 nM) or diluent for 30 minutes. The proteins were incubated with radiolabeled double stranded oligonucleotides encoding STAT5 consensus sequences in the rat beta casein promoter or the rat FoxO1 promoter **(Figure 6a)**. Parallel incubations contained a 200-fold excess of cold competitor (CC) double-stranded oligonucleotides encoding the STAT5 sequence in the rat beta casein gene or 2 μg of polyclonal anti-STAT5a antibody (Ab, **Figure 6b**). The protein-DNA complexes were separated by PAGE. The shifted bands (arrow) represent binding of STAT5 to DNA. The arrowhead (**Figure 6b**) represents the supershifted complex.

Figure 8. Over-expression of STAT5b mimics and potentiates the effects of PRL on beta cell gene expression. INS-1 832-13 cells were transfected with recombinant adenoviruses expressing a constitutively active, murine STAT5b adenovirus or green fluorescent protein (control). After 24 h of transduction, the conditioned media was analyzed (**Figure 8a**) for STAT5 protein using a polyclonal antibody to STAT5**.** The cells were washed with basal media and then incubated for an additional 24 hrs with PRL (20 nM) or diluent. mRNA levels were measured by Q-PCR. Values in diluent-treated control cells expressing green fluorescent protein (Control) were adjusted so that the mean equaled 1.0; values for STAT5b-expressing and hormone-treated cells were calculated as a function of the mean of control values. **Figure 8b** shows the means±SEM of all data from from 2 independent experiments, each of which contained 4 flasks per group. Statistically significant differences between the experimental (hormone-treated) and control groups are indicated by asterisks *$p<0.05$, **$p<0.01$, ***$p<0.001$. Statistical differences between the DEX group and the PRL + DEX group are indicated by the p values above the horizontal connecting lines.

Figure 9. Effects of PRL and DEX on INS-1 cell fatty acid oxidation and esterification. INS-1 cells were incubated for 24 h in serum-free RPMI (11.1 mM glucose, 0.1% HSA) in the presence or absence of rat PRL (20 nM), DEX (1 μM), a combination of the two, or diluent (Control). Rates of ^{14}C-palmitate oxidation and esterification were measured as described in Materials and Methods. Values, expressed as nmol/mg protein/hr, represent the means±SE of all data from two independent experiments, each of which contained 6 wells per group. Statistically significant differences between the experimental (hormone-treated) and control groups are indicated by asterisks *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Figure 10. Effects of PRL and DEX on glucose-stimulated insulin secretion in INS-1 832/13 cells. INS-1 832/13 cells were grown in RPMI 1640 (11.1 mM glucose) supplemented with 10% FBS; at 80% confluence the cells were washed and incubated for 16 hrs in basal medium (DMEM with 5.5 mM glucose) containing rat PRL (20 nM), DEX (0.1μM), a combination of the two, or diluent (Control), and insulin secretion was measured as described in Methods. Values represent the means±SE of all data from two independent experiments, each of which contained 5 samples per group. Statistically significant differences between the experimental (hormone-treated) and control groups are indicated by asterisks *$p<0.05$, **$p<0.01$.

Table 1. Analysis of gene expression in INS-1 cells by quantitative real time PCR.

Values were obtained in cells grown in RPMI 1640 (11.1 mM glucose) supplemented with 10 % FBS, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 10 mM HEPES, and 1% antibiotic/antimycotic solution

Gene	Accession #	Primer sequence (top forward, bottom reverse)	C_T
Rat PGC1α	**NM_031347**	**5′ GCT GGA TGG CTT GGG ACA T 3′** **5′ CAA CCA GGG CAG CAC ACT CT 3′**	**25**
Rat PPAR α	**NM_013196**	**5′ CCG TCG GGA TGT CAC ACA 3′** **5′ TTG CTT TCT CAG ATC TTG GCA TT 3′**	**24**
Rat PPAR γ	**AF246458**	**5′ GGA TGT CTC ACA ATG CCA TCA G 3′** **5′ CGC CAA CAG CTT CTC CTT CT 3′**	**29**
Rat FoxO1	**XM_001056726**	**5′ TGT GCC CTA CTT CAA GGA TAA GG 3′** **5′ GTG GCG AAT TGA ATT CTT CCA 3′**	**22**
Rat UCP2	**NM_019354**	**5′ AAG ACC ATT GCA CGA GAG GAA 3′** **5′ TGG CAT TTC GGG CAA CAT 3′**	**17**
Rat CPT1	**NM_031559**	**5′ AAT TGC AGT GGT ATT TGA AGC TAA AA 3′** **5′ GAT ATA TTC TTC CCA CCA GTC ACT CA 3′**	**22**
Rat ADD1	**AF286470**	**5′ GCT ACC GTT CCT CTA TCA ATG ACA 3′** **5′ GCA AGA CAG CAG ATT TAT TCA GCT T 3′**	**20**
Rat Glut2	**NM_012879**	**5′ GCT TCC AGT ACA TTG CGG ACT T 3′** **5′ AGG ACC ACC CCA GCA AAA A 3′**	**17**
Rat riboprotein	**X15096**	**5′ CCC AGA GGT GCT GGA CAT CA 3′** **5′ GCG GAC ACC CTC TAG GAA GC 3′**	**16**

