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Research Article

Pregnancy-associated Steroid Effects on Insulin Sensitivity, Adipogenesis, and Lipogenesis: Role of Wnt/β-Catenin Pathway

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Abstract

Context: The shift in maternal energy metabolism characteristic of pregnancy is thought to be driven by various hormonal changes, especially of ovarian and placental steroids. Imbalances in circulating estradiol (E_2) and progesterone (P_4) levels during this period are often associated with metabolic disturbances leading to the development of gestational diabetes mellitus (GDM). Since abnormalities in the Wnt pathway effector transcription factor 7-like 2 (TCF7L2) are commonly associated with the occurrence of GDM, we hypothesized that the canonical or β-catenin-dependent Wnt signaling pathway mediates the metabolic actions of E_2 and P_4.

Objective: Our study was aimed at elucidating the metabolic function of the steroids E_2 and P_4, and examining the role of the canonical Wnt signaling pathway in mediating the actions of these steroids.

Methods: The ovariectomized (OVX) rat was used as a model system to study the effect of known concentrations of exogenously administered E_2 and P_4. Niclosamide (Nic) was administered to block Wnt signaling. 3T3-L1 cells were used to analyze changes in differentiation in the presence of the steroids or niclosamide.

Results: In the present study, we observed that E_2 enhanced insulin sensitivity and inhibited lipogenesis while P_4 increased lipogenic gene expression—in 3T3-L1 adipocytes, and in adipose tissue and skeletal muscle of OVX rats when the dosage of E2 and P4 mimicked that of pregnancy. Both E_2 and P_4 were also found to upregulate Wnt signaling. Nic nhibited the steroid-mediated increase in Wnt signaling in adipocytes and OVX rats. The insulin-sensitizing and antilipogenic actions of E_2 were found to be mediated by the canonical Wnt pathway, but the effects of P_4 on lipogenesis appeared to be independent of it. Additionally, it was observed that inhibition of Wnt signaling by Nic hastened adipogenic differentiation, and the inhibitory effect of E_2 on differentiation was prevented by Nic.

Conclusion: The findings presented in this study highlight the role of steroids and Wnt pathway in glucose and lipid metabolism and are relevant to understanding the pathophysiology of metabolic disorders arising from hormonal disturbances.

Key Words: estradiol, progesterone, adipose tissue, skeletal muscle, lipid metabolism, insulin signaling

Abbreviations: *Acadm*, acyl-CoA dehydrogenase medium chain; *Adipoq*, adiponectin; ANOVA, analysis of variance; *Axin2*, axis inhibition protein 2; *B2m*, β-2 microglobulin; *Cebpa*, CCAAT/enhancer binding protein alpha; *Dbi*, diazepam binding inhibitor or acyl-CoA binding protein; *Dgat1*, diacylglycerol O-acyltransferase 1; DPBS, Dulbecco's phosphate-buffered saline; Dvl2, Dishevelled 2; E_2, estradiol; *Fabp4*, fatty acid binding protein 4; *Fasn*, fatty acid synthase; *Foxo1*, Forkhead box protein O1; GDM, gestational diabetes mellitus; GLUT4, glucose transporter 4; GWAT, gonadal white adipose tissue; HRP, horseradish peroxidase; *Insr*, insulin receptor; i.p., intraperitoneally; *Lep*, leptin; *Lpl*, lipoprotein lipase; *Lrp5*, low-density lipoprotein receptor-related protein 5; *Mogat1*, monoacylglycerol O-acyltransferase 1; Nic, niclosamide; NP, non-pregnant; OVX, ovariectomized; P_4, progesterone; *Ppard*, peroxisome proliferator-activated receptor delta; *Pparg*, peroxisome proliferator-activated receptor gamma; *Rpl19*, ribosomal protein L19; *Sfrp1*, secreted Frizzled-related protein 1; *Slc2a4*, solute carrier family 2 member 4 or GLUT4; *Slc27a1*, solute carrier family 27 member 1 or long-chain fatty acid transport protein 1; *Tcf7l2*, transcription factor 7-like 2; Veh, vehicle.

Estrogens, progestins, and androgens are vital to reproductive function and the sustenance of pregnancy. Hormones like 17β-estradiol (E_2), belonging to the estrogen class of steroids, and progesterone (P_4), the most prominent steroid of the progestin category, are additionally thought to influence energy metabolism and mediate metabolic adaptations in the mother's body to accommodate the energy demands of the developing fetus [1, 2]. Such adaptations typically involve decreasing the insulin sensitivity of maternal tissues like the skeletal muscle, adipose tissue, and liver [3, 4], thereby decreasing their glucose uptake and ensuring glucose availability for the uterus and placenta for fetal nourishment. The maternal fat depots also undergo a shift from a lipogenic state in early pregnancy to a lipolytic state in late pregnancy, thereby enabling the use of fatty acids as fuel [3]. It has been hypothesized that P_4 during pregnancy is the major contributing factor for maternal insulin resistance [5]. Although E_2 is generally associated with increased insulin sensitivity, exposure to very

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high levels of the hormone (like those characteristic of pregnancy) is reported to cause insulin resistance [5, 6]. Some studies have shown that disturbances in the ratios of these steroids during pregnancy perturb the metabolic balance in the maternal system and may contribute to the development of gestational diabetes mellitus (GDM) [1, 7].

GDM is a condition characterized by high blood sugar levels during pregnancy in women without preexisting diabetes. On average, it affects about 8% to 10% of pregnancies [8]. Reports suggest that the incidence of GDM has doubled over the last decade, much like the obesity epidemic [9]. Heightened insulin resistance of maternal tissues is believed to be the primary determinant in the development of this condition [10-12]. While the impaired insulin sensitivity characteristic of normal pregnancy is compensated for by the large quantities of insulin secreted by the pancreas, the GDM-affected maternal system fails to respond to even the increased insulin concentrations. GDM manifests typically during the second or third trimester of pregnancy, when insulin resistance is known to set in [13]. The condition usually corrects itself soon after the birth of the child, and maternal metabolism returns to its prepregnancy state [9]. However, uncontrolled glucose levels in circulation can cause complications in pregnancy, resulting in premature delivery, stillbirths and neonatal mortality, and macrosomia and hypoglycemia in the newborn [14]. The mother and child are at higher risk of developing type 2 diabetes, obesity, and other metabolic disorders later in life [13].

Apart from steroids, genetic factors also contribute to the risk of developing GDM. In the population, single-nucleotide variations (formerly single-nucleotide polymorphisms) in the transcription factor 7-like 2 (*TCF7L2*) gene (especially rs7903146) are reported to have the highest association with GDM and type 2 diabetes [15-17]. TCF7L2 is a transcription factor activated by the canonical Wnt signaling cascade. The Wnt pathway, an evolutionarily conserved pathway, is known to be involved in many developmental processes. Reports have suggested that Wnt signaling may modulate glucose uptake and insulin sensitivity in metabolically active tissues [18-20]. Studies have also reported that Wnt signaling represses adipogenesis and lipogenesis [21]. However, the influence of this pathway on the metabolic disturbances associated with GDM remains to be understood.

A few recent reports have implied that canonical or β-catenin-dependent Wnt signaling/the TCF7L2 gene product may be involved in mediating the effects of E_2 and P_4 on glucose and lipid metabolism in pancreatic islets and hepatocytes in vitro [22, 23]. It remains to be understood whether the effects of these steroids in the adipose tissue and skeletal muscle are dependent on the Wnt pathway. The adipose tissue is the primary storage site for energy in the body as fat. The uptake of fatty acids by this tissue and the process of lipogenesis are sensitive to insulin. The skeletal muscles also respond to insulin and collectively account for about 70% of the whole-body glucose uptake. These characteristics make the adipose tissue and skeletal muscle active players in energy metabolism.

In the present study, we investigated the involvement of the canonical Wnt signaling pathway in E_2- and P_4-mediated actions on insulin sensitivity, adipogenesis, and lipogenesis (i) in cultured adipocytes and (ii) in the adipose tissue and skeletal muscle of the female rat. We also examined whole-body parameters in the rat, like body weight, food consumption, and blood glucose levels. We demonstrate that the actions of E_2 on enhancing insulin sensitivity and repressing lipogenesis, as observed in our study, are mediated by the canonical Wnt signaling pathway. However, the effect of P_4 on lipogenesis did not appear to involve Wnt signaling. We also show that inhibiting Wnt signaling using the drug niclosamide (Nic) had adverse effects on pregnancy in rats.

Materials and Methods

Cell Culture

3T3-L1 mouse preadipocytes (ATCC CL-173; RRID: CVCL_0123) were obtained from American Type Culture Collection (ATCC) and were grown in complete medium consisting of Dulbecco's Modified Eagle's Medium containing 4500 mg/L glucose (Sigma-Aldrich Corporation), 10% fetal bovine serum (Gibco/Thermo Fisher Scientific), and 1X antibiotic-antimycotic (containing 100 U/mL penicillin, 0.1 mg/mL streptomycin and 0.25 µg/mL amphotericin B; Sigma-Aldrich Corporation) at 37 °C under a humidified atmosphere of 5% CO_2 in air. Cells used for experiments were transitioned to phenol red-free Dulbecco's Modified Eagle's Medium (Sigma-Aldrich Corporation) with fetal bovine serum and antibiotic-antimycotic, as phenol red is reported to have weak estrogenic activity [24]. Cells from passage numbers between 17 and 20 were used for experiments.

Experiments in preadipocytes

For experiments in preadipocytes, 3T3-L1 cells were seeded in 24- or 12-well plates (Corning Inc) at a density of 1.1×10^4 cells/cm^2. On reaching about 80% confluence, the monolayer was rinsed in Dulbecco's phosphate-buffered saline (DPBS; Sigma-Aldrich Corporation) to remove any traces of serum and incubated in serum-free medium for 6 hours. Cells were then incubated in serum-free media containing various combinations of the Wnt signaling inhibitor Nic, E_2, and P_4 for 18 hours. The treatment groups consisted of the following: Ctrl (no steroids or Nic), Nic, E_2, E_2 + Nic, P_4, P_4 + Nic, E_2 + P_4, and E_2 + P_4 + Nic. Nic, E_2 and P_4 (all purchased from Sigma-Aldrich Corporation) were dissolved in ethanol and used at concentrations of 1 µM, 100 and 1 µM, respectively. Ethanol was added to a final concentration of 0.03% in Ctrl wells. The 18 hours treatment duration was chosen based on time-course studies in adipocytes to determine the efficacy of Nic, E_2, and P_4 in initiating changes at the transcriptional level. Each treatment was carried out in triplicates. The monolayer was then processed for RNA isolation or protein lysate preparation.

Experiments in adipocytes

To differentiate preadipocytes into adipocytes, preadipocytes were seeded in 24- or 12-well plates at a density of 1.1×10^4 cells/cm^2. At this seeding density, cells would typically reach 100% confluence after about 48 hours, at which point the spent media was removed and fresh complete media was added to the monolayer. After another 48 hours of incubation, the media was changed to fresh complete medium supplemented with 0.5 mM 3-isobutyl 1-methyl xanthine (IBMX), 1 µM dexamethasone, and 9 µg/mL insulin (all

purchased from Sigma-Aldrich Corporation) to induce differentiation. The day on which this "differentiation media" was added to the 48 hour-confluent population was denoted as day 0 (D0) of differentiation. The cells were maintained in differentiation medium for 72 hours, after which the spent media was removed and fresh complete medium supplemented with 9 μg/mL insulin was added. Cells were maintained in this "adipocyte maintenance media" for 72 hours, after which this medium was replenished. The adipocyte maintenance media was replenished once every 72 hours until cells were completely differentiated into adipocytes, which could be visualized under an inverted microscope by the appearance of lipid droplets within all cells. Under the culture conditions in the present study, complete differentiation was evident about 11 to 12 days after the induction of differentiation. The differentiation protocol is represented in Supplementary Fig. S1A [25]. Completely differentiated adipocytes were then transitioned to complete medium without insulin and maintained for 24 hours, after which they were serum-starved for 6 hours. Nic or steroids in various combinations were then added to the media and adipocytes were incubated for 18 hours, after which they were processed for RNA isolation or protein lysate preparation. The treatment groups consisted of the following: Ctrl, Nic, E_2, E_2 + Nic, P_4, P_4 + Nic, E_2 + P_4, and E_2 + P_4 + Nic. The final concentrations of Nic, E_2, and P_4 in the wells were 1 μM, 100nM, and 1 μM, respectively. Ethanol was added to a final concentration of 0.03% in Ctrl wells. Each treatment was carried out in triplicate wells.

Experiments in differentiating adipocytes

Preadipocytes were seeded in 24- or 12-well plates at a density of 1.1×10^4 cells/cm^2. When cells reached 100% confluence after about 48 hours, spent media was removed and fresh complete media was added to the monolayer. After another 48 hours of incubation, the media was changed and differentiation was induced (D0) by adding fresh complete medium supplemented with 0.5 mM IBMX, 1 μM dexamethasone, and 9 μg/mL insulin. Nic, E_2, and P_4 in various combinations were also added to the differentiation media on D0. The cells were incubated in this medium for 72 hours, after which the spent media was removed and fresh complete medium supplemented with 9 μg/mL insulin was added. Nic and the steroids were again added to the adipocyte maintenance media at this stage (D3). Cells were incubated for another 72 hours, after which this medium was replenished (D6). The cells were processed for RNA isolation, protein lysate preparation, or Oil Red O staining 24 hours later (D7). The experimental protocol is represented in Supplementary Fig. S1B [25]. The treatment groups consisted of the following: Ctrl, Nic, E_2, E_2 + Nic, P_4, P_4 + Nic, E_2 + P_4, and E_2 + P_4 + Nic. Nic, E_2, and P_4 were used at concentrations of 1 μM, 100nM, and 1 μM, respectively. Ethanol was added to a final concentration of 0.03% in Ctrl wells. A set of wells containing cells that had not been induced on D0, but were rather maintained in complete media replenished every 2 to 3 days, was also maintained as a negative control for the differentiation process as measured by Oil Red O staining. Each treatment was carried out in triplicate wells.

Animals

Experimental protocols involving rats were approved by the Institutional Animal Ethics Committee, Indian Institute of Science. Harlan-Wistar rats were housed in a controlled environment and kept under 12-hour light and 12-hour dark cycles with ad libitum access to food and water. Virgin female rats aged 2 to 3 months were used for experiments.

Experiments in ovariectomized rats

To assess the influence of steroids as in pregnancy, rats were first ovariectomized to remove the source of endogenous steroids. Fallopian tubes and fat pads were ligated and both ovaries were surgically excised through small incisions on each flank after anaesthetizing rats with 40 mg of sodium pentobarbital per kg body weight. The skin incisions were closed with wound clips. Ovariectomized (OVX) rats were subcutaneously administered the vehicle, E_2, P_4, or E_2 + P_4 daily, for a period of 23 days from the day of ovariectomy. The treatment duration of 23 days was adopted to replicate the 23-day gestation period of our rat colony. The experimental group consisting of rats treated with E_2 + P_4 was incorporated to assess the combined action of these steroids as in pregnancy. To assess the involvement of Wnt signaling in mediating the metabolic actions of steroids, the Wnt inhibitor Nic was administered intraperitoneally (i.p.) in OVX rats, and in OVX rats along with E_2, P_4, and E_2 + P_4 treatments, for 23 days. Age-matched female rats with ovaries intact were also maintained during the course of the experiment for comparison. The experimental groups were denoted as Intact, OVX, OVX + Nic, OVX + E_2, OVX + Nic + E_2, OVX + P_4, OVX + Nic + P_4, OVX + E_2 + P_4, and OVX + Nic + E_2 + P_4. All injections were administered at 8 AM each day of the experiment. The experimental protocol is represented in Supplementary Fig. S2A [25].

E_2 (17β-estradiol), which was purchased from Sigma-Aldrich Corporation, was administered at a daily dose of 10-μg injections in refined groundnut oil. Susten 200 progesterone injection vials were purchased from Sun Pharma Laboratories Ltd and administered at a dose of 10 mg daily. A total of 200 μL of refined groundnut oil was administered to the control group. The dosages of E_2 and P_4 were previously standardized in the laboratory as the individual quantities required to maintain pregnancy in a pregnant OVX rat model. Radioimmunoassays of blood sampled from these rats on different days of treatment confirmed that the measured E_2 and P_4 concentrations in the serum were comparable to that recorded during pregnancy in rats (E_2: 150-250 pg/mL, P_4: 100-200 ng/mL). Nic, purchased from Sigma-Aldrich Corporation, was prepared in 10% Cremophor EL (Calbiochem/EMD Millipore Corporation) in saline (0.9% sodium chloride). The daily dosage of Nic was 20 mg per kg body weight of the rats, and was chosen based on reported doses that effectively block Wnt signaling in rodent models [26]. A total of 10% Cremophor EL in saline was administered to control rats.

The body weights and food consumption of rats from all experimental groups were recorded daily throughout the course of the experiment. At the end of treatment, fasting (4 hours) and nonfasting/random blood glucose levels of the rats were measured using an Accu-chek Active glucometer from Roche Diabetes Care, Inc in a 5-μL sample of blood that was collected from a nick made close to the tip of the tail. On the morning of the 23rd day (10 AM), the animals were anaesthetized using 40 mg per kg body weight i.p. injections of sodium pentobarbital to collect blood and then euthanized

by cervical dislocation to collect tissues (gonadal white adipose tissue or GWAT, soleus skeletal muscle, and liver). The GWAT was weighed before being processed for RNA isolation or protein lysate preparation.

Experiments in pregnant rats

For experiments involving pregnant rats, 2- to 3-month-old virgin female rats were cohabitated with adult male rats. The daily vaginal smear from the females were screened for the presence of sperm. The day of appearance of sperm in the smear was designated as day 1 of pregnancy. Pregnant rats were maintained for various durations, after which they were humanely killed for the collection of blood, GWAT, and soleus tissue. Pregnant rats maintained for 7, 10, 14, and 21 days into their pregnancy were denoted as P7, P10, P14, and P21, respectively. A group of age-matched nonpregnant (NP) female rats were also maintained as the control group in this experiment.