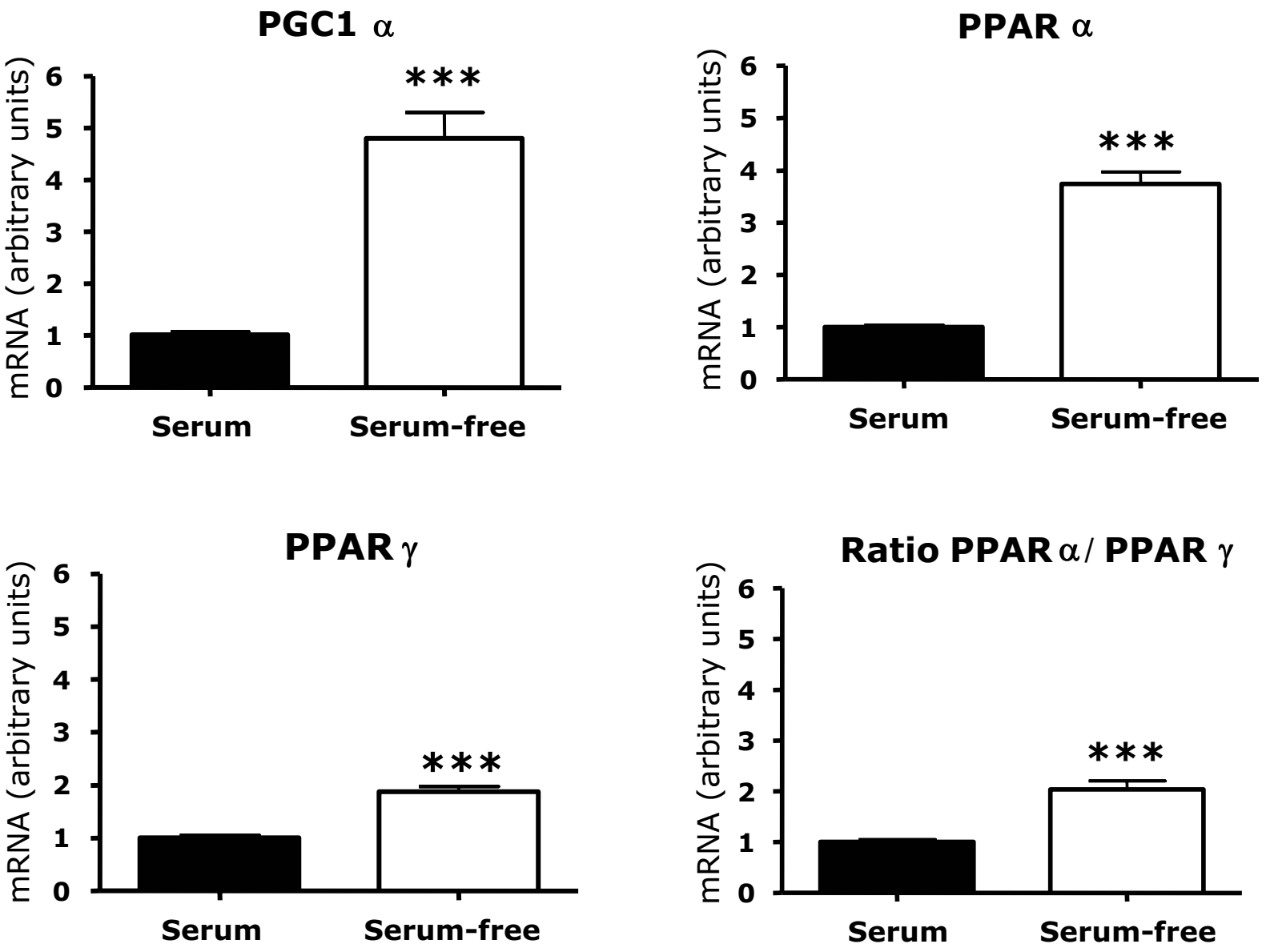

PGC1 α
mRNA (arbitrary units)
0
1
2
3
4
5
6
***
Serum
Serum-free
PPAR α
mRNA (arbitrary units)
0
1
2
3
4
5
6
***
Serum
Serum-free
PPAR γ
mRNA (arbitrary units)
0
1
2
3
4
5
6
***
Serum
Serum-free
Ratio PPAR α/ PPAR γ
mRNA (arbitrary units)
0
1
2
3
4
5
6
***
Serum
Serum-free

Figure 1a

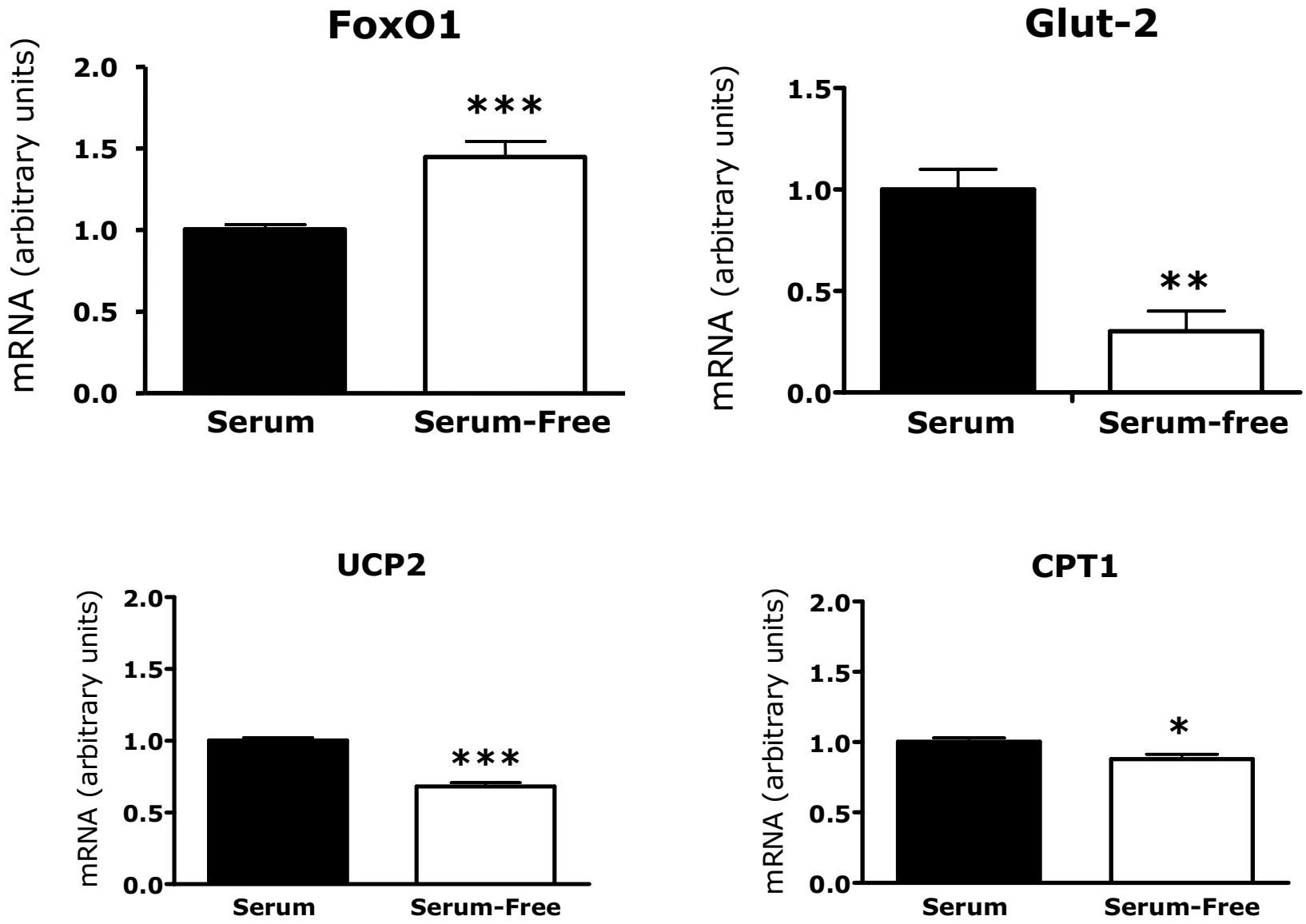

FoxO1
mRNA (arbitrary units)
2.0
1.5
1.0
0.5
0.0
***
Serum
Serum-Free
Glut-2
mRNA (arbitrary units)
1.5
1.0
0.5
0.0
**
Serum
Serum-free
UCP2
mRNA (arbitrary units)
2.0
1.5
1.0
0.5
0.0
***
Serum
Serum-Free
CPT1
mRNA (arbitrary units)
2.0
1.5
1.0
0.5
0.0
*
Serum
Serum-Free