In a separate experiment to assess the effect of Wnt signaling inhibition during pregnancy, pregnant rats were administered either Nic (20 mg/kg body weight, i.p.) or the vehicle (Veh: 10% Cremophor EL in saline, i.p.) daily from day 6 of pregnancy until day 21 (Supplementary Fig. S2B [25]). Treatment with the Wnt inhibitor was started on day 6 rather than day 1 of pregnancy because of the critical role of Wnt signaling in embryonic development and uterine processes leading up to implantation; these processes generally occur between days 1 and 5 of rat pregnancy. This ensured successful establishment of pregnancy before the start of the experiment. The body weights and food consumption of Veh and Nic-treated pregnant rats were recorded daily. At the end of treatment on day 21 of pregnancy, fasting (4 hours) and nonfasting/random blood glucose levels of the rats were measured. The animals were anaesthetized to collect blood and later euthanized to collect tissues (GWAT, soleus skeletal muscle, and liver). The whole liver was weighed before being processed for RNA isolation. The number of implantation sites (pups) in the uterus were also recorded. Since treatment with Nic in pregnant rats appeared to affect their survival, one group each of Veh and Nic-treated pregnant rats were maintained until parturition. At parturition, the number and weight of pups were recorded to assess whether the drug had any effects on fetal development.

Adiponectin Assay

Blood samples from rats were collected in glass tubes and stored at 4 °C overnight in a slanted position. The tubes were then centrifuged at 3000 rpm at 4 °C for 15 minutes and the supernatant (serum) was collected and stored at −20 °C until used for hormone assays. The adiponectin levels in serum were measured using an enzyme-linked immunosorbent assay (ELISA) kit (catalog No. AG-45A-0005Y; RRID:AB_2893402) from Adipogen Life Sciences according to the manufacturer's recommendations. The intra-assay and interassay variability was 4.8% and 4.9%, respectively.

RNA Isolation, Complementary DNA Preparation, and Real Time Reverse Transcription Polymerase Chain Reaction Analysis

Total RNA was isolated from cell monolayers and tissue samples using TRI Reagent (Sigma-Aldrich Corporation), as per the manufacturer's recommendations. The quality and quantity of RNA samples were assessed spectrophotometrically using the NanoDrop ND-1000 (Thermo Fisher Scientific) and the samples with A_{260}/A_{280} ratio greater than 1.8 were used for further analysis. Further, RNA quality was assessed by observing the integrity of the 28S and 18S ribosomal RNA strands by running samples on a 1% formaldehyde-agarose gel along with RNA samples of good quality and known concentration. A total of 1 µg of total RNA was reverse-transcribed into complementary DNA (cDNA) using a High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems. cDNA equivalent to 10 ng of total RNA was used for polymerase chain reaction (PCR).

Quantitative reverse transcription PCR (qRT-PCR) was performed on 96-well plates using a StepOnePlus Real-time PCR System (Applied Biosystems) with StepOne software. Each 10-µL reaction consisted of 5 µL of a 2X Power SYBR Green PCR Master Mix (Applied Biosystems), 1.25 µM each of forward and reverse primers, and cDNA equivalent to 10 ng of total RNA. Reactions were carried out in triplicates. Primers spanning exon-exon junctions were designed with the help of the Primer Express Software v2.0 (Applied Biosystems) based on sequences deposited in NCBI and Ensembl and were synthesized at Sigma-Aldrich Chemical Pvt Ltd, Bangalore. qRT-PCR was carried out with an initial denaturation step of 95 °C for 10 minutes. In each cycle of the reaction, denaturation was performed at 95 °C for 30 seconds, annealing at the specified temperature for each primer pair for 30 seconds, and extension at 72 °C for 30 seconds. A final extension step at 72 °C for 5 minutes was also carried out for each reaction. Expression levels of the genes analyzed in cell culture samples were normalized to the expression of the mouse 60S ribosomal protein L19 (*Rpl19*). Expression levels of the genes analyzed from rat tissue samples were normalized to the expression of rat β-2 microglobulin (*B2m*), which was used as the internal control. The internal controls chosen were constitutively expressed genes for each cell/tissue type, and their expression remained constant. The fold change in gene expression was determined using the $\Delta\Delta C_t$ (comparative C_t) method. The details of primers used for analyzing gene expression in cell culture samples are listed in Table 1, and the details of primers used for analyzing gene expression in rat tissue samples are listed in Table 2.

Preparation of Protein Lysates and Immunoblotting

Lysates from cell monolayers and tissue samples were prepared using the radioimmunoprecipitation assay buffer. Each 1 mL of radioimmunoprecipitation assay buffer used for the preparation of lysates consisted of 50 mM Tris, 10 µL IGEPAL (octylphenoxypolyethoxyethanol), 10 mg sodium deoxycholate, 150 mM NaCl, 1 mM EDTA (ethylenediaminetetraacetic acid) pH 8.0, 0.1% sodium dodecyl sulfate (SDS), 1 mM Na_3VO_4, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1X Protease Inhibitor Cocktail. All reagents used for preparation of the buffer were obtained from Sigma-Aldrich Corporation. Total protein content was estimated using the Bio-Rad Protein Assay (Bio-Rad Laboratories Inc) based on the method by Bradford [27, 28].

Protein lysates were resolved by SDS Tris-Glycine polyacrylamide gel electrophoresis (PAGE). All reagents used in the preparation of the gel and the lysates for electrophoresis were purchased from Sigma-Aldrich Corporation. The

Table 1. Primers used in quantitative reverse-transcription polymerase chain reaction for analyzing gene expression in cell culture samples

Gene	Sequence of primer pair (forward and reverse)	Annealing temperature, °C	Amplicon size, bp
Rpl19	Fwd: AGGCATATGGGCATAGGGAAG Rev: CCTTCAGGTACAGGCTGTGAT	61.3	160
Tcf7l2	Fwd: AACACCCCCACCATGTCCA Rev: GGCCTTGGGATTCCTGTTTTG	66.4	124
Lrp5	Fwd: AAGGGTCCACAAGGTCAAGG Rev: CACATGGGTTGGTTCCGACA	63.1	110
Axin2	Fwd: AAGCGACCCAGTCAATCCTT Rev: AGGGACTCCATCTACGCTAC	61.2	142
Ppard	Fwd: GGCCTTCTCTAAGCACATCTACA Rev: TGGATGACAAAGGGTGCGTTG	65.2	114
Sfrp1	Fwd: TACTGGCCCGAGATGCTCAA Rev: GGACACACGGTTGTACCTTGG	59.2	113
Slc2a4	Fwd: AATGTCTTGGCCGTGTTGGG Rev: CCCTGATGTTAGCCCTGAGTAG	64.8	117
Insr	Fwd: CTGGATTATTGTCTCAAAGGGCTG Rev: CTGGCCGAGTCGTCATACTC	59.6	110
Acadm	Fwd: AGATCGCAATGGGTGCTTTTG Rev: AAACTCCTTGGTGCTCCACTA	61.2	147
Foxo1	Fwd: TCGGAATGACCTCATGGATGG Rev: GGACTTTTAAATGTAGCCTGCTC	61.2	146
Adipoq	Fwd: CCCTCCACCCAAGGGAACTT Rev: TAGGACCAAGAAGACCTGCATC	59.2	143
Fabp4	Fwd: ATTTCCTTCAAACTGGGCGTG Rev: CTTTCATAACACATTCCACCACC	61.3	178
Cebpa	Fwd: CCGACTTCTACGAGGTGGAG Rev: CTATAGACGTCTCGTGCTCGC	56.7	177
Pparg	Fwd: GGTGTGATCTTAACTGCCGGA Rev: CCCAAACCTGATGGCATTGTG	60.6	110
Lpl	Fwd: AATTTGCCCTAAGGACCCCT Rev: ACATTCCCGTTACCGTCCA	60.6	144
Fasn	Fwd: TGTAAACGTCCTCACCCGAG Rev: GCTGGCCCATTTACCTGGAA	64.8	187
Slc27a1	Fwd: GTCAAGTACAATTGCACGGTAGT Rev: CGTAGAACTCGCCGATCTGT	62.8	184
Mogat1	Fwd: CCTTGACCCATGGTGCCAGTT Rev: AGTGGCAAGGCTACTCCCATT	60.6	142

Abbreviations: *Acadm*, acyl-CoA dehydrogenase medium chain; *Adipoq*, adiponectin; *Axin2*, axis inhibition protein 2; *B2m*, β-2 microglobulin; *Cebpa*, CCAAT/enhancer binding protein alpha; *Dbi*, diazepam binding inhibitor or acyl-CoA binding protein; *Dgat1*, diacylglycerol O-acyltransferase 1; *Fasn*, fatty acid synthase; *Insr*, insulin receptor; *Lpl*, lipoprotein lipase; *Lrp5*, low-density lipoprotein receptor-related protein 5; *Mogat1*, monoacylglycerol O-acyltransferase 1; *Pparg*, peroxisome proliferator-activated receptor gamma; *Rpl19*, ribosomal protein L19; *Slc2a4*, solute carrier family 2 member 4 or GLUT4; *Slc27a1*, solute carrier family 27 member 1 or long-chain fatty acid transport protein 1; *Tcf7l2*, transcription factor 7-like 2.

resolved proteins were then electroblotted onto a 0.2-µ Immobilon-P^{SQ} polyvinylidene fluoride membrane from Merck Millipore using a wet transfer unit (Bio-Rad Laboratories Inc). Nonspecific sites on the membrane were blocked by incubating it in 10% skimmed milk in 1X TBS-T at room temperature for 1 hour. The composition of 1X TBS-T used was 20 mM Tris pH 7.6, 137 mM NaCl and 0.1% Tween (Sigma-Aldrich Corporation). The milk solution was washed off using 1X TBS-T and the blot was incubated in the appropriate primary antibody overnight at 4 °C with gentle agitation on a rocker kept at very low speed. The primary antibodies were typically diluted in 2.5% bovine serum albumin (from Sigma-Aldrich Corporation) in 1X TBS-T. The primary antibodies used were purchased from Sigma-Aldrich Corporation, Santa Cruz Biotechnology, Inc, or Cell Signaling Technology, Inc; their specifications are listed in Table 3. The membrane was washed and then incubated in the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody for 2 hours at room temperature. HRP-conjugated antimouse and antirabbit immunoglobulin G secondary antibodies were obtained from Cell Signaling Technology, Inc (catalog No. 7076; RRID:AB_330924 and catalog No. 7074; RRID: AB_2099233, respectively). The HRP-conjugated antigoat immunoglobulin G secondary antibody was obtained from Merck Millipore (catalog No. AP106P; RRID:AB_92411). The secondary antibodies were typically used at a dilution of 1:3000 in 10% skimmed milk in 1X TBS-T. The membrane was again washed and then developed using Luminata Forte Western HRP substrate from Merck Millipore. The bands were visualized using a ChemiDoc XRS+ system from Bio-Rad Laboratories, Inc and were quantified using GeneTools image analysis software (Syngene International Ltd). β-Actin was

Table 2. Primers used in quantitative reverse-transcription polymerase chain reaction for analyzing gene expression in rat tissue samples

Gene	Sequence of primer pair (forward and reverse)	Annealing temperature, °C	Amplicon size, bp
B2m	Fwd: GCCATTCAGAAAACTCCCC Rev: AAGGACAGATCTGACATCTCGA	62.9	170
Tcf7l2	Fwd: AACGCGTCTATGTCTAGGTTCC Rev: GCTTCTTTATGTGGGGCTTTTT	60.1	181
Lrp5	Fwd: TTTACGCTGCGATGCTGTCT Rev: GTGTAACGCTGGCACATCAC	62.8	261
Axin2	Fwd: CAGTTTTGTGGCAGCAGA Rev: GACTCCAACGGGTAGCTCT	62.1	239
Ppard	Fwd: CACAACGCTATCCGCTTTGG Rev: TCGTGGATGATGAAGGGTGC	68.6	227
Sfrp1	Fwd: GAAGCCCCAAGGTACAACAG Rev: CTTCAGCTCCTTCTTCTTGATG	64.2	196
Slc2a4	Fwd: GTTGGTCTCGGTGCTCTTAG Rev: CCAAATATGGCCACGATG	54.5	159
Insr	Fwd: ACTGTCAAAATCGGAGACTTTG Rev: TGTTCATTAGACAGGCCTTGG	55	218
Acadm	Fwd: GATCTATCAGATTTACGAAGGTACTGCA Rev: TCATTTCTGTGGGAAGACAAGTAAAG	60.1	185
Foxo1	Fwd: GCTAGGAGTTAGTGAGCAGGC Rev: CAGGAACGTGTTTCTGCTG	65.1	167
Lpl	Fwd: AGAACATTCCCTTCACCCTGC Rev: GGAAGTAGGAGTCGTTCTTCCAC	64.7	126
Slc27a1	Fwd: GCCATTGTGGTGCACAGCAGGTAC Rev: CTGTCCCACGCCCATGATGTTC	66	138
Fasn	Fwd: GCAAGGAGCAAGGCGTGACAT Rev: GCTCTGGCGGAAAGCACACAG	65	188
Mogat1	Fwd: GCAGCCATTGGCCGCC Rev: CCAGAGTTTCATGCTCCGGAATAC	55	151
Adipoq	Fwd: GAAGGGAGACGCAGGTGTTC Rev: CGGCTTCTCCGGGCTCTC	60	122
Lep	Fwd: CAATGACATTTCACACACGCAGTC Rev: GGAAGGCAAGCTGGTGAGGATC	60.1	148
Dgat1	Fwd: ACAGCAATGATGGCTCAGGTCC Rev: CAGTGGGGGCATGAGCTACTG	50	178
Dbi	Fwd: AAGCTGAAAGGAACTTCCAAGG Rev: TCACAGGTATTATGTCACACATGTG	55	138

Abbreviations: *Acadm*, acyl-CoA dehydrogenase medium chain; *Adipoq*, adiponectin; *Axin2*, axis inhibition protein 2; *B2m*, β-2 microglobulin; *Cebpa*, CCAAT/enhancer binding protein alpha; *Dbi*, diazepam binding inhibitor or acyl-CoA binding protein; *Dgat1*, diacylglycerol O-acyltransferase 1; *Fasn*, fatty acid synthase; *Insr*, insulin receptor; *Lpl*, lipoprotein lipase; *Lrp5*, low-density lipoprotein receptor-related protein 5; *Mogat1*, monoacylglycerol O-acyltransferase 1; *Pparg*, peroxisome proliferator-activated receptor gamma; *Rpl19*, ribosomal protein L19; *Slc2a4*, solute carrier family 2 member 4 or GLUT4; *Slc27a1*, solute carrier family 27 member 1 or long-chain fatty acid transport protein 1; *Tcf7l2*, transcription factor 7-like 2.

used as the loading control for cell lysates and rat GWAT lysates. Actin was used as the loading control for soleus muscle lysates. The protein levels were thus normalized to the levels of the loading controls in each case.

Oil Red O Staining of Differentiating Adipocytes

Media was aspirated from the wells containing D7 differentiating adipocytes and the wells were rinsed in 1X DPBS. Ten percent formalin (prepared from 37%-41% formaldehyde [Merck] in DPBS) was then added to the wells and the plate was covered and incubated at room temperature for 45 minutes. Formalin was removed and wells were rinsed consecutively in distilled water and 60% v/v isopropanol (Merck) in distilled water. Once the isopropanol was removed, wells were allowed to dry for 2 to 3 minutes. The working solution of Oil Red O was added to the wells to just cover the bottom surface (200 µL for each well of a 24-well plate, 400 µL for each well of a 12-well plate). The plate was covered and incubated at room temperature for 10 minutes. The stain was then removed and the wells were rinsed with distilled water 3 to 4 times to remove any excess stain. Care was taken to ensure that the stained monolayer was never left dry and the wells always contained distilled water. Stained wells were visualized under an inverted microscope in bright field and images were captured at 20× magnification. The images were analyzed for the area occupied by the Oil Red O stained vesicles using the image analysis software ImageJ (US National Institutes of Health). The working solution of Oil Red O was prepared fresh just before use by mixing 3 parts of the stock (3-mg/mL Oil Red O [Sigma-Aldrich Corporation] prepared in 100% isopropanol) with 2 parts of distilled water,

Table 3. Primary antibodies used in immunoblotting

Antibody against protein	Antibody source/ RRID/catalog No.	Size of protein, kDa	Dilution of antibody used	Antibody raised in
β-actin	Sigma-Aldrich Inc (RRID:AB_262011) A3854	42	1:10 000	Mouse
Actin	Santa Cruz Biotechnology Inc (RRID:AB_630836) sc-1616	43	1:1000	Goat
β-Catenin	Cell Signaling Technology Inc (RRID:AB_11127203) No. 8814	92	1:500	Rabbit
Dvl2	Cell Signaling Technology Inc (RRID:AB_2093338) No. 3216	90-95	1:500	Rabbit
Insulin receptor-β	Santa Cruz Biotechnology Inc (RRID:AB_631835) sc-711	95 (mature) 200 (precursor)	1:500	Rabbit
phospho-Akt	Cell Signaling Technology Inc (RRID:AB_2255933) No. 2965	60	1:1000	Rabbit
GLUT4	Santa Cruz Biotechnology Inc (RRID:AB_629533) sc-53566	49	1:500	Mouse
Fatty acid synthase	Cell Signaling Technology Inc (RRID:AB_2100796) No. 3180	273	1:1000	Rabbit

letting it stand for about 10 minutes, and filtering it using Whatman filter paper.

After imaging, distilled water was completely aspirated from the wells and the Oil Red O stain was eluted using 100% isopropanol. The plate was covered and incubated with isopropanol for 10 to 15 minutes at room temperature with gentle rocking. A total of 100 µL of the solution from each well was then transferred to a 96-well plate and absorbance was measured at 520 nm using a microplate reader. One hundred percent isopropanol was used as the blank, and the absorbance values obtained from the blank was subtracted from the values obtained from other wells to obtain corrected values. These values were proportional to the intensity of the stain in each well.

Statistical Analyses

Wherever applicable, data are expressed as mean ± SEM. The graphs were plotted and data analyzed using GraphPad Prism 8 software (GraphPad Software Inc). Comparisons between 2 groups were analyzed using the 2-tailed paired or unpaired *t* test with 95% CIs. Comparisons of 3 or more groups were analyzed by one-way analysis of variance (ANOVA) and Bonferroni post tests with 95% CIs. The graphs of body weights against day of treatment were analyzed by 2-way repeated-measures ANOVA to compare the trend seen between groups. A *P* value less than .05 was considered to be statistically significant. *, **, and *** represent statistically significant differences between groups, where *P* less than .05, *P* less than .01, and *P* less than .001, respectively. #, ##, and ### represent statistically significant differences in comparison to the Ctrl or OVX group, where *P* less than .05, *P* less than .01, and *P* less than .001, respectively.

Results

Effect of Steroids and Niclosamide on Wnt Signaling in 3T3-L1 cells

3T3-L1 adipocytes were incubated with E_2, P_4, or $E_2 + P_4$ in the absence or presence of Nic. The steroids E_2 and P_4, as well as their combination, increased the expression of the transcription factor *Tcf7l2*—an effector of canonical Wnt signaling (Fig. 1A), the Wnt coreceptor *Lrp5* (low-density lipoprotein receptor-related protein 5; Fig. 1B), and Wnt signaling target genes *Axin2* (axis inhibition protein 2; Fig. 1C) and *Ppard* (peroxisome proliferator-activated receptor delta; Fig. 1D) by approximately 2-fold. E_2 and $E_2 + P_4$ decreased the expression of the Wnt signaling repressor *Sfrp1* (secreted frizzled-related protein 1; Fig. 1E). The steroids also brought about an increase in the protein levels of active or non-phosphorylated β-catenin (Fig. 1F and 1G) and signal transducer Dvl2 (disheveled segment polarity protein 2; Fig. 1F and 1H). These observations suggest that both E_2 and P_4 upregulate Wnt signaling in adipocytes. Although Nic did not significantly decrease the expression of Wnt signaling pathway marker genes in untreated adipocytes, it inhibited the increase in Wnt signaling in the presence of E_2 and P_4, thereby confirming its efficiency as a Wnt pathway inhibitor. Similar effects of steroids and Nic were observed in preadipocytes and differentiating adipocytes (Supplementary Figs. S3 and S4 [25]).

Effect of Steroids and Niclosamide on Insulin Sensitivity in 3T3-L1 Cells

The steroid E_2 brought about an increase in the expression of the GLUT4 glucose transporter gene *Slc2a4* (2-fold; $P = .0028$; Fig. 2A), the insulin receptor gene *Insr* (2.5-fold; $P = .0008$; Fig. 2B), the insulin signaling target gene *Acadm*

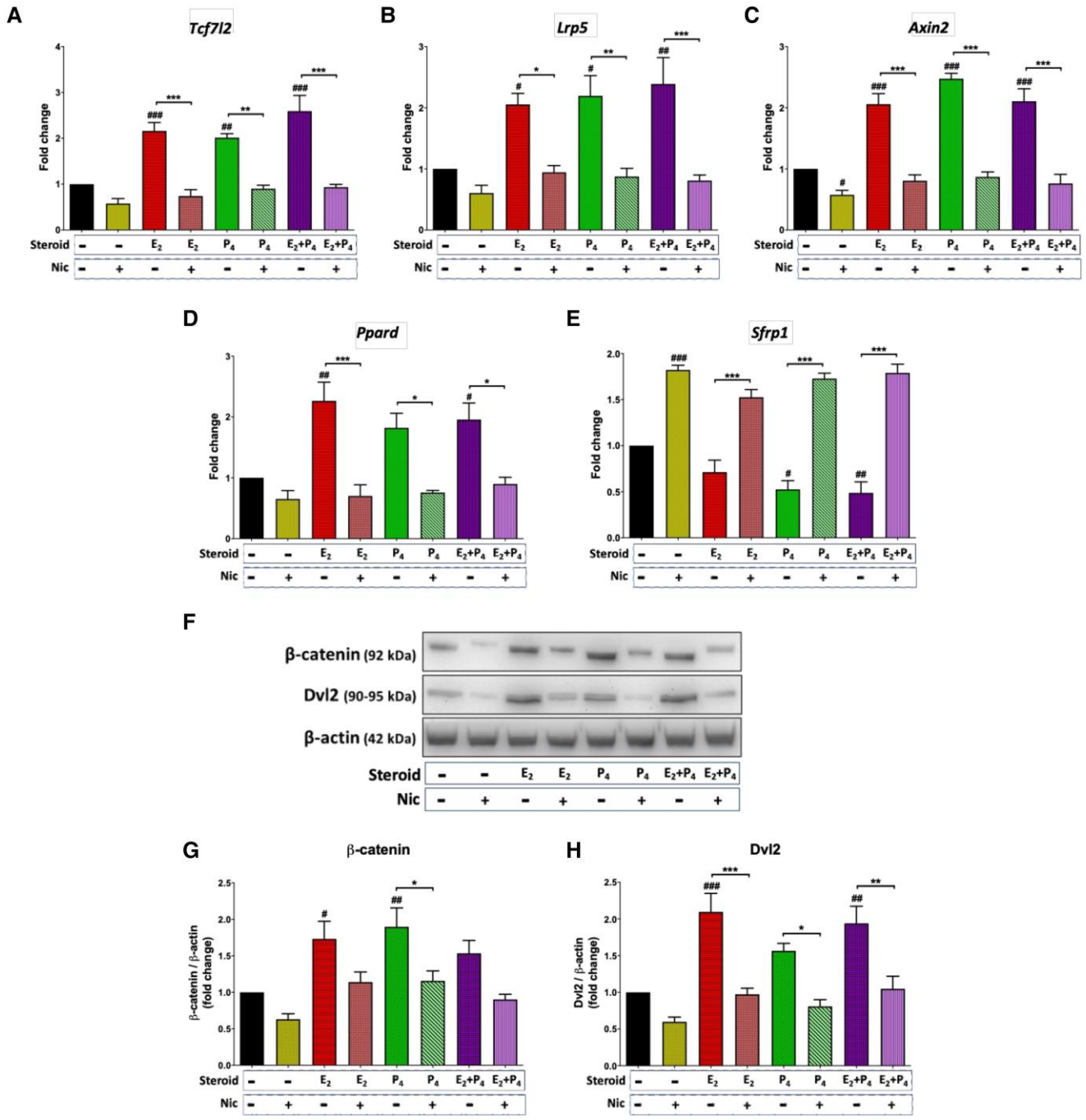

Figure 1. Effect of steroids and niclosamide (Nic) on canonical Wnt signaling in mature/differentiated adipocytes. 3T3-L1 adipocytes were serum-starved for 6 hours and incubated with Nic (1 μM), estradiol (E_2; 100 nM), progesterone (P_4; 1 μM), or their combinations for 18 hours (n = 3). RNA isolated from these cells was analyzed for the expression of canonical Wnt signaling pathway genes A, *Tcf7l2*; and B, *Lrp5*; target genes C, *Axin2*; and D, *Ppard*; and the negative regulator of Wnt signaling E, *Sfrp1* by qRT-PCR. Gene expression was normalized to the expression of *Rpl19*. Cell lysates were analyzed for the active form of β-catenin and Dvl2 protein by F, immunoblotting. β-actin was used as the loading control. G and H, Densitometric analysis was performed. Data are represented as mean ± SEM of fold change compared to the expression in untreated cells, in A to E, G, and H. Statistical significance among treatment groups was determined by one-way analysis of variance and Bonferroni post test. *, **, and *** represent statistically significant differences of *P* less than .05, *P* less than .01, and *P* less than .001, respectively. #, ##, and ### represent statistically significant differences of *P* less than .05, *P* less than .01, and *P* less than .001, respectively, with respect to untreated cells. *Axin2*, axis inhibition protein 2; Dvl2, disheveled segment polarity protein 2; *Lrp5*, low-density lipoprotein receptor-related protein 5; *Ppard*, peroxisome proliferator-activated receptor delta; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; *Rpl19*, 60S ribosomal protein L19; *Sfrp1*, secreted frizzled-related protein 1; *Tcf7l2*, transcription factor 7-like 2.

(acyl-CoA dehydrogenase medium chain; 2.1-fold; $P = .0018$; Fig. 2C), the insulin receptor β-subunit protein ($P = .0082$; Fig. 2E and 2F), and the phosphorylated form of protein kinase B/Akt that is activated during insulin signaling (phospho-Akt; $P = .0032$; Fig. 2E and 2G) in adipocytes. These observations indicate that E_2 enhances insulin sensitivity in 3T3-L1 cells. P_4, on the other hand, did not have any effect on insulin signaling pathway markers in these cells. In cells treated with both E_2 and P_4, the presence of E_2 resulted in an increase in the expression of insulin signaling markers

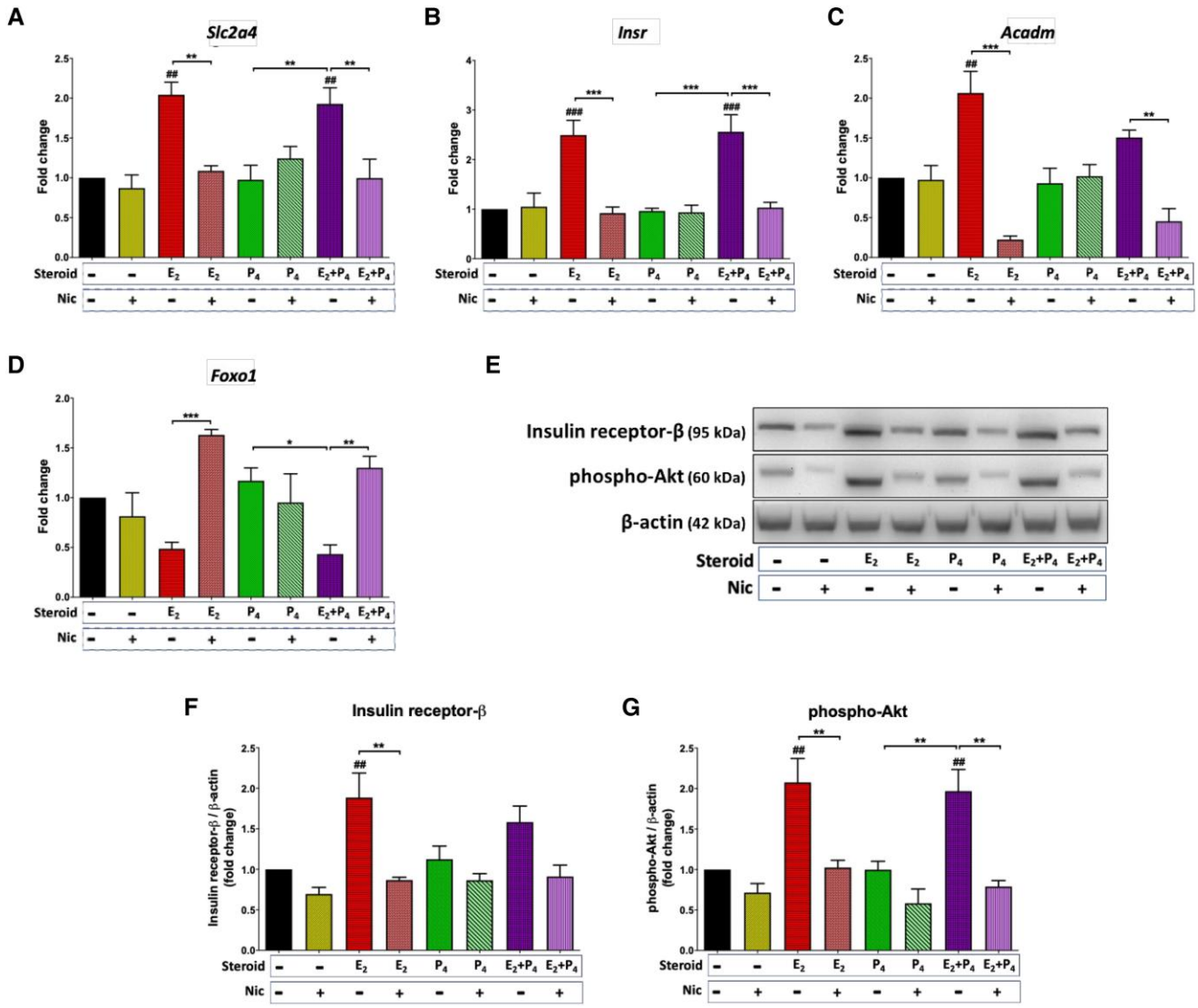

Figure 2. Effect of steroids and niclosamide (Nic) on insulin signaling in mature/differentiated adipocytes. 3T3-L1 adipocytes were serum-starved for 6 hours and incubated with Nic (1 μM), estradiol (E_2; 100 nM), progesterone (P_4; 1 μM), or their combinations for 18 hours (n = 3). RNA isolated from these cells was analyzed for the expression of insulin sensitivity marker genes like A, *Slc2a4*; B, *Insr*; C, *Acadm*; and D, *Foxo1* by qRT-PCR. Gene expression was normalized to the expression of *Rpl19*. Cell lysates were analyzed for the expression of insulin signaling pathway proteins insulin receptor-β and phospho-Akt by E, immunoblotting. β-actin was used as the loading control. F and G, Densitometric analysis was performed. Data are represented as mean ± SEM of fold change compared to the expression in untreated cells, in A to D, F, and G. Statistical significance among treatment groups was determined by one-way analysis of variance and Bonferroni post test. *, **, and *** represent statistically significant differences of *P* less than .05, *P* less than .01, and *P* less than .001, respectively. #, ##, and ### represent statistically significant differences of *P* less than .05, *P* less than .01, and *P* less than .001, respectively, with respect to untreated cells. *Acadm*, acyl-CoA dehydrogenase medium chain; *Foxo1*, forkhead box O1; *Insr*, insulin receptor; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; *Rpl19*, 60S ribosomal protein L19; *Slc2a4*, solute carrier family 2 member 4 or facilitated glucose transporter GLUT4.

Slc2a4 (1.9-fold; $P = .0079$), *Insr* (2.6-fold; $P = .0005$), and phospho-Akt ($P = .0081$). Although inhibition of Wnt signaling by Nic did not bring about any significant changes in insulin sensitivity by itself, it prevented the increase in insulin sensitivity in cells treated with E_2 and $E_2 + P_4$. The steroids and Nic had similar effects on the insulin signaling pathway in preadipocytes (Supplementary Fig. S5 [25]).

Effect of Steroids and Niclosamide on Adipogenesis in 3T3-L1 Preadipocytes

To analyze the extent of differentiation of preadipocytes into adipocytes following inhibition of Wnt signaling in the absence and presence of steroids, preadipocytes were incubated with Nic, E_2, P_4, or their combinations during the differentiation process. Cells were stained with Oil Red O on day 7 of differentiation (D7) to visualize lipid vesicles. Cells that were not induced to differentiate did not pick up much of the Oil Red O stain as evident in the image seen in the left panel of Fig. 3A. A stark difference in the intensity of staining between uninduced cells and differentiating cells was evident ($P < .0001$; Fig. 3A, 3C, and 3D).

Cells in wells treated with Nic were stained red to a greater extent than untreated cells ($P < .0001$), as evident from the images seen in the far-left panel of Fig. 3B and from calculation of the area occupied by the stained vesicles, shown in Fig. 3C.

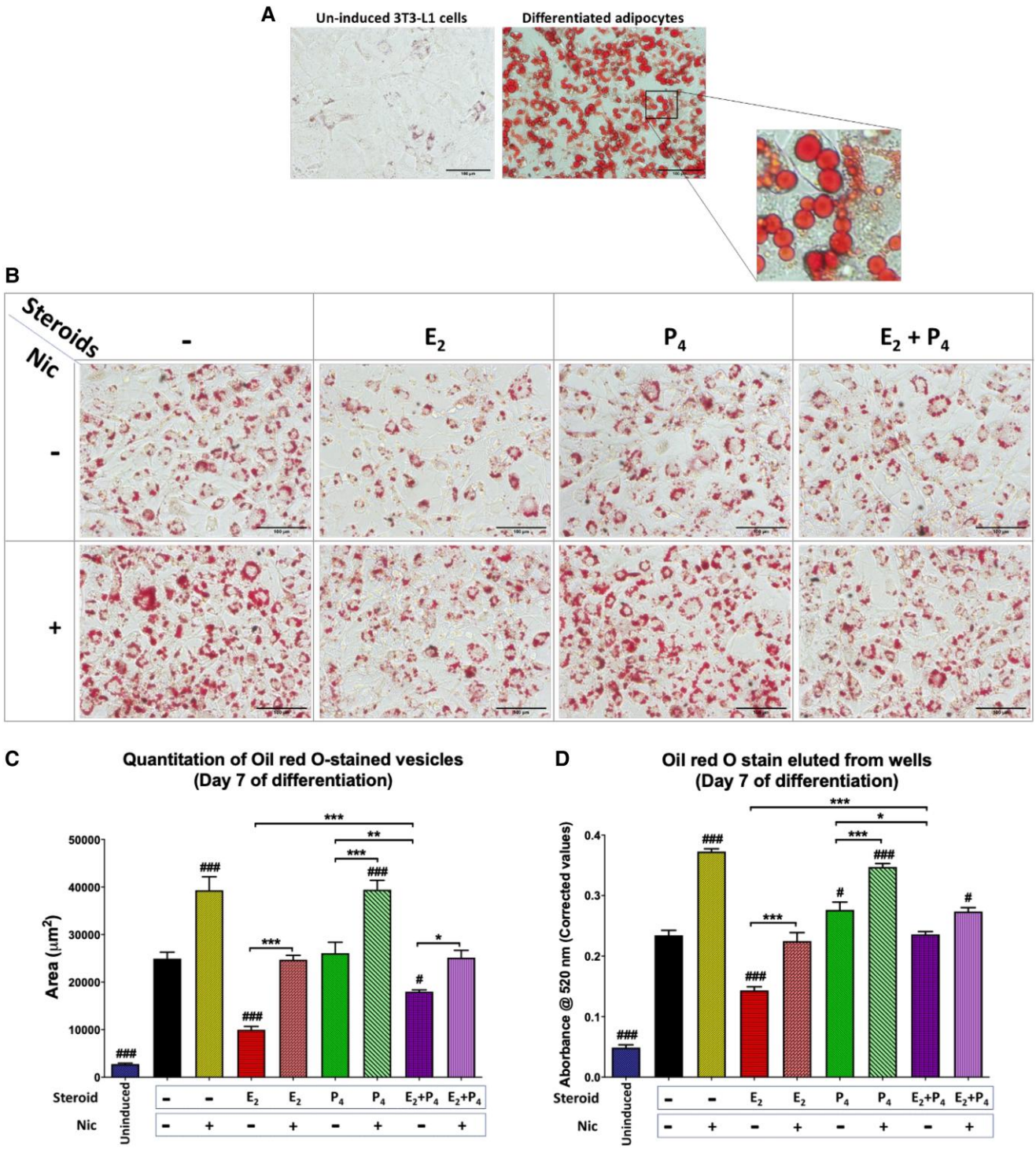

Figure 3. Effect of steroids and niclosamide (Nic) on adipogenic differentiation in 3T3-L1 cells. Preadipocytes were grown in 24- or 12-well plates and induced to differentiate into adipocytes. On day 7 of differentiation, wells were stained with Oil Red O to visualize lipid droplets and characterize the extent of differentiation. The images in A represent the appearance of preadipocytes in which differentiation had not been induced (on the left panel) and completely differentiated adipocytes in which lipid droplets can be visualized by the red color (on the right panel). Representative images of stained wells containing adipocytes differentiated in the presence of Nic (1 μM), estradiol (E_2; 100 nM), progesterone (P_4; 1 μM), or their combinations (n = 3) are shown in B. Representative images in A and B were captured at 20× magnification in bright field using an inverted microscope. The scale bars represent a distance of 100 μm. C, The images captured were quantified for the area occupied by the red stain in different treatment groups using image analysis software. D, Additionally, the Oil Red O stain was eluted from wells and intensity of the color was measured at 520 nm. Data are represented as mean ± SEM in C and D. Statistical significance among treatment groups was determined by one-way analysis of variance and Bonferroni post test. *, **, and *** represent statistically significant differences of *P* less than .05, *P* less than .01, and *P* less than .001, respectively. # and ### represent statistically significant differences of *P* less than .05 and *P* less than .001, respectively, with respect to untreated differentiating cells.