Figure 1b

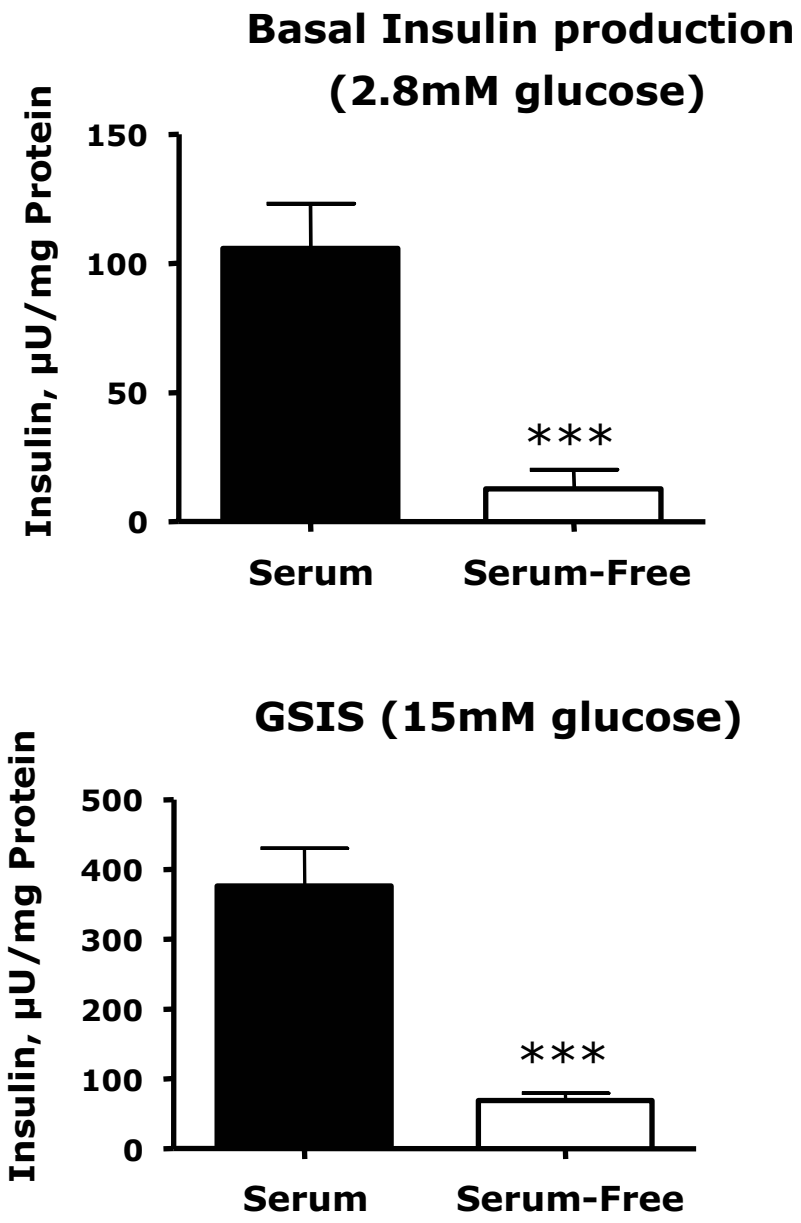

Basal Insulin production
(2.8mM glucose)
Insulin, µU/mg Protein
150
100
50
0
***
Serum
Serum-Free
GSIS (15mM glucose)
Insulin, µU/mg Protein
500
400
300
200
100
0
***
Serum
Serum-Free

Figure 2

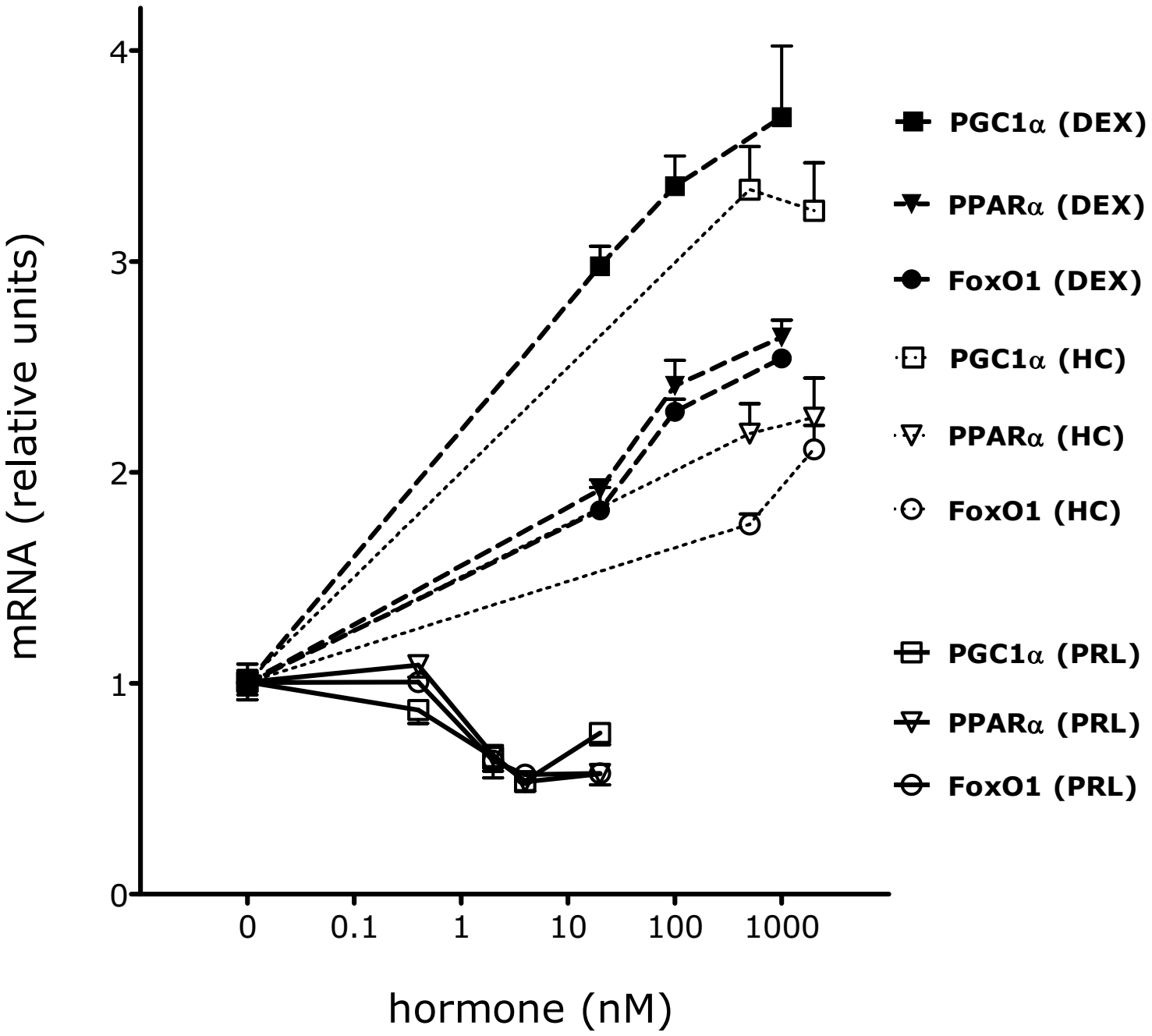

mRNA (relative units)
0
1
2
3
4
0
0.1
1
10
100
1000
hormone (nM)
PGC1α (DEX)
PPARα (DEX)
FoxO1 (DEX)
PGC1α (HC)
PPARα (HC)
FoxO1 (HC)
PGC1α (PRL)
PPARα (PRL)
FoxO1 (PRL)