The intensity of the stain eluted from wells treated with Nic was also higher than in untreated wells ($P < .0001$; Fig. 3D), suggesting that inhibition of Wnt signaling by Nic hastens the process of adipogenic differentiation. On the other hand, incubation of differentiating adipocytes with E_2 resulted in decreased adipogenic differentiation on D7 compared to untreated wells ($P < .0001$), as evident from the image presented in Fig. 3B. Further, quantitation of images and measurement of the intensity of eluted stain from the E_2-treated wells confirmed this finding (Fig. 3C and 3D). Treatment with Nic in the presence of E_2, however, negated this effect ($P < .0001$), and the intensity of the stain in wells treated with both Nic and E_2 was similar to that in untreated wells. The presence of P_4 did not appear to affect the differentiation process, as evident from the images captured of P_4-treated wells and their quantitation (Fig. 3B and 3C). The presence of Nic with P_4 enhanced adipogenic differentiation ($P \leq .0001$), much like that observed with Nic treatment in the absence of P_4. The presence of both E_2 and P_4 in the differentiation media appeared to inhibit differentiation as observed in the images of stained wells on D7 ($P = .0229$). However, the extent of inhibition was not as pronounced as in the presence of E_2 alone (Fig. 3B and 3C). The intensity of stain eluted from $E_2 + P_4$-treated wells was similar to that of untreated wells (Fig. 3D), suggesting that the presence of P_4 dulls the antiadipogenic effect of E_2 on differentiating adipocytes. Nic in combination with E_2 and P_4 appeared to repress the inhibitory effect of $E_2 + P_4$ on adipogenic differentiation ($P = .0172$; Fig. 3C).

The expression of adipogenesis marker genes was analyzed in differentiating adipocytes on D7. Incubation with Nic increased the expression of *Adipoq* (adiponectin; Fig. 4A), *Fabp4* (fatty acid binding protein 4; Fig. 4B), *Cebpa* (CCAAT/enhancer binding protein alpha; Fig. 4C), and *Pparg* (peroxisome proliferator-activated receptor gamma; Fig. 4D) by more than 2-fold ($P \leq .0001$). It also appeared to increase the protein levels of the fatty acid synthase (FASN) enzyme complex ($P = .0854$; Fig. 4I and 4J). E_2-treated adipocytes, on the other hand, showed a trend of decreased expression of these genes (nonsignificant) and decreased FASN protein levels, supporting the observation that it inhibits adipogenic differentiation. The presence of Nic prevented the E_2-mediated decrease in *Fabp4* expression ($P = .0051$). While P_4 did not cause any significant change in the expression of *Adipoq*, *Pparg*, or FASN, it increased the expression of *Fabp4* and *Cebpa* by approximately 2-fold in differentiating adipocytes ($P = .0009$ and $P = .0211$, respectively). The presence of Nic in combination with P_4 in the media resulted in a marked increase in the expression of *Adipoq* ($P = .0041$), *Fabp4* ($P = .0107$), and *Cebpa* ($P = .0167$) with respect to cells treated with P_4 alone.

Although adipogenesis and lipogenesis are distinct processes, the expression of lipogenic genes is thought to be upregulated during adipogenic differentiation to aid in the synthesis of triglycerides for storage within lipid vesicles. Therefore, the expression of a few lipogenesis marker genes was analyzed in differentiating adipocytes on D7. It was observed that the effects of Nic and/or steroids on the expression of these genes were similar to their effects on adipogenesis. Nic caused an equal to or greater than 2-fold increase in the expression of *Lpl* (lipoprotein lipase; $P = .0031$; Fig. 4E), *Fasn* (fatty acid synthase; $P = .0011$; Fig. 4F), and *Mogat1* (monoacylglycerol O-acyltransferase 1; $P = .0197$; Fig. 4H) in differentiating adipocytes. Treatment of differentiating adipocytes with P_4 increased the expression of *Slc27a1* (fatty acid transport protein 1; $P = .0201$; Fig. 4G), and *Mogat1* ($P = .009$) by approximately 2-fold. The effect of P_4 on *Mogat1* was enhanced further in the presence of Nic.

Overall, the data suggest that inhibition of Wnt signaling by Nic hastens adipogenic differentiation in 3T3-L1 cells. E_2 inhibits differentiation, an effect that appears to be dependent on the Wnt pathway. While P_4 alone did not bring about any visible changes in adipogenesis, inhibition of Wnt signaling by Nic in the presence of P_4 enhanced the process. P_4 also appeared to dull the effect of E_2 on adipogenesis when cells were treated with both steroids.

Effect of Steroids and Niclosamide on Body Weight and Food Consumption in Ovariectomized Rats

The body weights were monitored in OVX rats treated with E_2, P_4, Nic, and their combinations over the course of the 23-day treatment period. The loss of ovarian steroids by ovariectomy was found to increase the body weight gain ($122.7 \pm 2.1\%$ in intact rats vs $147.2 \pm 1.8\%$ in OVX rats on day 23 of treatment; $P < .0001$; Fig. 5B). Replacement of the steroid E_2 in OVX rats was able to reduce and thereby regulate body weight gain ($147.2 \pm 1.8\%$ in OVX rats vs $108.3 \pm 0.8\%$ in OVX + E_2 rats on day 23 of treatment; $P < .0001$; Fig. 5C). Replacement of P_4, however, brought about no changes in the pattern of body weight gain in OVX rats (Fig. 5D). Replacement of both E_2 and P_4 in OVX rats also reduced the body weight gain ($147.2 \pm 1.8\%$ in OVX rats vs $126.9 \pm 1.8\%$ in OVX + E_2 + P_4 rats on day 23 of treatment; $P < .0001$; Fig. 5E), although not to the same extent as E_2 alone. The pattern of body weight gain in E_2 + P_4-treated OVX rats closely resembled that of intact rats (Fig. 5A). Treatment of OVX rats with Nic brought about a reduction in the body weight gain over the course of the treatment period ($147.2 \pm 1.8\%$ in OVX rats vs $135.4 \pm 3.0\%$ in OVX + Nic rats on day 23 of treatment; Fig. 5F). Nic treatment in OVX + P_4 rats also brought about a reduction in the body weight gain ($147.2 \pm 1.8\%$ in OVX rats vs $133.0 \pm 3.0\%$ in OVX + P_4 rats on day 23 of treatment; Fig. 5H)—the differences were, however, not significant. Nic treatment in OVX + E_2 and OVX + E_2 + P_4 rats did not affect the pattern of body weight gain (Fig. 5G and 5I, respectively).

Food consumption was also monitored during the treatment period (Fig. 5J). The total food consumption of OVX rats over 23 days (393 ± 13 g) was found to be greater ($P = .0263$) than that of intact rats (333 ± 3 g). E_2 treatment in OVX rats considerably reduced the food consumption (from 393 ± 13 g to 313 ± 9 g; $P < .0001$), while P_4 and $E_2 + P_4$ treatment had no such effect. Nic treatment in OVX, OVX + P_4, and OVX + E_2 + P_4 rats also appeared to (nonsignificantly) decrease food consumption, but Nic treatment in OVX + E_2 rats did not reduce food consumption further.

Effect of Steroids and Niclosamide on Wnt Signaling in the Gonadal White Adipose Tissue and Soleus Muscle of Ovariectomized Rats

The expression of canonical Wnt signaling pathway marker genes *Tcf7l2* (Fig. 6A and 6F), *Lrp5* (Fig. 6B and 6G), *Axin2* (Fig. 6C and 6H), and *Ppard* (Fig. 6D and 6I), and active β-catenin protein (Fig. 6E and 6J), both in the GWAT and the soleus skeletal muscle of intact rats was higher than that in

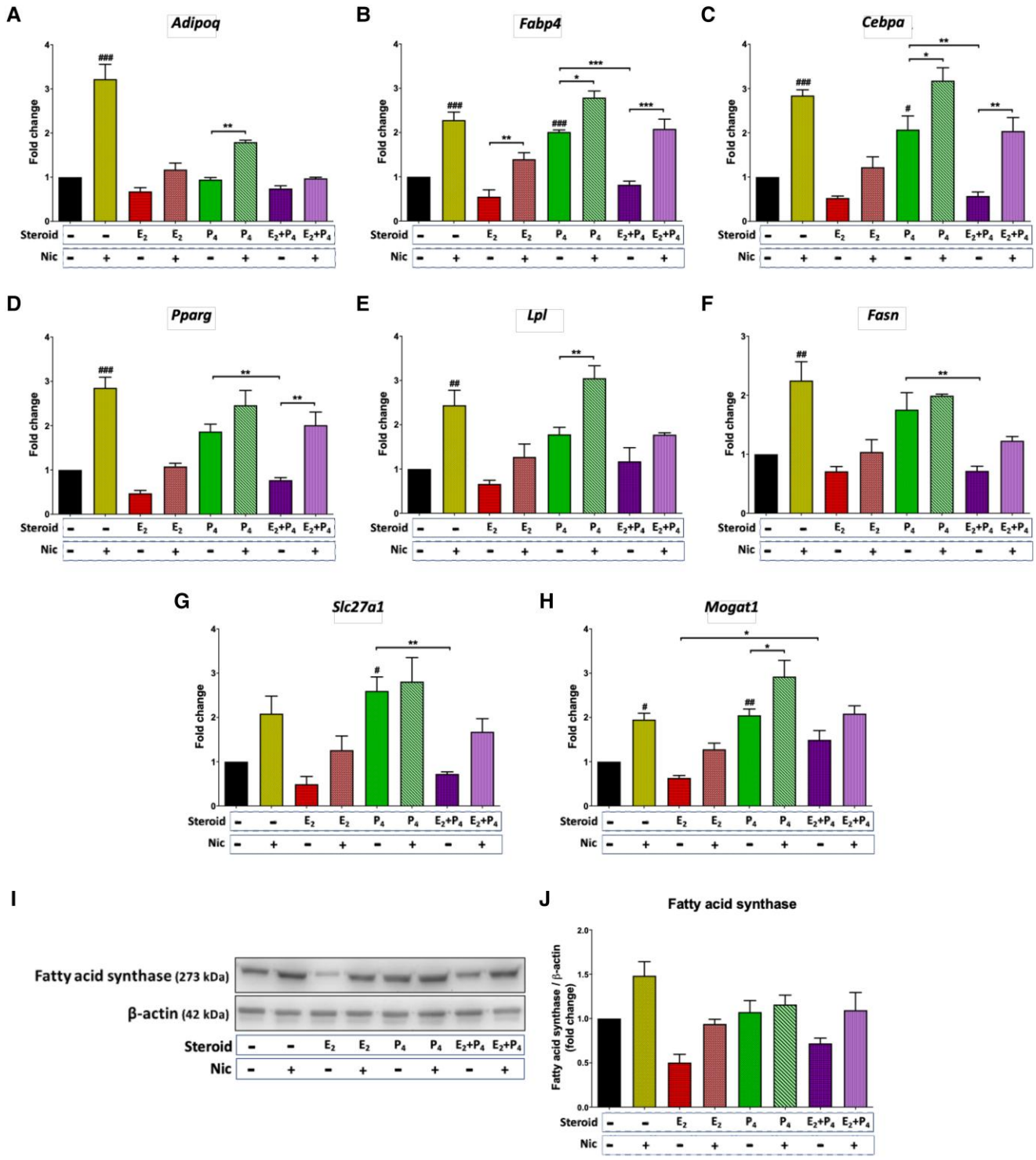

Figure 4. Effect of steroids and niclosamide (Nic) on expression of adipogenesis and lipogenesis markers during 3T3-L1 differentiation. Preadipocytes were induced to differentiate into adipocytes in the presence of Nic (1 μM), estradiol (E_2; 100 nM), progesterone (P_4; 1 μM), or their combinations (n = 3). On day 7 of differentiation, RNA isolated from these cells was analyzed for the expression of adipogenesis markers A, *Adipoq*; B, *Fabp4*; C, *Cebpa*; and D, *Pparg*; and lipogenesis markers E, *Lpl*; F, *Fasn*; G, *Slc27a1*; and H, *Mogat1* by qRT-PCR. Gene expression was normalized to the expression of *Rpl19*. I, Cell lysates were analyzed for the expression of the fatty acid synthase enzyme complex by immunoblotting. β-actin was used as the loading control. J, Densitometric analysis was performed. Data are represented as mean ± SEM of fold change compared to the expression in untreated cells, in A to H and J. Statistical significance among treatment groups was determined by one-way analysis of variance and Bonferroni post test. *, **, and *** represent statistically significant differences of *P* less than .05, *P* less than .01, and *P* less than .001, respectively. #, ##, and ### represent statistically significant differences of *P* less than .05, *P* less than .01, and *P* less than .001, respectively, with respect to untreated cells. *Adipoq*, adiponectin; *Cebpa*, CCAAT/enhancer binding protein alpha; *Fabp4*, fatty acid binding protein 4; *Lpl*, lipoprotein lipase; *Fasn*, fatty acid synthase; *Mogat1*, monoacylglycerol O-acyltransferase 1; *Pparg*, peroxisome proliferator-activated receptor gamma; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; *Rpl19*, 60S ribosomal protein L19; *Slc27a1*, solute carrier family 27 member 1 or long-chain fatty acid transport protein 1.

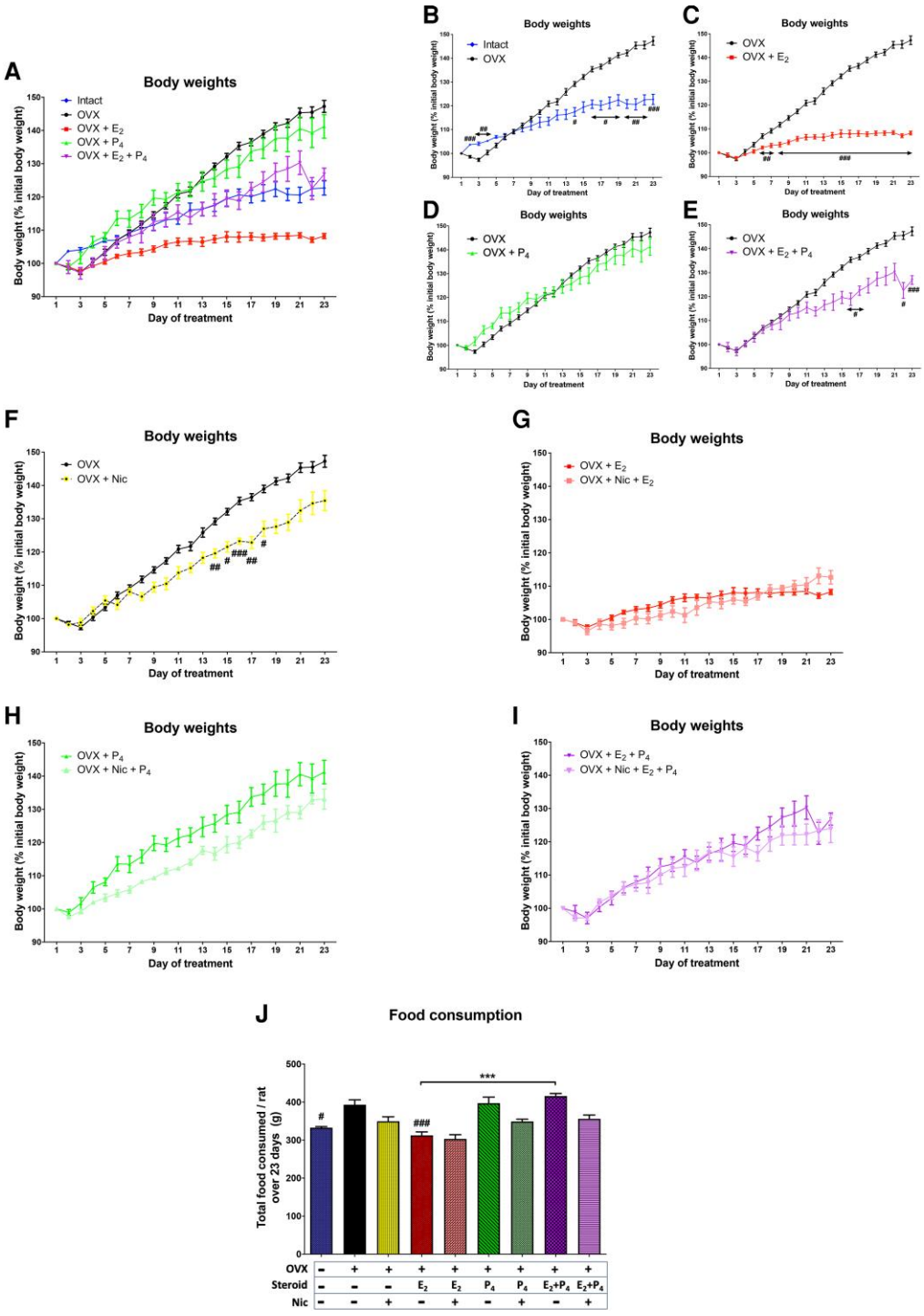

Figure 5. Effect of steroids and niclosamide (Nic) on body weight and food consumption in ovariectomized (OVX) rats. OVX rats were treated with the steroids—estradiol (E_2; 10 μg/day), progesterone (P_4; 10 mg/day), and their combination—in the absence and presence of the Wnt inhibitor Nic (20 mg/kg body weight/day). Body weights of rats from the various experimental groups were recorded on a daily basis during the 23-day treatment period (n≥6). The body weight on each day is expressed as a percentage of the body weight on day 1 of treatment (considered 100%) for each rat in each group. Mean ± SEM of the percentage of initial body weight of animals in each group is plotted against the day of treatment. A, Body weight gain in intact rats, OVX rats, and OVX rats treated with E_2, P_4, and E_2 + P_4 was compared. To appreciate the differences between groups, graphs showing body weight gain of each group in relation to the control OVX group were plotted: B, Intact vs OVX rats; C, OVX vs OVX + E_2 rats; D, OVX vs OVX + P_4 rats; and E, OVX vs OVX + E_2 + P_4 rats. To assess the effect of Nic, comparisons of body weight gain were made between the following groups: F, OVX vs OVX + Nic rats; G, OVX + E_2 vs OVX + Nic + E_2 rats; H, OVX + P_4 vs OVX + Nic + P_4 rats; and I, OVX + E_2 + P_4 vs OVX + Nic + E_2 + P_4 rats. Statistical significance among groups was determined by 2-way analysis of variance (ANOVA) and Bonferroni post test. #, ##, and ### represent statistically significant differences of *P* less than .05, *P* less than .01, and *P* less than .001, respectively, between the percentage body weight gain of the treatment group and the percentage body weight gain of the OVX group on a particular day. J, Total food consumption of rats from the various experimental groups was recorded over the course of the 23-day treatment period. Data are represented as mean ± SEM. Statistical significance among treatment groups was determined by one-way ANOVA and Bonferroni post test. *** represents a statistically significant difference of *P* less than .001. # and ### represent statistically significant differences of *P* less than .05 and *P* less than .001, respectively, with respect to untreated OVX rats.

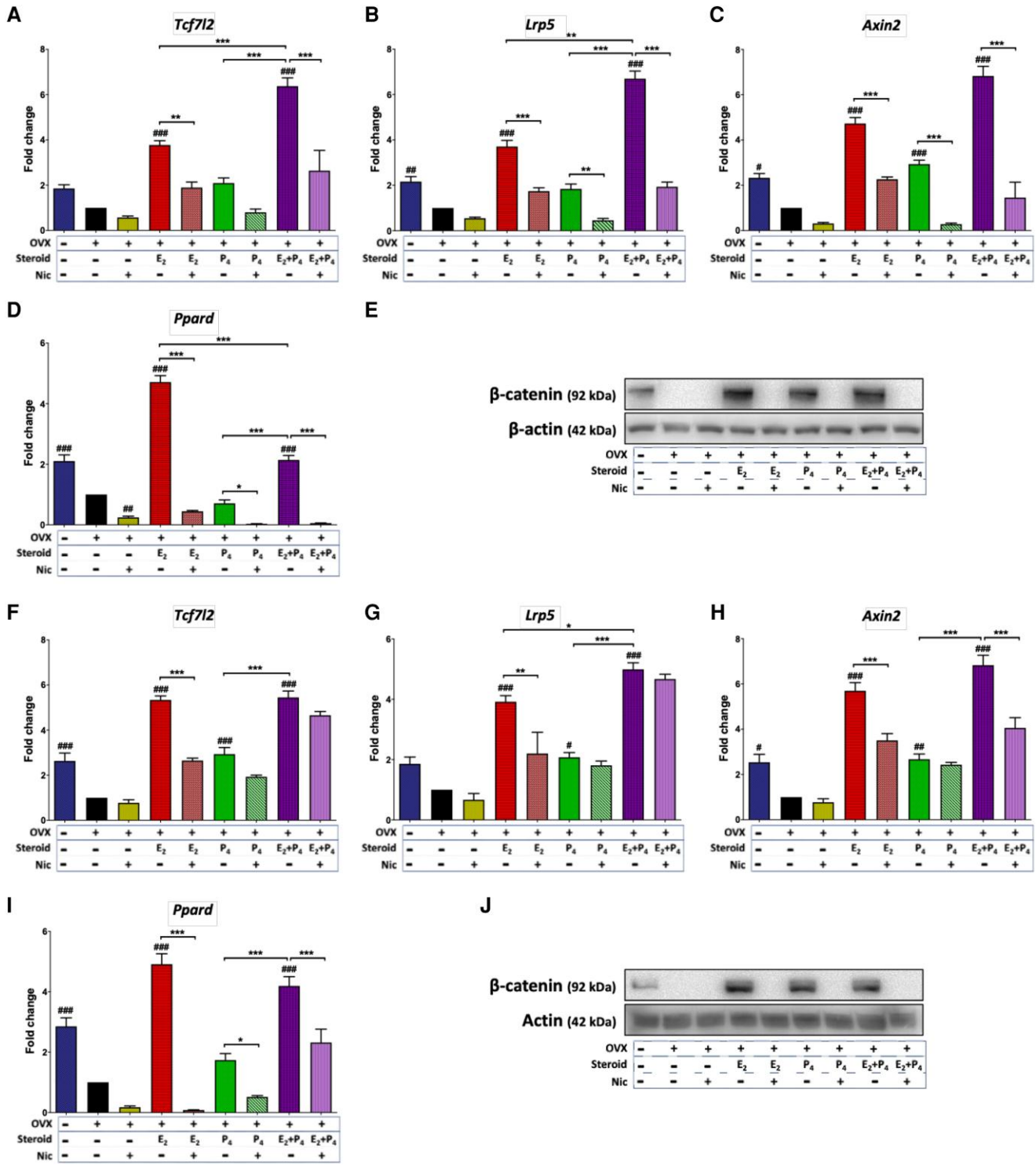

Figure 6. Effect of steroids and niclosamide (Nic) on canonical Wnt signaling in the gonadal white adipose tissue (GWAT) and soleus skeletal muscle of ovariectomized (OVX) rats. OVX rats were treated with Nic (20 mg/kg body weight/day), estradiol (E_2; 10 μg/day), progesterone (P_4; 10 mg/day), or their combinations, for a period of 23 days. The GWAT and soleus muscle collected from these rats (n = 4 each) were analyzed for the expression of canonical Wnt signaling pathway genes *Tcf7l2* (A, GWAT; F, soleus) and *Lrp5* (B, GWAT; G, soleus), and target genes *Axin2* (C, GWAT; H, soleus) and *Ppard* (D, GWAT; I, soleus) by qRT-PCR analysis. Gene expression was normalized to the expression of *B2m*. GWAT and soleus lysates (n = 3 each) were also analyzed for the active form of β-catenin by immunoblotting (E, GWAT; J, soleus). β-actin and actin were used as loading controls for GWAT and soleus lysates, respectively. Data is represented as mean ± SEM of fold change compared to the untreated OVX control group in A to D and F to I. Statistical significance among treatment groups was determined by one-way analysis of variance and Bonferroni post test. *, **, and *** represent statistically significant differences of *P* less than .05, *P* less than .01, and *P* less than .001, respectively. #, ##, and ### represent statistically significant differences of *P* less than .05, *P* less than .01, and *P* less than .001, respectively, with respect to untreated OVX rats. *Axin2*, axis inhibition protein 2; *B2m*, β-2 microglobulin; *Lrp5*, low-density lipoprotein receptor-related protein 5; *Ppard*, peroxisome proliferator-activated receptor delta; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; *Tcf7l2*, transcription factor 7-like 2.

OVX rats, suggesting that the loss of steroids decreases Wnt signaling in these tissues. E_2, P_4, and $E_2 + P_4$ treatment in OVX rats upregulated Wnt signaling in the GWAT and soleus muscle, as evident from the increased expression of marker genes and β-catenin. These results mirror the observations made in adipocytes incubated with steroids. However, the increase in gene expression due to P_4 was not as pronounced as with E_2. In the GWAT and soleus of OVX rats and OVX rats treated with Nic, the active form of the β-catenin protein was undetectable with the quantity of protein lysate that was used for Western blotting (120 μg of GWAT lysate and 200 μg of soleus lysate). Nic was able to inhibit the upregulation of Wnt signaling due to E_2, P_4, and $E_2 + P_4$ treatment in both GWAT and soleus of OVX rats. These observations confirm the inhibitory action of Nic on Wnt signaling.

Effect of Steroids and Niclosamide on Blood Glucose Levels and Insulin Sensitivity of the Gonadal White Adipose Tissue and Soleus Muscle in Ovariectomized Rats

The nonfasting blood glucose levels of intact rats, OVX rats, and OVX rats treated with E_2, P_4, and $E_2 + P_4$ did not differ significantly (Fig. 7A). However, Nic treatment in OVX + E_2, OVX + P_4, and OVX + E_2 + P_4 rats appeared to increase the average glucose levels in circulation. The fasting blood glucose levels remained the same in all treatment groups, except in $E_2 + P_4$-treated OVX rats, in which the levels were slightly higher ($P = .0232$; Fig. 7B).

In the GWAT and soleus muscle of OVX rats, the expression of *Slc2a4* (Fig. 7C and 7H), *Insr* (Fig. 7D and 7I), and *Acadm* (Fig. 7E and 7J) were found to be lower compared to that in intact rats. Similarly, the insulin receptor β-subunit, phospho-Akt, and GLUT4 proteins—whose levels were detectable in the GWAT and soleus of intact rats—were not picked up in these tissues from OVX rats (Fig. 7G and 7L). These observations indicate that ovariectomy leads to reduced insulin sensitivity in female rats. Based on the expression of the aforementioned markers, the steroid E_2 was found to enhance insulin sensitivity both in the GWAT and soleus muscle of OVX rats, whereas P_4 did not appear to affect insulin sensitivity. $E_2 + P_4$ treatment enhanced insulin sensitivity in the GWAT of OVX rats. It, however, failed to bring about any increase in the expression of *Slc2a4*, *Insr*, and *Acadm* in the soleus muscle tissue. However, an increase in the insulin receptor-β protein levels ($P = .0063$) was evident in the soleus of OVX rats following $E_2 + P_4$ treatment.

Inhibition of Wnt signaling by Nic in OVX rats did not appear to affect insulin sensitivity of the GWAT and soleus muscle. Inhibiting Wnt signaling in OVX rats in the presence of P_4 also did not significantly alter the expression of insulin signaling pathway markers in these tissues. However, inhibition of the Wnt pathway in the presence of E_2 decreased insulin sensitivity—which was heightened in the presence of E_2 alone—in the GWAT and soleus. Nic also decreased insulin sensitivity in the GWAT of OVX + E_2 + P_4 rats in a similar manner. While Nic did not affect the expression of *Slc2a4*, *Insr*, and *Acadm* in the soleus muscle of OVX + E_2 + P_4 rats, it increased the expression of *Foxo1* ($P = .0131$; Fig. 7F and 7K), a transcription factor that is negatively regulated by insulin signaling, and decreased insulin receptor-β ($P = .0152$) protein levels.

Effect of Steroids and Niclosamide on Fat Accumulation and Lipogenesis in the Gonadal White Adipose Tissue of Ovariectomized Rats

The loss of steroids by ovariectomy led to an increase ($P < .0001$) in the accumulation of GWAT, as evident from the weight of GWAT in intact (1.5 ± 0.2 g) and OVX rats (3.3 ± 0.2 g; Fig. 8A). Replacement of the steroid E_2 in OVX rats decreased GWAT accumulation (to 1.1 ± 0.1 g; $P < .0001$), but replacement of P_4 did not affect the quantity of GWAT. Replacement of both steroids ($E_2 + P_4$) in OVX rats also decreased GWAT (to 2.4 ± 0.3 g; $P = .0159$), although not to the same extent as E_2 alone. Although Nic treatment in OVX and OVX + P_4 rats did not affect GWAT accumulation, Nic treatment in the presence of E_2 appeared to negate the inhibitory effect of E_2 on fat accumulation—the weight of GWAT in E_2 + Nic-treated OVX rats (2.7 ± 0.2 g) was significantly ($P < .0001$) higher than in E_2-treated OVX rats (1.1 ± 0.1 g), and that in $E_2 + P_4$ + Nic-treated OVX rats (2.9 ± 0.2 g) was slightly (but nonsignificantly) higher than in $E_2 + P_4$-treated OVX rats (2.4 ± 0.2 g).

Much like the effect on GWAT accumulation, OVX mice showed a trend of increased expression of lipogenesis marker genes *Lpl* (Fig. 8B), *Slc27a1* (Fig. 8C), *Fasn* (Fig. 8D), and *Mogat1* (Fig. 8E), and the FASN protein (Fig. 8F and 8G). Although E_2 and $E_2 + P_4$ treatment showed a trend of inhibiting the expression of these markers in OVX rats, these effects were nonsignificant. P_4 treatment increased ($P < .0001$) the expression of *Lpl*, *Slc27a1*, *Fasn*, and *Mogat1*. While Nic treatment in OVX and OVX + P_4 rats did not significantly alter lipogenesis, Nic treatment in the presence of E_2 (in OVX + E_2 and OVX + E_2 + P_4 rats) enhanced the expression of lipogenesis markers in the GWAT.

Effect of Steroids and Niclosamide on Adipokines in Ovariectomized Rats

Adiponectin, a hormone secreted by the adipose tissue, is known to play an important role in sensitizing tissues to insulin [29]. Treatment with E_2 and $E_2 + P_4$ increased the circulating concentrations of adiponectin ($P < .0001$; Fig. 9A) and GWAT *Adipoq* expression (Fig. 9B) in OVX rats, while treatment with P_4 had no effect. This observation further confirms the insulin-sensitizing effect of E_2 in metabolically active tissues of the OVX rat. Treatment with Nic showed a trend of decreased serum adiponectin in OVX rats both in the absence and presence of steroids.

The adipokine leptin serves as a signal of "satiety." The expression of the leptin gene *Lep* in the GWAT of OVX rats was approximately 2-fold lower than that in intact rats ($P = .0174$; Fig. 9C). E_2 increased *Lep* expression 4.5-fold ($P < .0001$), but treatment with P_4 had no effect. In OVX rats treated with $E_2 + P_4$, P_4 inhibited the E_2-mediated increase in *Lep* expression in the GWAT ($P < .0001$). Nic treatment in OVX, OVX + P_4, and OVX + E_2 + P_4 rats also had no effect on *Lep* gene expression. However, Nic was able to inhibit the E_2-mediated increase in *Lep* expression ($P < .0001$).

Changes in Wnt Signaling in the Gonadal White Adipose Tissue and Soleus of Rats During Pregnancy

We analyzed the expression of canonical Wnt signaling pathway markers in GWAT and soleus muscle tissue collected from rats at various stages of pregnancy in comparison to

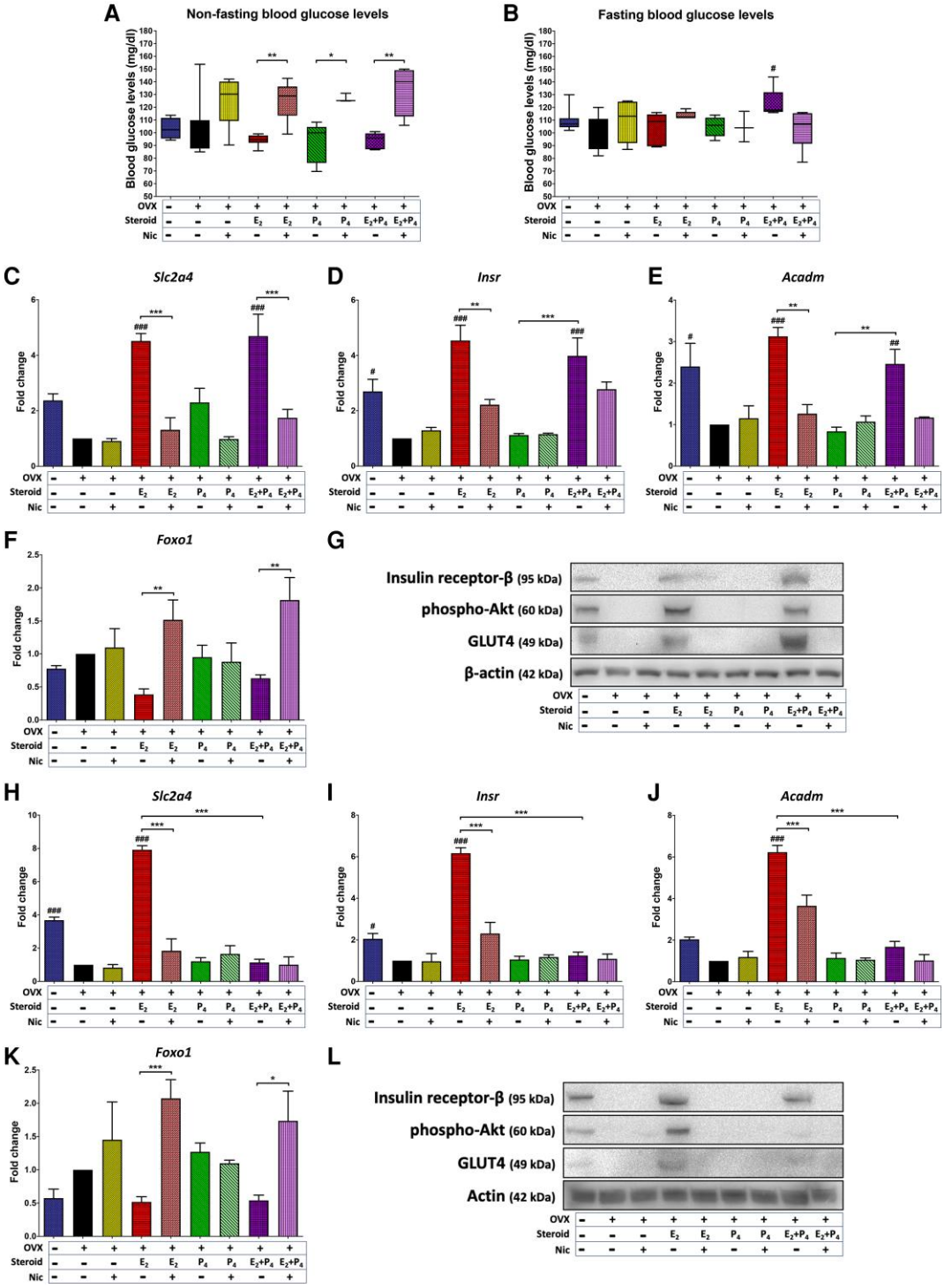

Figure 7. Effect of steroids and niclosamide (nic) on blood glucose levels and insulin sensitivity in the gonadal white adipose tissue (GWAT) and soleus skeletal muscle muscle of ovariectomized (OVX) rats. OVX rats were treated with Nic (20 mg/kg body weight/day), estradiol (E_2; 10 μg/day), progesterone (P_4; 10 mg/day), or their combinations, for a period of 23 days. A, Nonfasting and B, fasting (4 hours) glucose levels were measured in rats from each experimental group (n≥6) using blood collected from a tail nick. Data are plotted as mean ± SEM in A and B. GWAT and soleus muscle collected from these rats (n = 4 each) were analyzed for expression of insulin sensitivity marker genes like *Slc2a4* (C, GWAT; H, Soleus), *Insr* (D, GWAT; I, soleus), *Acadm* (E, GWAT; J, soleus), and *Foxo1* (F, GWAT; K, Soleus) by qRT-PCR analysis. Gene expression was normalized to the expression of *B2m*. GWAT and soleus lysates ($n = 3$ each) were also analyzed for expression of insulin signaling pathway proteins insulin receptor-β and phospho-Akt, and the facilitated glucose transporter GLUT4 by immunoblotting (G, GWAT; L, soleus). β-actin and actin were used as loading controls for GWAT and soleus lysates, respectively. Data is represented as mean ± SEM of fold change compared to the untreated OVX control group in C to F and H to K. Statistical significance among treatment groups was determined by one-way analysis of variance and Bonferroni post test. *, **, and *** represent statistically significant differences of *P* less than .05, *P* less than .01, and *P* less than .001, respectively. #, ##, and ### represent statistically significant differences of *P* less than .05, *P* less than .01, and *P* less than .001, respectively, with respect to untreated OVX rats. *Acadm*, acyl-CoA dehydrogenase medium chain; *B2m*, β-2 microglobulin; *Foxo1*, forkhead box O1; *Insr*, insulin receptor; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; *Slc2a4*, solute carrier family 2 member 4 or GLUT4.