Fig 3

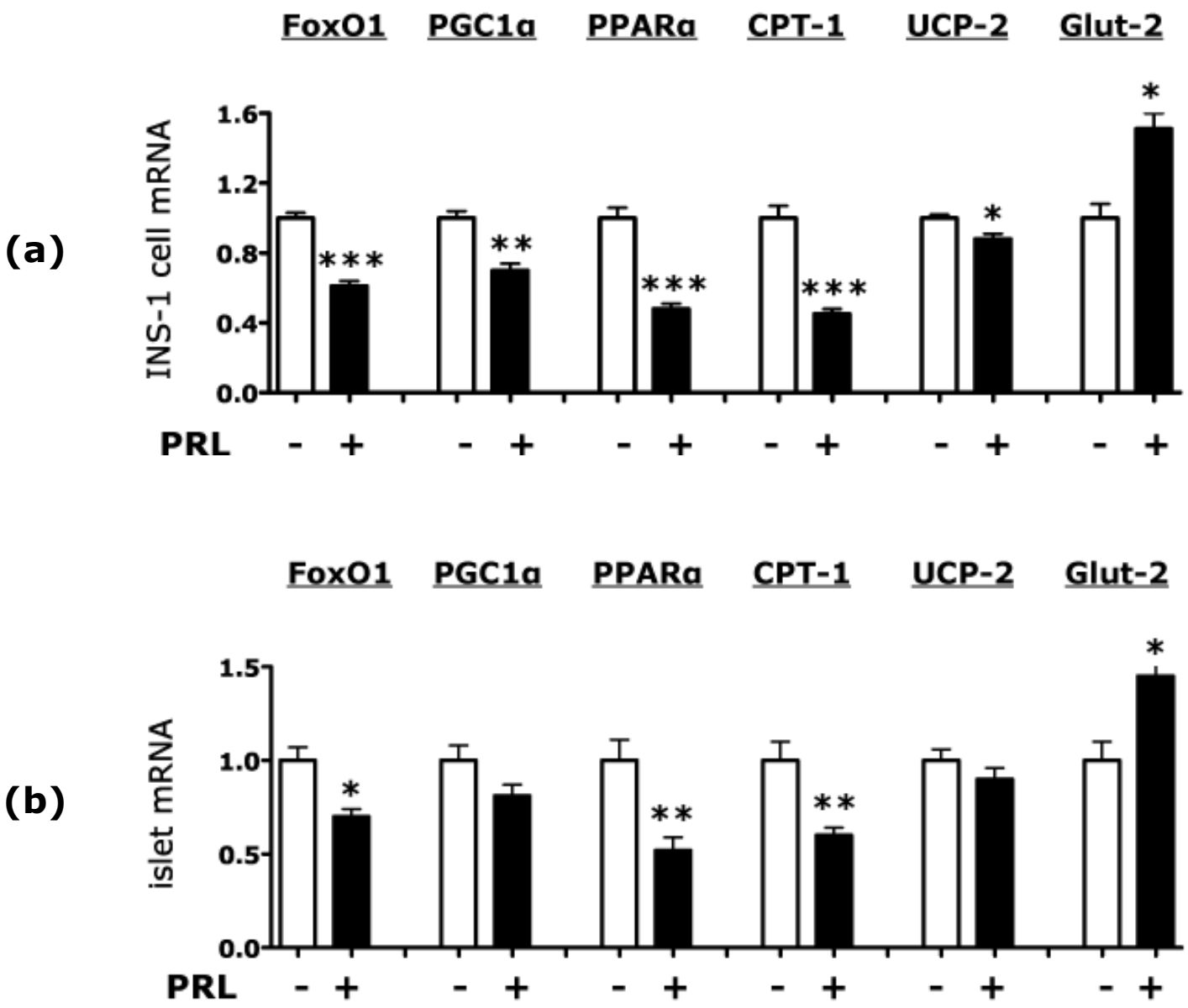

(a)
FoxO1
PGC1α
PPARα
CPT-1
UCP-2
Glut-2
INS-1 cell mRNA
1.6
1.2
0.8
0.4
0.0
***
**
***
***
*
*
PRL
- + - + - + - + - + - +
(b)
FoxO1
PGC1α
PPARα
CPT-1
UCP-2
Glut-2
islet mRNA
1.5
1.0
0.5
0.0
*
**
**
*
PRL
- + - + - + - + - + - +