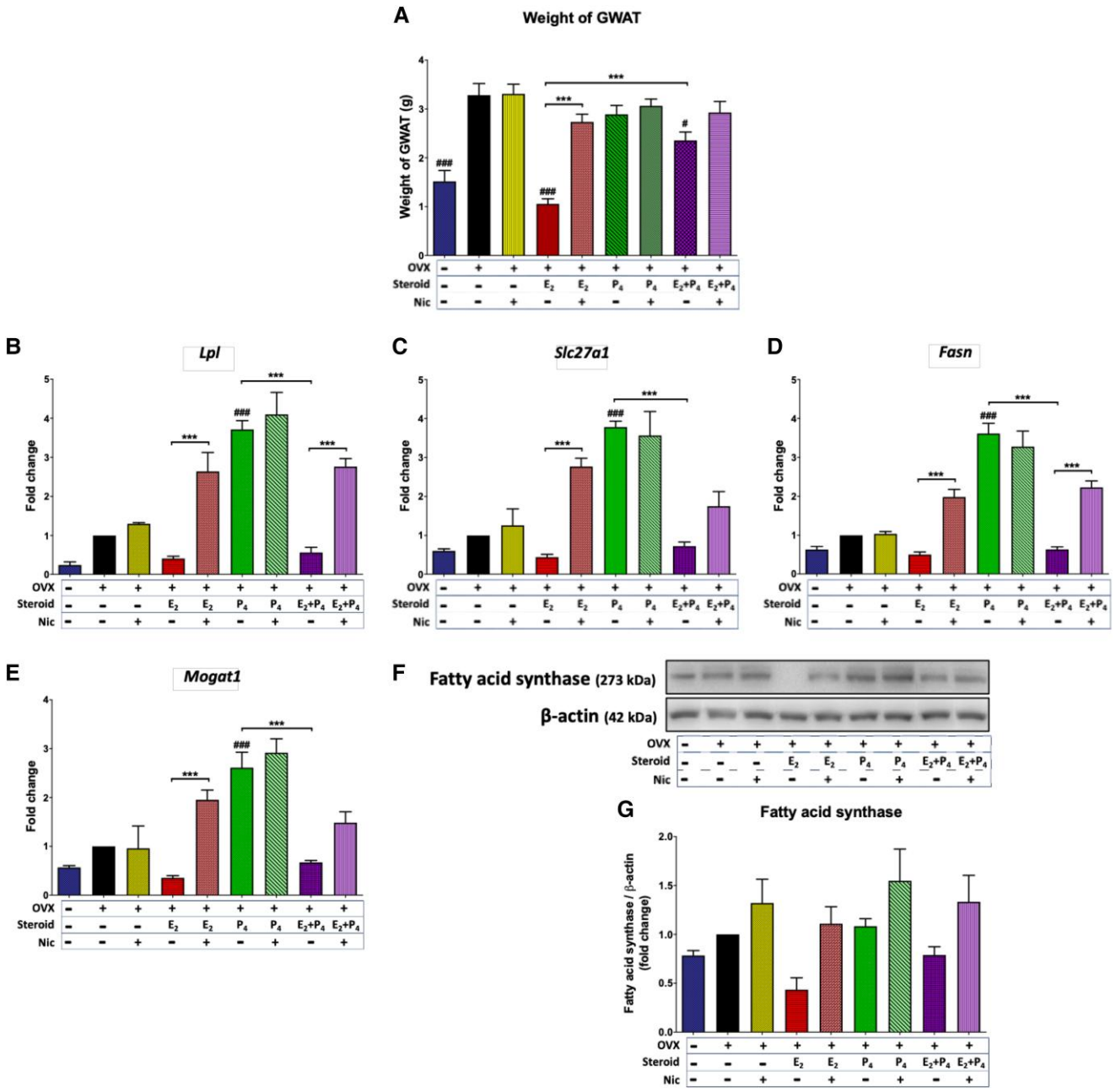

Figure 8. Effect of steroids and niclosamide (Nic) on fat accumulation and lipogenesis in the gonadal white adipose tissue (GWAT) of ovariectomized (OVX) rats. OVX rats were treated with Nic (20 mg/kg body weight/day), estradiol (E_2; 10 μg/day), progesterone (P_4; 10 mg/day), or their combinations, for a period of 23 days. A, GWAT collected from these rats (n≥6) was weighed and plotted as mean ± SEM. RNA isolated from GWAT (n = 4) was analyzed for the expression of lipogenesis markers B, *Lpl*; C, *Fasn*; D, *Slc27a1*; and E, *Mogat1*, by qRT-PCR analysis. Gene expression was normalized to the expression of *B2m*. F, GWAT lysates (n = 3) were also analyzed for expression of the fatty acid synthase enzyme complex by immunoblotting. β-actin was used as the loading control. G, Densitometric analysis was performed. Data are represented as mean ± SEM of fold change compared to the untreated OVX control group in B to E and G. Statistical significance among treatment groups was determined by one-way analysis of variance and Bonferroni post test. *, **, and *** represent statistically significant differences of *P* less than .05, *P* less than .01, and *P* less than .001, respectively. #, ##, and ### represent statistically significant differences of *P* less than .05, *P* less than .01, and *P* less than .001, respectively, with respect to untreated OVX rats. *B2m*, β-2 microglobulin; *Fasn*, fatty acid synthase; *Lpl*, lipoprotein lipase; *Mogat1*, monoacylglycerol O-acyltransferase 1; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; *Slc27a1*, solute carrier family 27 member 1 or long-chain fatty acid transport protein 1.

that in NP rats. The active form of β-catenin in the GWAT (Fig. 10A and 10B) appeared to increase during the initial stages of pregnancy until about day 14, and later decreased. The expression of *Lrp5* ($P = .0505$; Fig. 10D), *Axin2* ($P = .0046$; Fig. 10E), and *Ppard* ($P = .0444$; Fig. 10F), also indicated that Wnt signaling was upregulated in the GWAT during pregnancy. On the other hand, the expression of *Tcf7l2* ($P = .0016$; Fig. 10J), *Lrp5* ($P = .0270$; Fig. 10K), and *Axin2* ($P = .0092$; Fig. 10L), suggested that Wnt signaling was downregulated in the soleus during pregnancy.

Effect of Niclosamide Treatment During Pregnancy in Rats

Pregnant rats were treated with Nic to assess the effect of inhibiting Wnt signaling on energy metabolism during

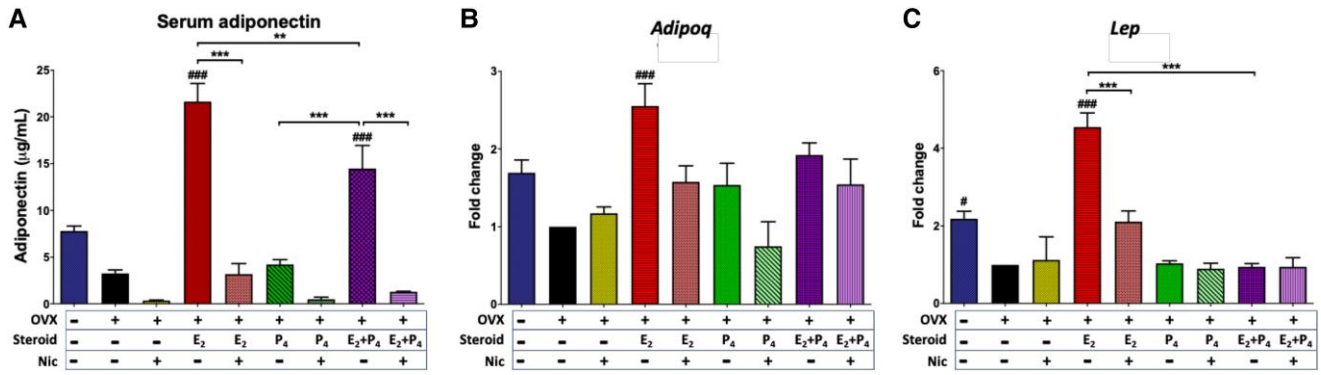

Figure 9. Effect of steroids and niclosamide (Nic) on adipokines in ovariectomized (OVX) rats. OVX rats were treated with Nic (20 mg/kg body weight/day), estradiol (E_2; 10 µg/day), progesterone (P_4; 10 mg/day), or their combinations, for 23 days. A, Blood collected from these rats (n = 5) was used to determine serum adiponectin concentrations by ELISA. Data are represented as mean ± SEM of the absolute values. The GWAT collected from these rats (n = 4) was analyzed for the expression of adipokines B, *Adipoq* and C, *Lep* by qRT-PCR analysis. Gene expression was normalized to the expression of *B2m*. Data are represented as mean ± SEM of fold change compared to the untreated OVX control group in B and C. Statistical significance among treatment groups was determined by one-way analysis of variance and Bonferroni post-test. ** and *** represent statistically significant differences of *P* less than .01 and *P* less than .001, respectively. # and ### represent statistically significant differences of *P* less than .05 and *P* less than .001, respectively, with respect to untreated OVX rats. *Adipoq*, adiponectin; *B2m*, β-2 microglobulin; ELISA, enzyme-linked immunosorbent assay; *Lep*, leptin; GWAT, gonadal white adipose tissue.

pregnancy. Nic inhibited Wnt signaling both in the GWAT and soleus muscle of pregnant rats (Supplementary Fig. S6 [25]). As with OVX rats, Nic treatment in pregnant rats caused a trend of decreased body weight gain (150.7 ± 5.0% in Veh-treated rats vs 127.5 ± 7.0% in Nic-treated rats on day 21 of pregnancy; Fig. 11A) and food consumption (303 ± 12 g in Veh-treated rats vs 228 ± 24 g in Nic-treated rats over 21 days of pregnancy; $P = .0083$; Fig. 11B). There was no significant difference in nonfasting (Fig. 11C) and fasting blood glucose levels (Fig. 11D) with Nic treatment in pregnant rats. Nic decreased the expression of genes *Slc2a4* ($P = .0011$ in GWAT; $P = .0003$ in soleus; Fig. 11E and 11J), *Insr* ($P = .0043$ in GWAT; $P = .0077$ in soleus; Fig. 11F and 11K), *Acadm* ($P = .0113$ in GWAT; $P = .0014$ in soleus; Fig. 11G and 11L), and insulin receptor-β ($P = .0179$ in GWAT; $P = .0116$ in soleus), phospho-Akt ($P = .0338$ in GWAT; $P = .0262$ in soleus), and GLUT4 proteins ($P = .0850$ in GWAT; $P = .0296$ in soleus; Fig. 11I and 11N), and increased the expression of *Foxo1* in the GWAT ($P = .0197$) and soleus ($P = .0343$; Fig. 11H and 11M), indicating that inhibition of Wnt signaling decreased insulin sensitivity in pregnant rats. Inhibition of Wnt signaling by Nic increased lipogenesis in the GWAT of pregnant rats, as evident from the expression of marker genes *Lpl* ($P = .0002$; Fig. 11O), *Slc27a1* ($P = .0071$; Fig. 11P), *Fasn* ($P = .0011$; Fig. 11Q), *Mogat1* ($P = .0134$; Fig. 11R), and the FASN protein ($P = .0412$; Fig. 11S). The expression of the adipokines *Adipoq* ($P = .0141$; Fig. 11U) and *Lep* ($P = .0013$; Fig. 11V) was lower in the GWAT of Nic-treated pregnant rats, compared to that in Veh-treated pregnant rats. Serum adiponectin levels, which were found to decrease through pregnancy in the rat (Fig. 11W), were further decreased with Nic treatment ($P = .0016$; Fig. 11T).

We also observed that the liver of Nic-treated pregnant rats were much larger than that of Veh-treated pregnant rats, and appeared to have a whitish layer of fat lining the surface (Fig. 12A). The weight of the whole liver was significantly higher in Nic-treated pregnant rats (Fig. 12B and 12C). Analysis of expression of liver-specific lipogenic genes *Fasn* ($P = .0095$; Fig. 12D), *Mogat1* ($P = .0083$; Fig. 12E), *Dgat1* (diacylglycerol O-acyltransferase 1; $P = .0129$; Fig. 12F), and *Dbi* (diazepam binding inhibitor or acyl-CoA binding protein; $P = .0494$; Fig. 12G), also revealed that Nic treatment increased lipogenesis in the liver of pregnant rats. Remarkably, such an effect on the liver was not observed with Nic treatment in OVX rats in the absence or presence of steroids (Supplementary Fig. S7 [25]).

Surprisingly, in addition to the observations mentioned earlier, we found that treatment with 20-mg Nic per kg body weight in pregnant rats affected survival of pregnant rats, whereas no deleterious effects were observed in OVX rats treated with Nic. Out of 10 pregnant rats for which Nic administration was initiated beginning day 6 of pregnancy, only 6 survived until parturition. In contrast, all 10 pregnant rats that were administered Veh survived until parturition. Nic treatment was also found to delay parturition by 3 to 4 days in pregnant rats. While the Veh-treated rats parturiated on day 22 to 23 of pregnancy, the Nic-treated rats parturiated only by day 26. Another striking observation made was the smaller litter size (9-10 pups) of Nic-treated rats, compared to that of Veh-treated rats (13-15 pups). In rats euthanized on day 21 of pregnancy, the number of implants was also found to be lesser in Nic-treated rats than in the controls. While the pups of Veh-treated rats were healthy and normal, all the pups of Nic-treated rats were either dead at birth or died within a day or two of birth. Therefore, Nic appears to have a negative effect on pregnancy and fetal development in rats.

Discussion

The endocrine system plays a crucial role in the maintenance of energy homeostasis in the body. During pregnancy, steroid hormones secreted by the ovaries and placenta are believed to be involved in the manifestation of metabolic changes characteristic of this period. Although the primary functions of these steroids in most mammals is in the manifestation of primary and secondary sexual characteristics and in the process of reproduction (gametogenesis), hormones like E_2 and P_4 have been found to affect energy metabolism to varying degrees. However, the actions of these hormones are often dependent

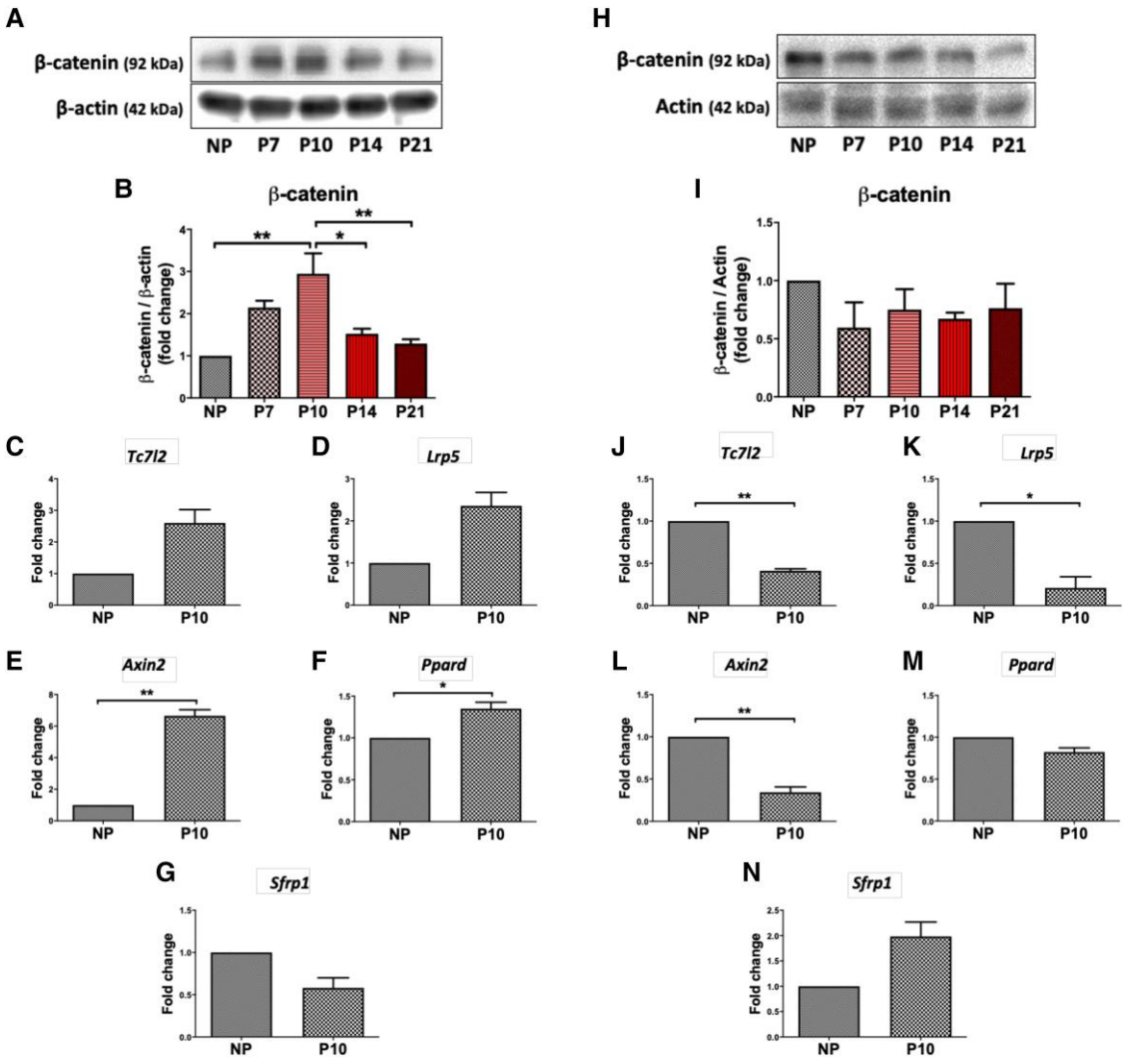

Figure 10. Expression of wnt signaling pathway markers in the gonadal white adipose tissue (GWAT) and soleus skeletal muscle of nonpregnant and pregnant rats. GWAT and soleus muscle collected from nonpregnant (NP) rats and from pregnant rats humanely killed on day 7 (P7), day 10 (P10), day 14 (P14), and day 21 (P21) of their pregnancy (n = 3 each) was analyzed for the active form of β-catenin by immunoblotting (A, GWAT; H, soleus). β-Actin and actin were used as loading controls for GWAT and soleus lysates, respectively. B, Densitometric analysis was performed (GWAT; I, soleus). GWAT and soleus muscle from NP and P10 rats (n = 4 each) were also analyzed for the expression of canonical Wnt signaling pathway genes *Tcf7l2* (C, GWAT; J, Soleus) and *Lrp5* (D, GWAT; K, Soleus), target genes *Axin2* (E, GWAT; L, Soleus) and *Ppard* (F, GWAT; M, Soleus), and the negative regulator of Wnt signaling *Sfrp1* (G, GWAT; N, Soleus) by qRT-PCR analysis. Gene expression was normalized to the expression of *B2m*. Data are represented as mean ± SEM of fold change compared to the NP control group, in B to G and I to N. Statistical significance was determined by one-way analysis of variance and Bonferroni post test in B and I and by *t* test in C to G and J to N. * and ** represent statistically significant differences of *P* less than .05 and *P* less than .01, respectively. *Axin2*, axis inhibition protein 2; *B2m*, β-2 microglobulin; *Lrp5*, low-density lipoprotein receptor-related protein 5; *Ppard*, peroxisome proliferator-activated receptor delta; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; *Sfrp1*, secreted frizzled-related protein 1; *Tcf7l2*, transcription factor 7-like 2.

on the system in which it is present, with effects being specific to tissue or cell type, specific to sex, and also dependent on other factors that may influence their function. In the present study, experiments were conducted to assess the effects of E_2 and P_4 on glucose and lipid metabolism and ascertain the role of the canonical Wnt signaling pathway in mediating these effects, employing both in vitro (an adipocyte cell line) and in vivo (an OVX rat model) approaches. In OVX rats, we specifically examined the GWAT because it represents abdominal fat depots in humans (abdominal fat is commonly associated with the incidence of metabolic disorders), and the soleus skeletal muscle of the hind limb, because it is rich in insulin-sensitive type I oxidative muscle fibers.

We observed that the steroids E_2 and P_4 positively influence canonical Wnt signaling and increase active β-catenin levels in 3T3-L1 adipocytes and preadipocytes, and in the GWAT and soleus muscle of OVX rats. Treatment with both E_2 and P_4 appeared to synergistically enhance Wnt signaling. In several other studies, E_2 has been reported to increase the expression of Wnt ligands, inactivate glycogen synthase kinase 3β (GSK3β), stabilize the β-catenin protein, and enhance the expression of T-cell factors/lymphoid enhancer factors (TCF/LEF) [30-35]. These observations were, however, made in uterine stromal cells, endometrial biopsies, mouse blastocysts, osteoblasts, and neural cells. Similarly, P_4 has also been shown to increase the expression of Wnt ligands and β-catenin in uterine stromal cells, breast cancer cells, mammary epithelial cells, and in the dorsal hippocampus of mice [30, 36, 37]. Both E_2 and P_4 have been demonstrated to increase the expression of *Tcf7l2* and β-catenin in human liver cancer cells,

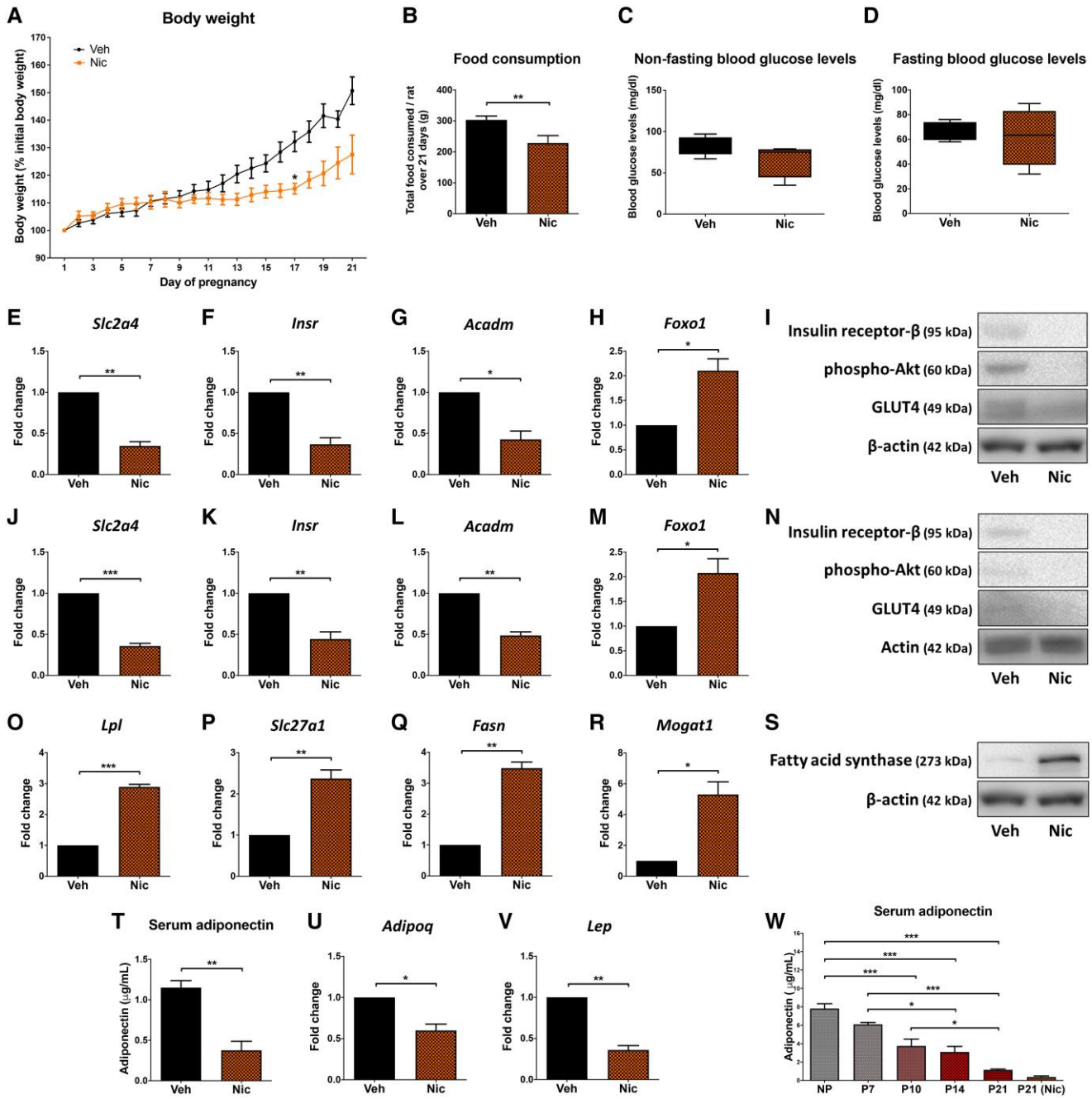

Figure 11. Effect of niclosamide (Nic) treatment in pregnant rats. Rats were treated with either the vehicle (Veh; 10% Cremophor EL) or Nic (20 mg/kg body weight/day) during pregnancy. Body weights of vehicle (Veh) and Nic-treated pregnant rats were recorded on a daily basis (n≥6). Body weight on each day is expressed as a percentage of the body weight on day 1 of treatment (considered 100%) for each rat in each group. A, Mean ± SEM of the percentage of initial body weight of animals in each group is plotted against the day of treatment. Statistical significance between the groups was determined by 2-way analysis of variance and Bonferroni post test. B, Total food consumption of Veh- and Nic-treated pregnant rats was recorded over the course of the treatment period. C, Nonfasting and D, fasting (4 hours) glucose levels were measured in rats at the end of treatment (n≥6) using blood collected from a tail nick. Data are represented as mean ± SEM in B to D. Gonadal white adipose tissue (GWAT) collected from Veh- and Nic-treated pregnant rats (n = 4) was analyzed for the expression of insulin sensitivity marker genes like E, *Slc2a4*; F, *Insr*; G, *Acadm*; and H, *Foxo1* for the expression of lipogenesis markers O, *Lpl*; P, *Fasn*; Q, *Slc27a1*; and R, *Mogat1* and for the expression of adipokines U, *Adipoq* and V, *Lep* by qRT-PCR analysis. Similarly, the soleus skeletal muscle (n = 4) was analyzed for the expression of insulin sensitivity marker genes J, *Slc2a4*; K, *Insr*; L, *Acadm*; and M, *Foxo1*. Gene expression was normalized to the expression of *B2m*. GWAT lysates (n = 3) were analyzed for expression of insulin signaling pathway proteins insulin receptor-β and phospho-Akt; I, the facilitated glucose transporter GLUT4; and S, the fatty acid synthase enzyme complex, by immunoblotting. Soleus lysates (n = 3) were analyzed for expression of N, insulin receptor-β, phospho-Akt, and GLUT4 by immunoblotting. β-Actin and actin were used as loading controls for GWAT and soleus lysates, respectively. Data are represented as mean ± SEM of fold change compared to the Veh-treated group in E to H, J to M, O to R, and U to V. T, Blood collected from Veh- or Nic-treated pregnant rats (n = 5) was used to determine serum adiponectin concentrations by ELISA. W, Adiponectin levels were also measured in blood collected from nonpregnant rats (NP) and from pregnant rats humanely killed on day 7 (P7), day 10 (P10), day 14 (P14), and day 21 (P21) of their pregnancy (n = 4) for comparison. Data are represented as mean ± SEM of the absolute values in T and W. Statistical significance between treatment groups was determined by *t* test. *, **, and *** represent statistically significant differences of *P* less than .05, *P* less than .01, and *P* less than .001, respectively. *Acadm*, acyl-CoA dehydrogenase medium chain; *Adipoq*, adiponectin; *B2m*, β-2 microglobulin; ELISA, enzyme-linked immunosorbent assay; *Fasn*, fatty acid synthase; *Foxo1*, forkhead box O1; *Insr*, insulin receptor; *Lep*, leptin; *Lpl*, lipoprotein lipase; *Mogat1*, monoacylglycerol O-acyltransferase 1; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; *Slc2a4*, solute carrier family 2 member 4 or GLUT4; *Slc27a1*, solute carrier family 27 member 1 or long-chain fatty acid transport protein 1.

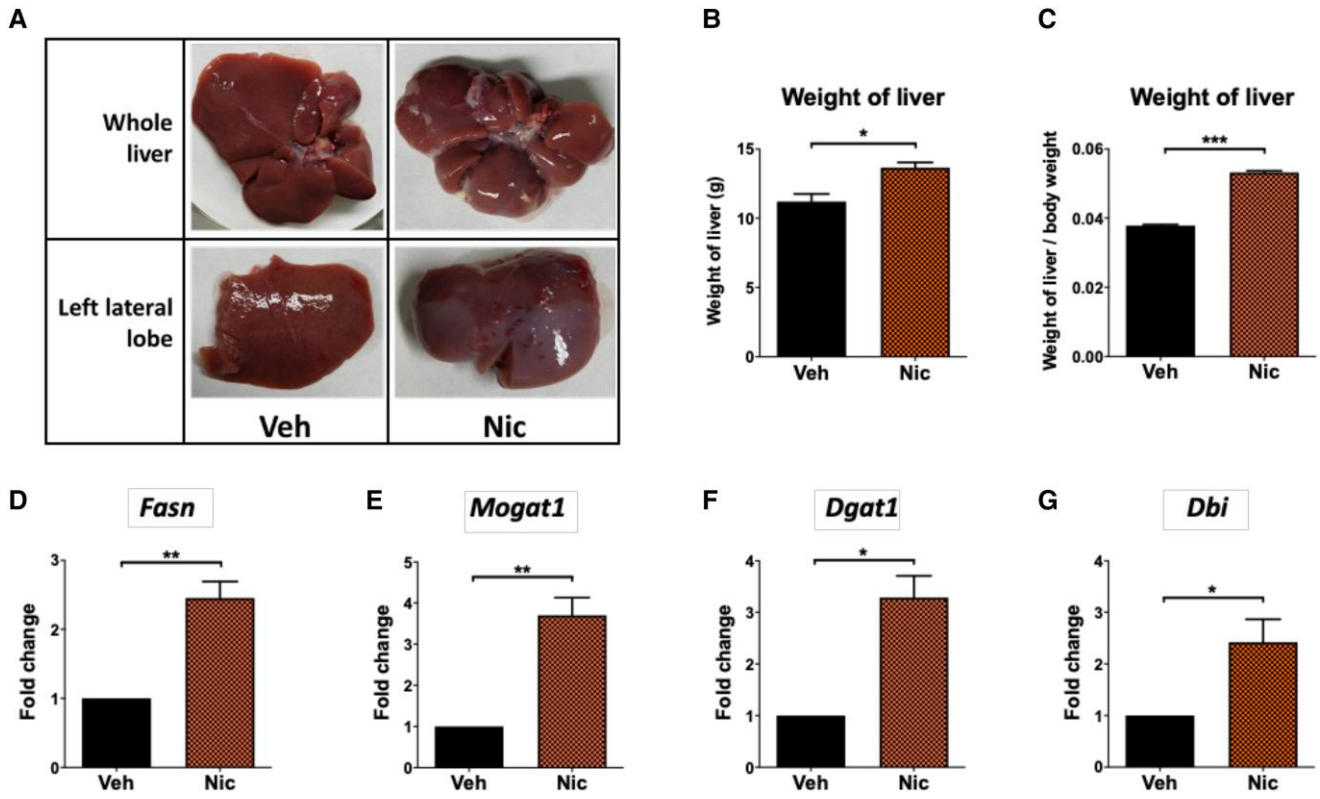

Figure 12. Effect of niclosamide (Nic) on weight of liver and expression of lipogenic genes in liver tissue of pregnant rats. Whole liver collected from pregnant rats treated with the vehicle (Veh; 10% Cremophor EL) or Nic (20 mg/kg body weight/day) (n≥6) was A, photographed and B, weighed. C, The weight of the liver is also expressed as a fraction of the body weight of the rat. The whitish appearance of the liver from Nic-treated pregnant rats in A denotes the accumulation of fat around the organ. Data are represented as mean ± SEM in B and C. The liver collected from Veh- and Nic-treated pregnant rats (n = 4) was analyzed for the expression of liver-specific lipogenesis markers D, *Fasn*; E, *Mogat1*; F, *Dgat1*; and G, *Dbi* by qRT-PCR analysis. Gene expression was normalized to the expression of *B2m*. Data are represented as mean ± SEM of fold change compared to the Veh-treated group in D to G. Statistical significance between treatment groups was determined by *t* test. *, **, and *** represent statistically significant differences of *P* less than .05, *P* less than .01, and *P* less than .001, respectively. *B2m*, β-2 microglobulin; *Dbi*, diazepam binding inhibitor or acyl-CoA binding protein; *Dgat1*, diacylglycerol O-acyltransferase 1; *Fasn*, fatty acid synthase; *Mogat1*, monoacylglycerol O-acyltransferase 1; qRT-PCR, quantitative reverse-transcription polymerase chain reaction.

mouse primary hepatocytes, and mouse insulinoma cells [22, 23]. The present study is, therefore, the first to demonstrate steroid-mediated effects on the Wnt signaling pathway in metabolically active adipocytes, and in the adipose tissue and skeletal muscle.

While E_2 enhanced insulin signaling in adipocytes and in the adipose tissue and skeletal muscle of OVX rats, P_4 had no effect on insulin sensitivity. The observations made by incubation of adipocytes and treatment of OVX rats both with E_2 and P_4 suggest that the actions of E_2 on insulin sensitivity in the GWAT and soleus are blunted or inhibited in the presence of P_4. In contrast, an early study has reported that a combination of these steroids in a manner simulating pregnancy diminishes whole-body insulin sensitivity in OVX mice [38]. A more recent study has also demonstrated insulin resistance in OVX mice and hepatocytes from treatment with E_2 and P_4 at concentrations compatible with pregnancy [5]. On the other hand, another report suggests that both E_2 and P_4 individually improve insulin sensitivity of the skeletal muscle in streptozotocin-induced diabetic rats and their treatment in combination has a synergistic effect [39]. Treatment with both E_2 and P_4 in postmenopausal women has been shown to increase free fatty acid–induced insulin resistance, in contrast to treatment with E_2 alone [40]. High circulating concentrations of both E_2 and P_4 are associated with insulin resistance in women prior to the onset of menopause [41]. However, other reports suggest that hormone replacement therapy including both E_2 and P_4 improves insulin sensitivity in women post menopause [42]. Therefore, the actions of E_2 and P_4 in combination appear to be dependent on their individual concentrations or their ratio to one another. We were unable to mimic the insulin-resistant state as in pregnancy through $E_2 + P_4$ treatment in OVX rats. Treating OVX rats with various dosages of E_2 and P_4 would have been ideal in determining the relationship between steroid ratio and metabolic disturbances.

Although inhibition of canonical Wnt signaling by Nic did affect insulin sensitivity of 3T3-L1 preadipocytes and adipocytes, Nic abrogated the increased insulin signaling brought about by the presence of E_2 in the media (as observed with E_2 alone or with $E_2 + P_4$). These observations are indicative of the involvement of the Wnt pathway in the insulin-sensitizing effect of E_2 in adipocytes, and also support similar findings in the GWAT and soleus muscle of the OVX rat model. Although other studies have reported the involvement of Wnt pathway proteins in mediating the actions of E_2 in the mouse liver and islet cells [22, 23], this is the first report demonstrating such an effect in the adipose tissue and skeletal muscle.

Blocking Wnt signaling in differentiating preadipocytes using 1-μM Nic was found to hasten the process of adipogenesis.