Figure 4

Serum-free medium

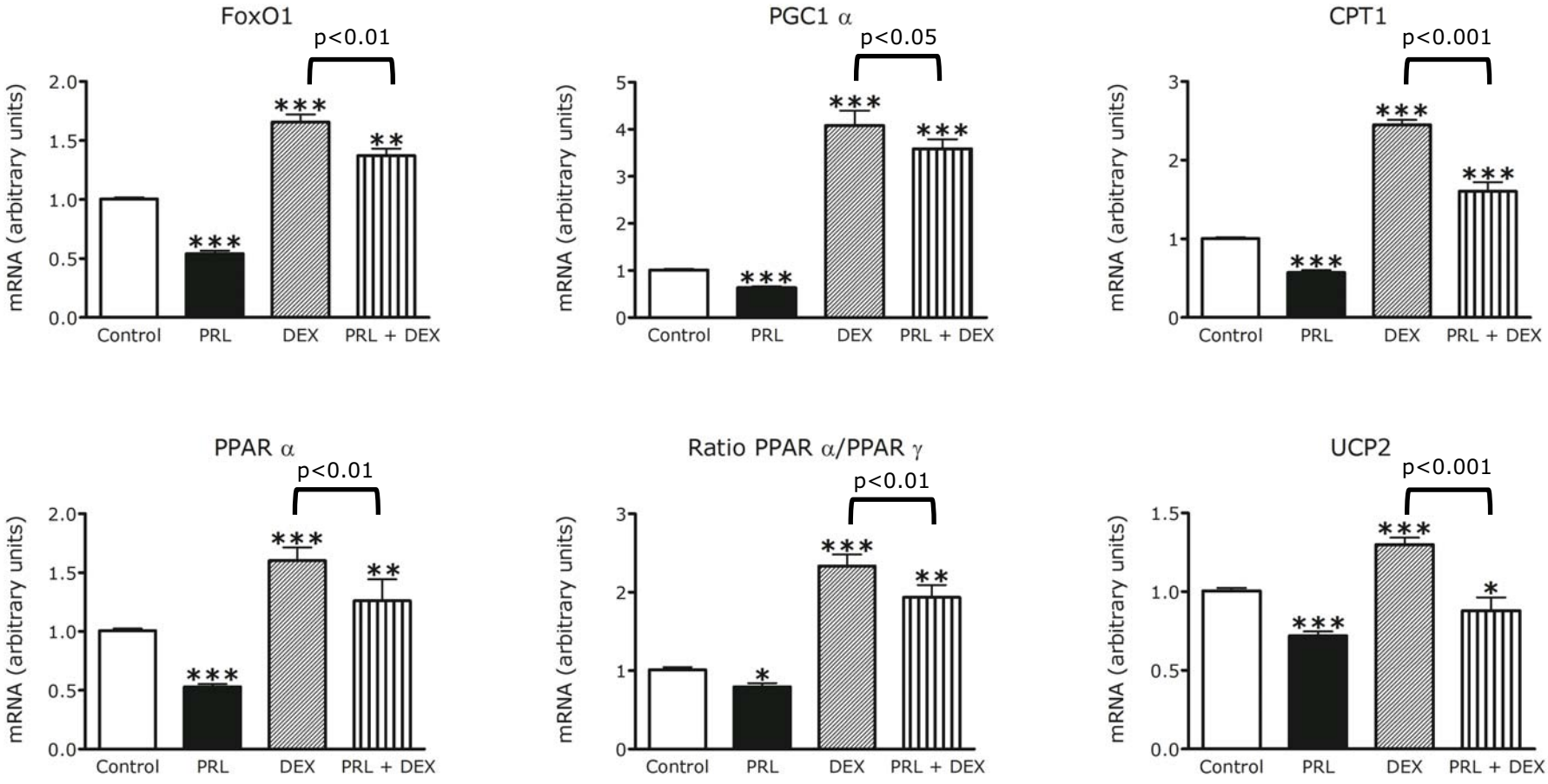


Figure 5a

1% calf serum

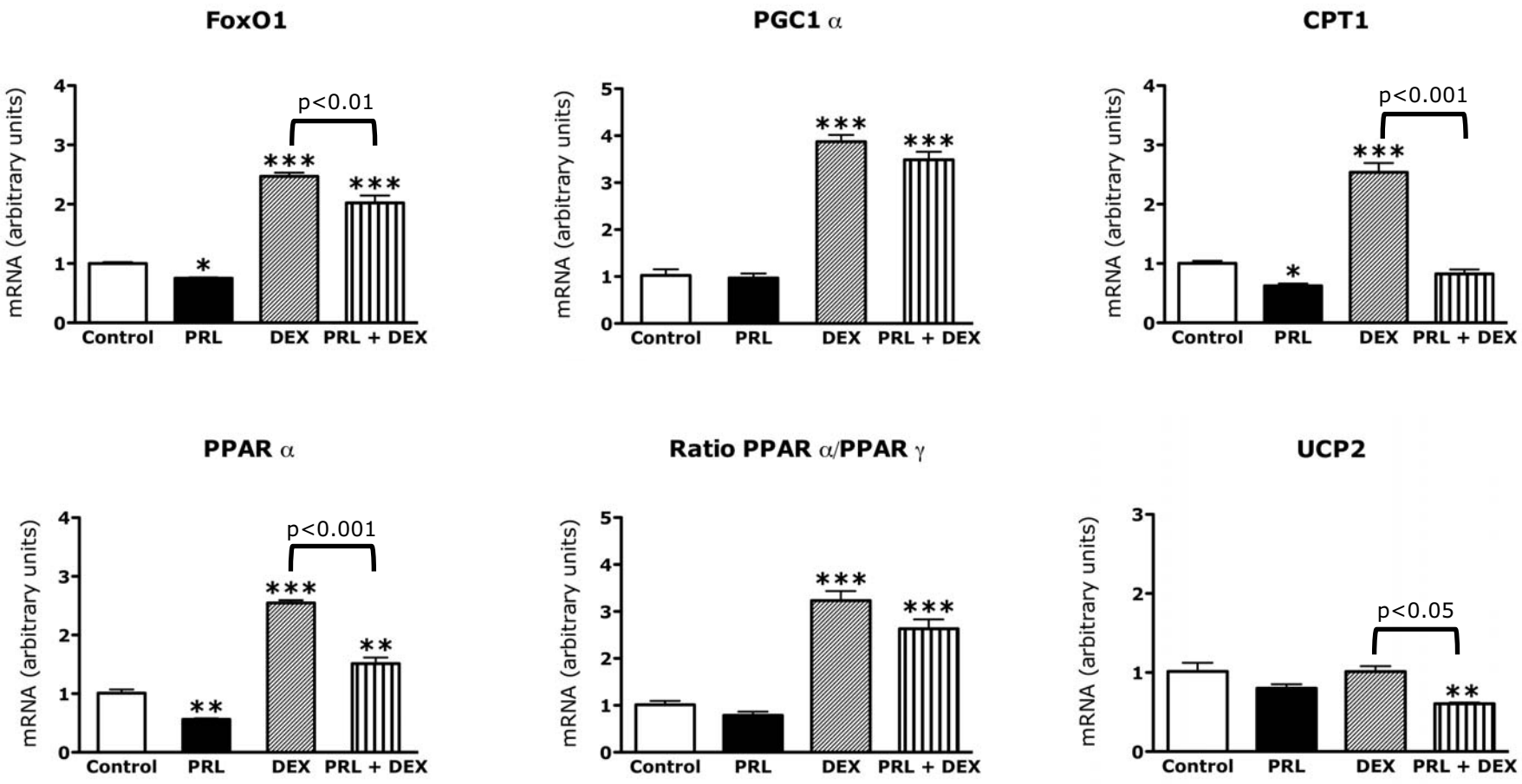


Figure 5b

10% calf serum

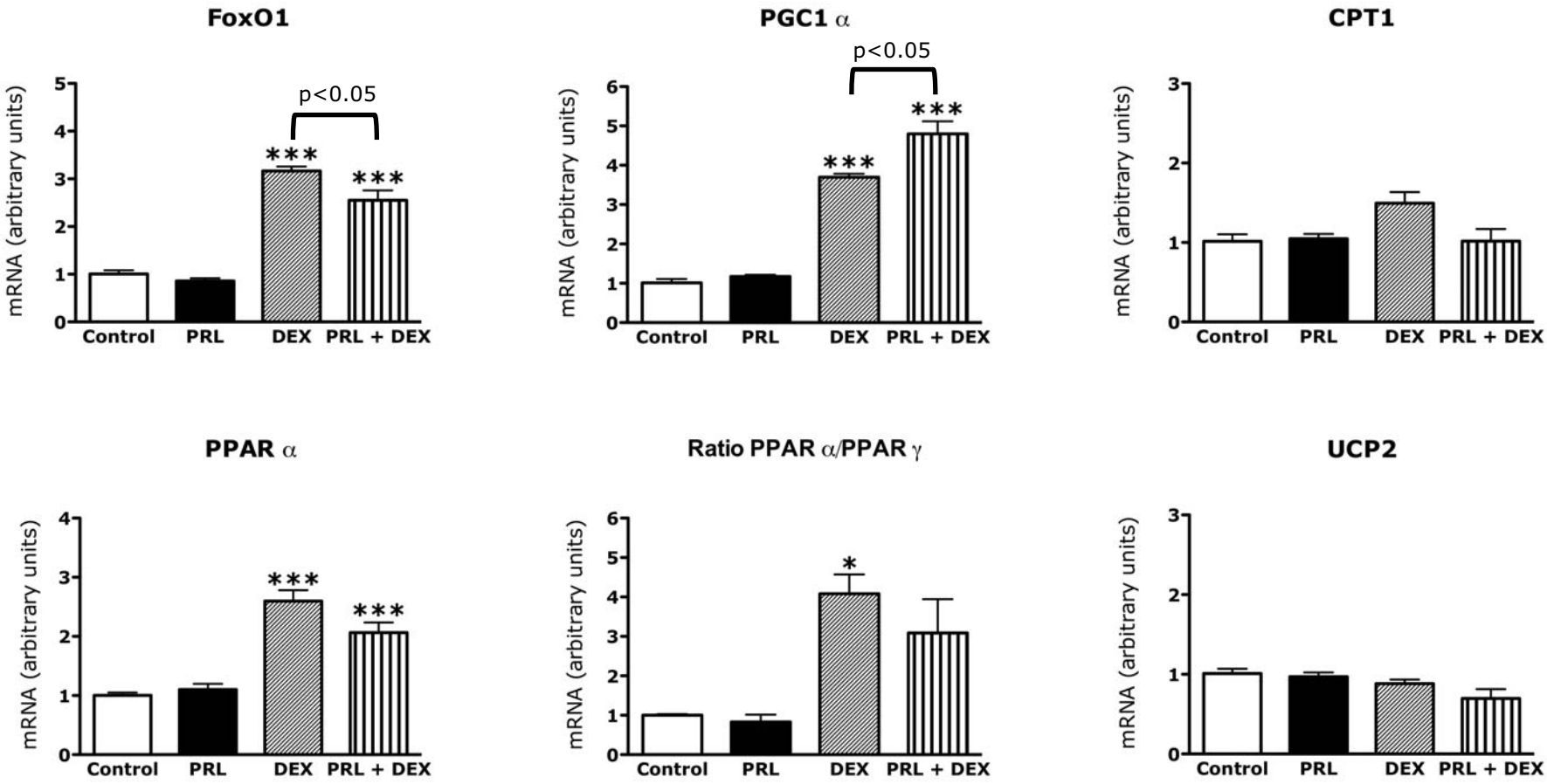
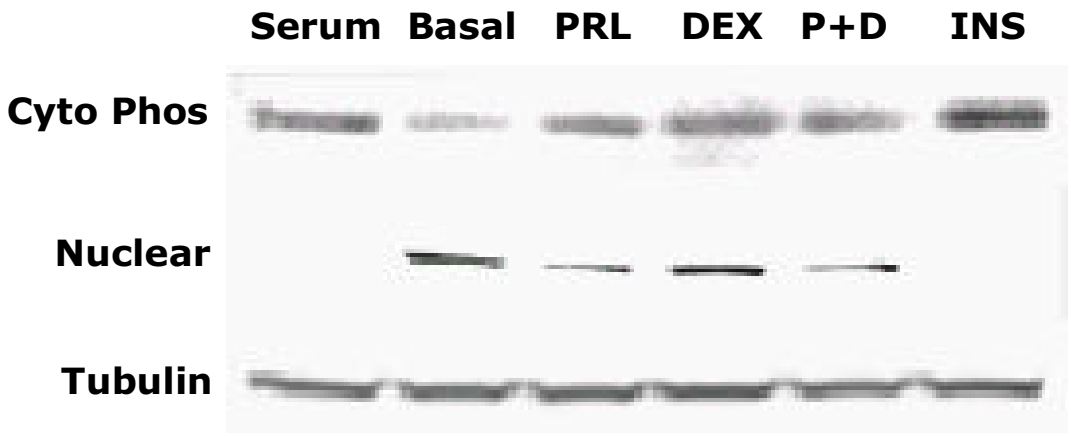


Figure 5c

15 min



16 hr

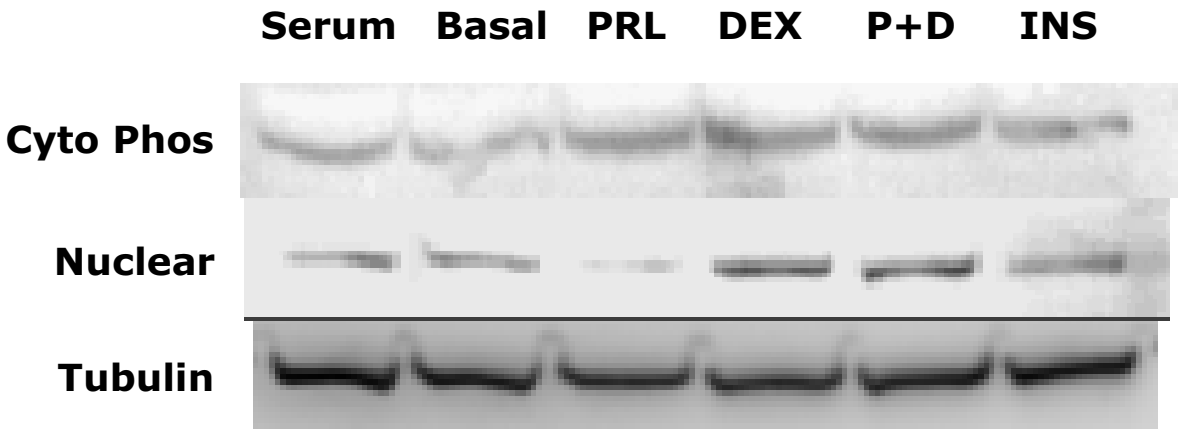


Figure 6

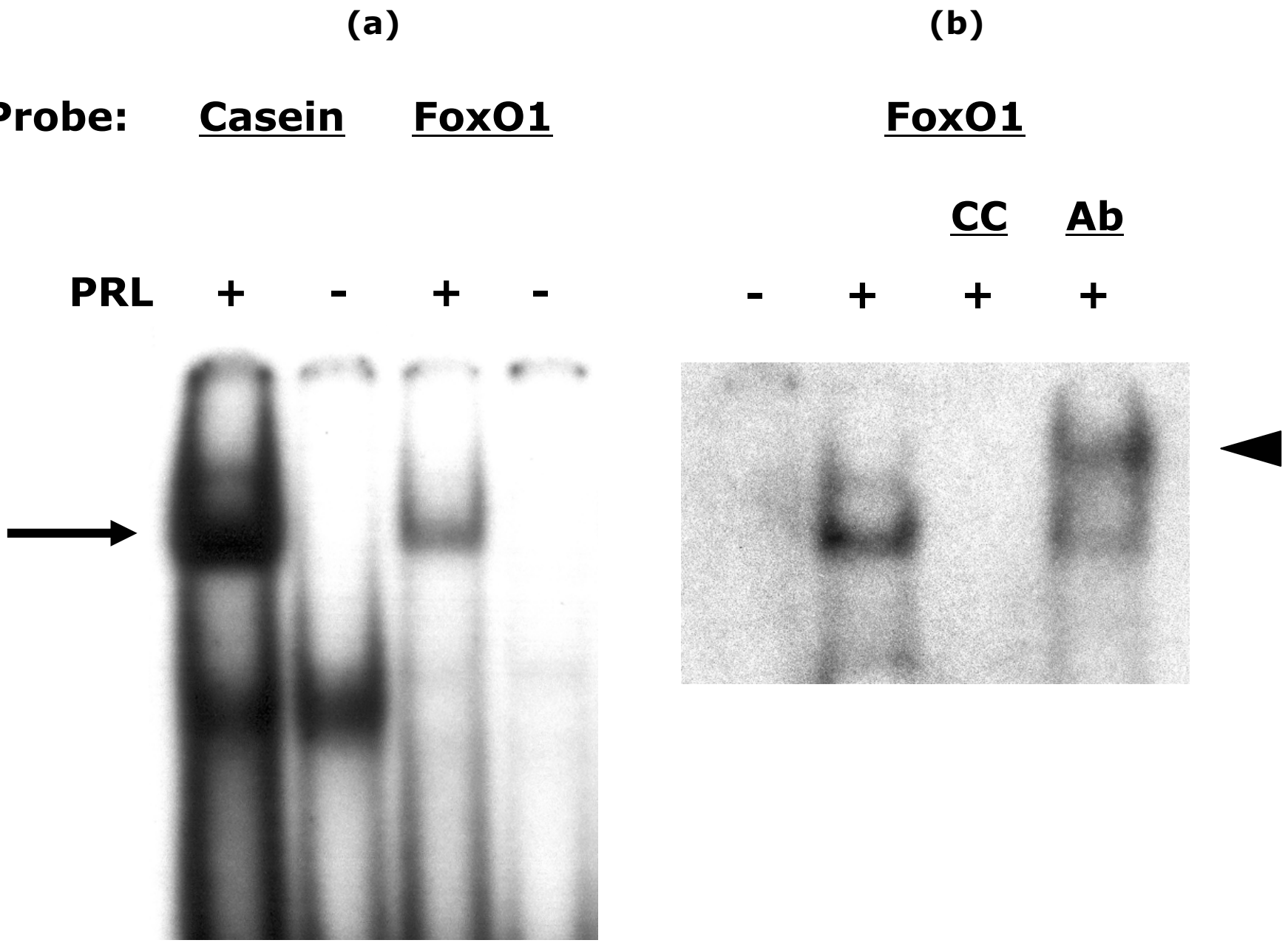
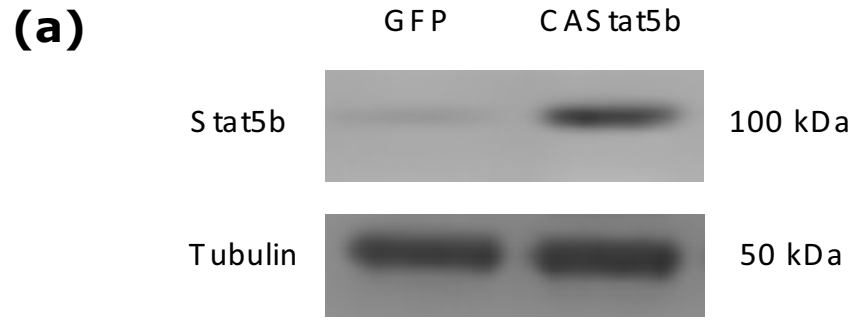
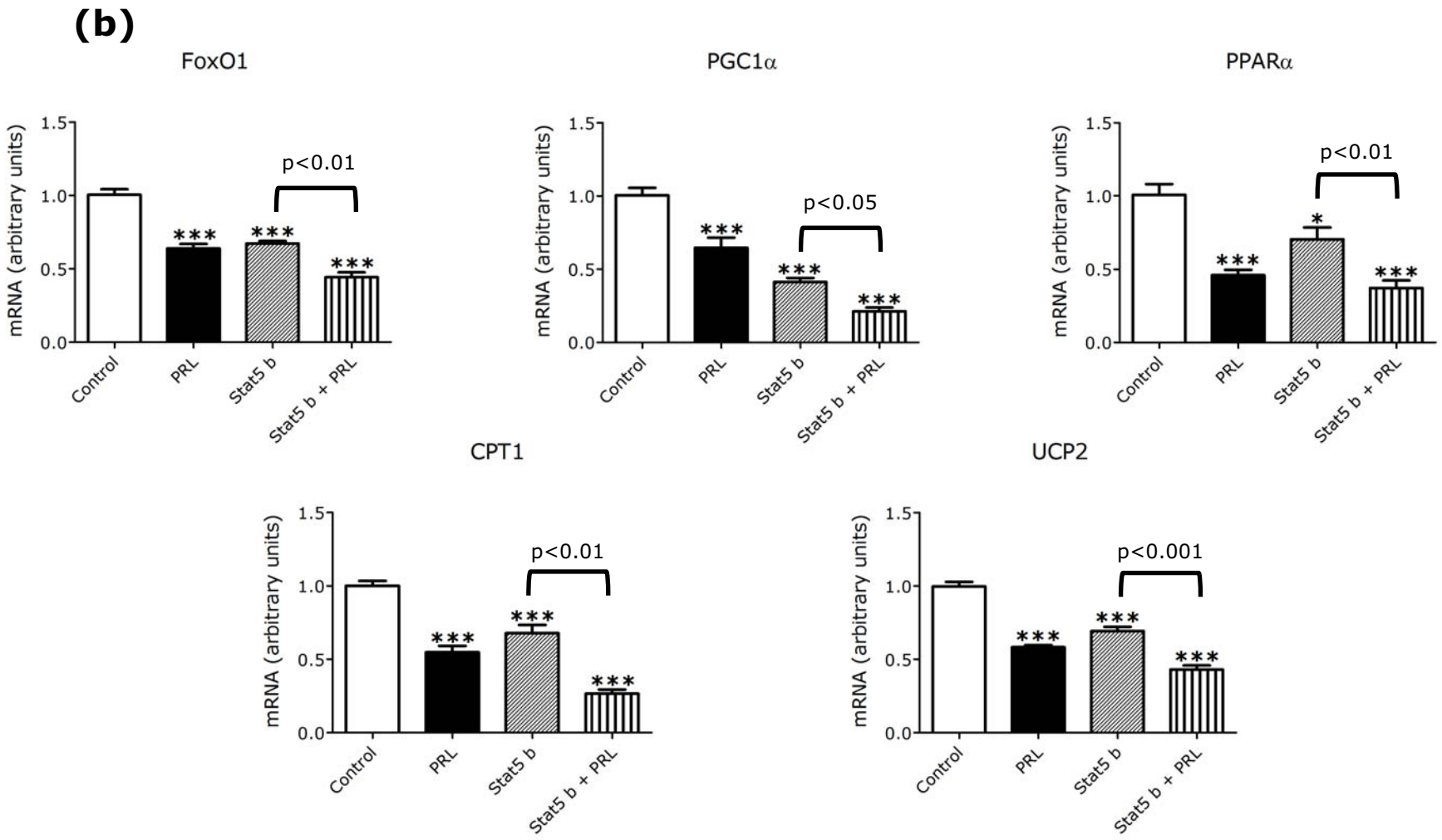


Figure 7

Figure 8


(a)
GFP
CAStat5b
Stat5b
100 kDa
Tubulin
50 kDa


(b)
FoxO1
PGC1α
PPARα
CPT1
UCP2
mRNA (arbitrary units)
Control
PRL
Stat5 b
Stat5 b + PRL
p<0.01
p<0.05
p<0.001
***
*

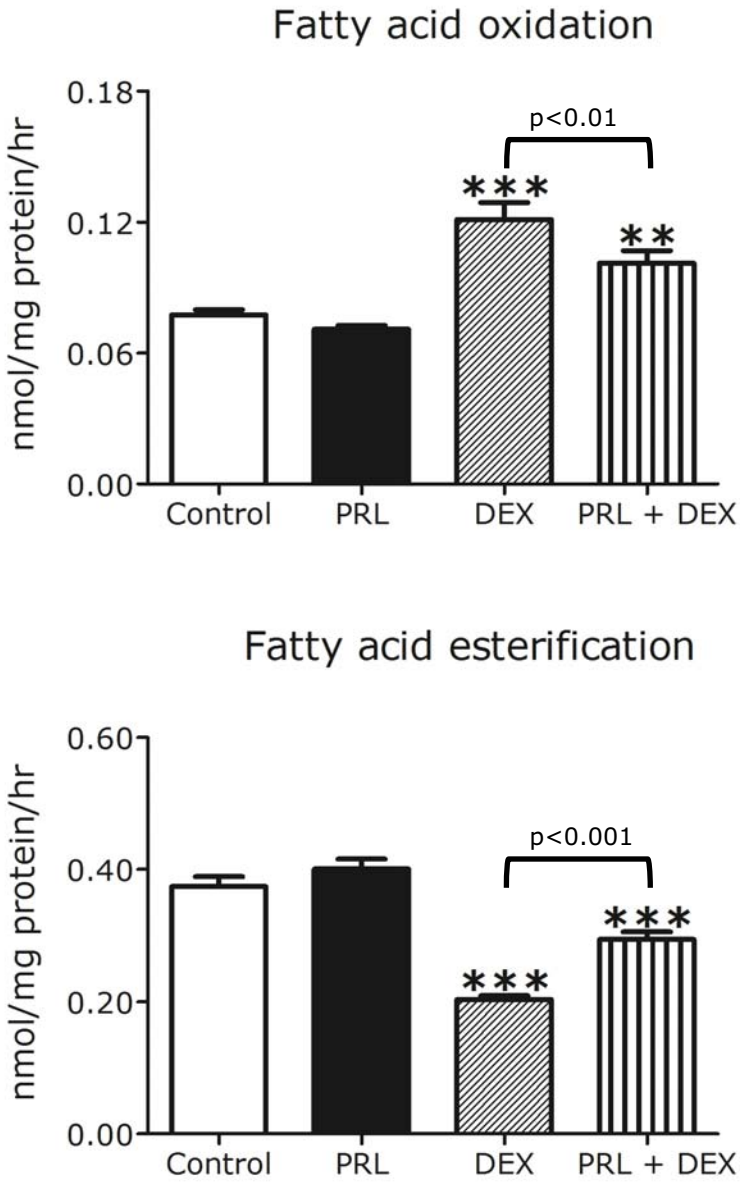

Fatty acid oxidation
nmol/mg protein/hr
0.18
0.12
0.06
0.00
p<0.01
***
**
Control
PRL
DEX
PRL + DEX
Fatty acid esterification
nmol/mg protein/hr
0.60
0.40
0.20
0.00
p<0.001
***
***
Control
PRL
DEX
PRL + DEX

Figure 9

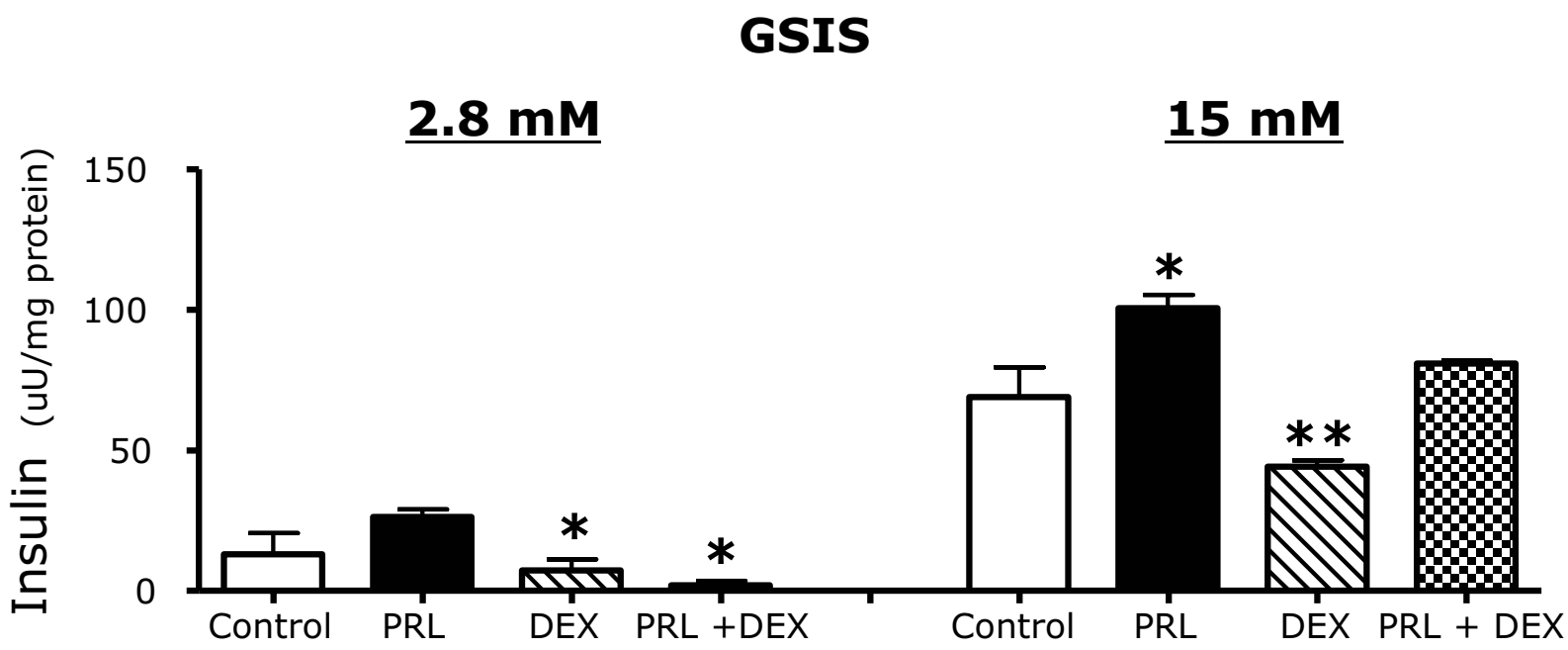

GSIS
2.8 mM
15 mM
Insulin (uU/mg protein)
150
100
50
0
*
*
*
**
Control
PRL
DEX
PRL +DEX
Control
PRL
DEX
PRL + DEX

Figure 10