These findings are in concurrence with several other reports indicating that Wnt signaling inhibits CEBPA and PPARG and represses adipogenic differentiation [21, 43]. Although most reports support the idea that β-catenin–mediated Wnt signaling inhibits adipogenesis in vitro, Gyamfi and colleagues [44] have shown that Nic concentrations greater than 1 μM inhibited adipogenic differentiation in primary human preadipocytes. Concomitant with the increase in adipogenesis in our study, the expression of lipogenic genes was also increased by Nic. However, in OVX rats, Nic did not affect GWAT accumulation and lipogenesis. In contrast, a recent study suggests that Wnt signaling is required for the process of lipogenesis and adipose tissue-specific knockout of β-catenin in mice promotes resistance to diet-induced obesity [45]. Although there are no reports detailing changes in adipose tissue lipogenesis with Nic, numerous studies have demonstrated that Nic and its derivatives prevent high-fat diet– and high-carbohydrate diet–induced lipogenesis in the liver and have a protective function against the development of hepatic steatosis and nonalcoholic fatty liver disease in mouse models [46-49]. Another study suggests that Nic corrects the harmful effects of a high-fat diet on the lipid profile in mice [50].

E_2 was found to inhibit adipogenic differentiation. In the female rat, exogenous administration of E_2 following OVX repressed fat accumulation in GWAT depots. P_4 did not significantly alter the process of adipogenesis. Although there was no measurable change in the quantity of GWAT in OVX rats treated with P_4, it enhanced the expression of lipogenic genes in the GWAT. Incubation of differentiating adipocytes both with E_2 and P_4 inhibited adipogenic differentiation, although not to the same degree as incubation with E_2 alone. $E_2 + P_4$ treatment in OVX rats also reduced the accumulation of GWAT, but not to the same extent as treatment with E_2 alone. These observations indicate that the actions of E_2 on adipogenesis are blunted in the presence of P_4, much like its effects on insulin sensitivity. Inhibition of Wnt signaling by Nic appeared to block the antiadipogenic action of E_2 in adipocytes and in the GWAT of OVX rats, both in the absence and presence of P_4, suggesting that the inhibitory effect of E_2 on adipogenic differentiation and fat accumulation is dependent on the Wnt signaling pathway.

The loss of E_2 following ovariectomy brought about a remarkable increase in the body weight gain over the course of the 23-day treatment period, which could be controlled by the administration of exogenous E_2. The modulation of body weight gain in E_2-treated OVX rats can be attributed to decreased adiposity (evident from the suppressed accumulation of fat in GWAT depots) as well as reduced food intake. E_2 has been hypothesized to modulate food intake by its stimulatory effects on anorectic hormones like leptin [51, 52]. We found that treatment with E_2 promoted *Lep* gene expression in the GWAT of OVX rats, a finding that may be well correlated with the decrease in food intake. Unlike E_2, exogenous administration of P_4 in OVX rats did not bring about any changes in body weight and food consumption. P_4, however, appeared to diminish the effect of E_2 on these indicators of whole-body metabolism when both steroids were administered to OVX rats.

As aberrations in the *Tcf7l2* gene and defects in the Wnt signaling cascade are known to be strongly associated with GDM risk during pregnancy, the expression of Wnt pathway genes and β-catenin in the GWAT and soleus of pregnant and NP rats was examined with the objective to understand its regulation during pregnancy. Canonical Wnt signaling was found to be upregulated in the GWAT during pregnancy but downregulated in the soleus muscle. Since Wnt signaling is reported to decrease insulin signaling and glucose uptake [19], the suppression of Wnt signaling in the skeletal muscle may be correlated with the decrease in insulin sensitivity characteristic of maternal tissues during the course of pregnancy. On the other hand, our study supports the premise that Wnt signaling inhibits adipogenesis and lipogenesis. In this context, the increase in Wnt signaling observed in the GWAT may be associated with the increased lipolysis and breakdown of fat depots due to higher energy demand in the latter half of pregnancy. Moreover, the levels of the steroids continuously increase during the course of pregnancy in rats, up until parturition. The upregulation of Wnt signaling in the GWAT of rats during the early part of pregnancy, as observed in our study, may be correlated with the increase in circulating E_2 and P_4 concentrations. However, during late pregnancy, the expression of Wnt pathway markers decreases while steroid levels continue to increase. This may be due to the contribution of various other hormones and factors important for the process of parturition that influence Wnt signaling during this period.

In pregnant rats, inhibiting Wnt signaling decreased insulin sensitivity of the GWAT and the soleus muscle, and decreased the expression of lipogenesis markers in the GWAT. These observations are in agreement with reports suggesting a positive effect of Wnt signaling on insulin sensitivity and glucose uptake [18, 19, 53] and a negative effect on lipogenesis [54]. Serum adiponectin levels, indicative of whole-body insulin sensitivity, also decreased with Nic treatment. The increase in lipogenesis, however, was not accompanied by increased body weight gain in Nic-treated pregnant rats. In fact, the body weight gain in Nic-treated pregnant rats appeared to be lower than that in Veh-treated pregnant rats. The decline in body weight gain can be attributed to reduced food consumption with Nic treatment in pregnant rats, but could not be correlated with the decrease in GWAT *Lep* expression. Further, inhibition of Wnt signaling by Nic enhanced lipogenesis in the liver of pregnant rats. Such an effect was, however, not evident in the liver tissue of OVX rats treated with Nic in the absence or presence of steroids, suggesting that this action of Nic may be dependent on the presence of other factors during pregnancy.

Surprisingly, Nic treatment (20 mg/kg body weight i.p.) during the course of pregnancy was found to negatively affect survival of pregnant rats and their pups. The decreased body weight gain of Nic-treated pregnant rats can, therefore, additionally be attributed to the lower weight of the gravid uterus due to lesser number of implants and smaller size of pups. Nic was also found to delay parturition in pregnant rats in the present study. These findings are contrary to another report that indicates that Nic (at doses as high as 200 mg/kg body weight, oral) does not affect reproductive function and does not affect the number, size, or development of pups in pregnant mice [55]. An early report in rats also suggests that Nic treatment (80 mg/kg body weight, oral) in pregnant rats does not greatly affect the development of fetuses [56]. Moreover, the World Health Organization data sheet on Nic indicates that doses of up to 44 mg/kg body weight i.p. do not have any adverse effects in rodents [57]. Nic has been reported to be safe for use in humans and in pregnant women when administered orally. The deleterious effects of Nic in pregnant rats observed in

the present study may, therefore, be attributed to the systemic mode of administration employed.

In summary, the present study reports that the steroids E_2 and P_4 upregulate canonical Wnt signaling in 3T3-L1 adipocytes, and in the GWAT and soleus muscle of OVX rats. E_2 improves insulin sensitivity in the adipose tissue and skeletal muscle, represses fat accumulation, and represses adipogenic differentiation in vitro. These actions of E_2 appear to be mediated by the β-catenin-dependent Wnt signaling pathway. The presence of P_4 dampens the observed metabolic effects of E_2. Inhibition of Wnt signaling, independently, was found to hasten the process of adipogenesis. During pregnancy, Wnt signaling is upregulated in the GWAT, but downregulated in the soleus muscle of rats. Inhibition of Wnt signaling by Nic in pregnant rats, which had deleterious effects during pregnancy, decreased insulin sensitivity of the adipose tissue and skeletal muscle, and increased lipogenesis in the adipose tissue and liver. The Wnt/β-catenin signaling pathway, therefore, plays an important role in the maintenance of energy homeostasis in several tissues and cell types. Since abnormal circulating steroid concentrations in various contexts (pregnancy, polycystic ovary disease) are often linked to metabolic disorders, the findings presented in this study are relevant in understanding the pathophysiology of such conditions and devising methods to manage them.

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Author Contributions

N.S.A. and R.M. conceived and designed the study. N.S.A. performed the experiments and acquired and analyzed data. N.S.A. and H.R.K. performed the animal experiments. N.S.A. and R.M. interpreted the results. N.S.A. wrote the manuscript. R.M., S.G.R., and H.R.K. reviewed and approved the final version of the manuscript.

Disclosures

The authors have nothing to disclose. The authors declare no conflicts of interest.

Data Availability

Original data generated and analyzed during this study are included in this published article or in the data repositories listed in "References."

References

1. Vejrazkova D, Vcelak J, Vankova M, *et al.* Steroids and insulin resistance in pregnancy. *J Steroid Biochem Mol Biol*. 2014;139:122-129.
2. Napso T, Yong HEJ, Lopez-Tello J, Sferruzzi-Perri AN. The role of placental hormones in mediating maternal adaptations to support pregnancy and lactation. *Front Physiol*. 2018;9:1091.
3. Parrettini S, Caroli A, Torlone E. Nutrition and metabolic adaptations in physiological and complicated pregnancy: focus on obesity and gestational diabetes. *Front Endocrinol (Lausanne)*. 2020;11:937.
4. Del Rincon JP, Iida K, Gaylinn BD, *et al.* Growth hormone regulation of p85α expression and phosphoinositide 3-kinase activity in adipose tissue: mechanism for growth hormone-mediated insulin resistance. *Diabetes*. 2007;56(6):1638-1646.
5. Masuyama H, Hiramatsu Y. Potential role of estradiol and progesterone in insulin resistance through constitutive androstane receptor. *J Mol Endocrinol*. 2011;47(2):229-239.
6. Gupte AA, Pownall HJ, Hamilton DJ. Estrogen: an emerging regulator of insulin action and mitochondrial function. *J Diabetes Res*. 2015;2015:916585.
7. Villarroel C, Salinas A, López P, *et al.* Pregestational type 2 diabetes and gestational diabetes exhibit different sexual steroid profiles during pregnancy. *Gynecol Endocrinol*. 2017;33(3):212-217.
8. Ferrara A. Increasing prevalence of gestational diabetes mellitus: a public health perspective. *Diabetes Care*. 2007;30(Suppl 2):S141-S146.
9. McIntyre HD, Catalano P, Zhang C, Desoye G, Mathiesen ER, Damm P. Gestational diabetes mellitus. *Nat Rev Dis Prim*. 2019;5(1):47.
10. Barbour LA, McCurdy CE, Hernandez TL, Kirwan JP, Catalano PM, Friedman JE. Cellular mechanisms for insulin resistance in normal pregnancy and gestational diabetes. *Diabetes Care*. 2007;30(Suppl 2):S112-S119.
11. Baz B, Riveline JP, Gautier JF. Endocrinology of pregnancy: gestational diabetes mellitus: definition, aetiological and clinical aspects. *Eur J Endocrinol*. 2016;174(2):R43-R51.
12. Powe CE, Allard C, Battista MC, *et al.* Heterogeneous contribution of insulin sensitivity and secretion defects to gestational diabetes mellitus. *Diabetes Care*. 2016;39(6):1052-1055.
13. Buchanan TA, Xiang AH. Gestational diabetes mellitus. *J Clin Invest*. 2005;115(3):485-491.
14. Coustan DR. Gestational diabetes mellitus. *Clin Chem*. 2013;59(9):1310-1321.
15. Grant SFA, Thorleifsson G, Reynisdottir I, *et al.* Variant of transcription factor 7-like 2 (*TCF7L2*) gene confers risk of type 2 diabetes. *Nat Genet*. 2006;38(3):320-323.
16. Lin PC, Lin WT, Yeh YH, Wung SF. Transcription factor 7-like 2 (TCF7L2) rs7903146 polymorphism as a risk factor for gestational diabetes mellitus: a meta-analysis. *PLoS One*. 2016;11(4):e0153044.
17. Saxena R, Gianniny L, Burtt NP, *et al.* Common single nucleotide polymorphisms in *TCF7L2* are reproducibly associated with type 2 diabetes and reduce the insulin response to glucose in nondiabetic individuals. *Diabetes*. 2006;55(10):2890-2895.
18. Yoon JC, Ng A, Kim BH, Bianco A, Xavier RJ, Elledge SJ. Wnt signaling regulates mitochondrial physiology and insulin sensitivity. *Genes Dev*. 2010;24(14):1507-1518.
19. Abiola M, Favier M, Christodoulou-Vafeiadou E, Pichard AL, Martelly I, Guillet-Deniau I. Activation of Wnt/β-catenin signaling increases insulin sensitivity through a reciprocal regulation of Wnt10b and SREBP-1c in skeletal muscle cells. *PLoS One*. 2009;4(12):e8509.
20. Palsgaard J, Emanuelli B, Winnay JN, Sumara G, Karsenty G, Kahn CR. Cross-talk between insulin and Wnt signaling in preadipocytes: role of Wnt co-receptor low density lipoprotein receptor-related protein-5 (LRP5). *J Biol Chem*. 2012;287(15):12016-12026.
21. Prestwich TC, MacDougald OA. Wnt/β-catenin signaling in adipogenesis and metabolism. *Curr Opin Cell Biol*. 2007;19(6):612-617.
22. Dong F, Ling Q, Ye D, *et al.* TCF7L2 involvement in estradiol- and progesterone-modulated islet and hepatic glucose homeostasis. *Sci Rep*. 2016;6(1):24859.
23. Tian L, Shao W, Ip W, Song Z, Badakhshi Y, Jin T. The developmental Wnt signaling pathway effector β-catenin/TCF mediates hepatic functions of the sex hormone estradiol in regulating lipid metabolism. *PLoS Biol*. 2019;17(10):e3000444.
24. Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. *Proc Natl Acad Sci U S A*. 1986;83(8):2496-2500.

25. Alex NS, Khan HR, Ramachandra SG, Medhamurthy R. Supplementary material for "Pregnancy-associated steroid effects on insulin sensitivity, adipogenesis and lipogenesis: Role of Wnt/β-catenin pathway." Uploaded June 14, 2023. doi: 10.6084/m9.figshare.23505909
26. Sack U, Walther W, Scudiero D, *et al.* Novel effect of antihelminthic niclosamide on s100a4-mediated metastatic progression in colon cancer. *J Natl Cancer Inst*. 2011;103(13):1018-1036.
27. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976;72(1-2):248-254.
28. Kruger NJ. The Bradford method for protein quantitation. In: Walker JM, ed. *The Protein Protocols Handbook*. Springer Protocols Handbooks: Humana Press; 2003:15-22.
29. Tschritter O, Fritsche A, Thamer C, *et al.* Plasma adiponectin concentrations predict insulin sensitivity of both glucose and lipid metabolism. *Diabetes*. 2003;52(2):239-243.
30. Rider V, Isuzugawa K, Twarog M, *et al.* Progesterone initiates Wnt-β-catenin signaling but estradiol is required for nuclear activation and synchronous proliferation of rat uterine stromal cells. *J Endocrinol*. 2006;191(3):537-548.
31. Xiong W, Zhang L, Yu L, *et al.* Estradiol promotes cells invasion by activating β-catenin signaling pathway in endometriosis. *Reproduction*. 2015;150(6):507-516.
32. Hou X, Tan Y, Li M, Dey SK, Das SK. Canonical Wnt signaling is critical to estrogen-mediated uterine growth. *Mol Endocrinol*. 2004;18(12):3035-3049.
33. Mohamed OA, Dufort D, Clarke HJ. Expression and estradiol regulation of Wnt genes in the mouse blastocyst identify a candidate pathway for embryo-maternal signaling at implantation. *Biol Reprod*. 2004;71(2):417-424.
34. Gao B, Huang Q, Lin YS, *et al.* Dose-dependent effect of estrogen suppresses the osteo-adipogenic transdifferentiation of osteoblasts via canonical Wnt signaling pathway. *PLoS One*. 2014;9(6):e99137.
35. Varea O, Garrido JJ, Dopazo A, Mendez P, Garcia-Segura LM, Wandosell F. Estradiol activates β-catenin dependent transcription in neurons. *PLoS One*. 2009;4(4):e5153.
36. Rajaram RD, Buric D, Caikovski M, *et al.* Progesterone and Wnt4 control mammary stem cells via myoepithelial crosstalk. *EMBO J*. 2015;34(5):641-652.
37. Fortress AM, Heisler JD, Frick KM. The mTOR and canonical Wnt signaling pathways mediate the mnemonic effects of progesterone in the dorsal hippocampus. *Hippocampus*. 2015;25(5):616-629.
38. González C, Alonso A, Grueso NA, *et al.* Role of 17β-estradiol administration on insulin sensitivity in the rat: implications for the insulin receptor. *Steroids*. 2002;67(13-14):993-1005.
39. Ordóñez P, Moreno M, Alonso A, Llaneza P, Díaz F, González C. 17β-Estradiol and/or progesterone protect from insulin resistance in STZ-induced diabetic rats. *J Steroid Biochem Mol Biol*. 2008;111(3-5):287-294.
40. Archer JS, Coffler M, Yu J, Wachs D, Yoo R, Chang R. Effect of estrogen and progesterone on free fatty acid-induced insulin resistance in postmenopausal women. *Fertil Steril*. 2005;84(1):S117-S118.
41. Yeung EH, Zhang C, Mumford SL, *et al.* Longitudinal study of insulin resistance and sex hormones over the menstrual cycle: the BioCycle study. *J Clin Endocrinol Metab*. 2010;95(12):5435-5442.
42. Mauvais-Jarvis F, Manson JAE, Stevenson JC, Fonseca VA. Menopausal hormone therapy and type 2 diabetes prevention: evidence, mechanisms, and clinical implications. *Endocr Rev*. 2017;38(3):173-188.
43. Ross SE, Hemati N, Longo KA, *et al.* Inhibition of adipogenesis by Wnt signaling. *Science*. 2000;289(5481):950-953.
44. Gyamfi J, Lee YH, Min BS, Choi J. Niclosamide reverses adipocyte induced epithelial-mesenchymal transition in breast cancer cells via suppression of the interleukin-6/STAT3 signalling axis. *Sci Rep*. 2019;9(1):11336.
45. Bagchi DP, Nishii A, Li Z, *et al.* Wnt/β-catenin signaling regulates adipose tissue lipogenesis and adipocyte-specific loss is rigorously defended by neighboring stromal-vascular cells. *Mol Metab*. 2020;42:101078.
46. Guo J, Tao H, Alasadi A, Huang Q, Jin S. Niclosamide piperazine prevents high-fat diet-induced obesity and diabetic symptoms in mice. *Eat Weight Disord*. 2019;24(1):91-96.
47. Tao H, Zhang Y, Zeng X, Shulman GI, Jin S. Niclosamide ethanolamine–induced mild mitochondrial uncoupling improves diabetic symptoms in mice. *Nat Med*. 2014;20(11):1263-1269.
48. Bhagat HA, Compton SA, Musso DL, *et al.* N-substituted phenylbenzamides of the niclosamide chemotype attenuate obesity related changes in high fat diet fed mice. *PLoS One*. 2018;13(10):e0204605.
49. Park JS, Lee YS, Lee DH, Bae SH. Repositioning of niclosamide ethanolamine (NEN), an anthelmintic drug, for the treatment of lipotoxicity. *Free Radic Biol Med*. 2019;137:143-157.
50. Al-Gareeb AI, Aljubory KD, Alkuraishy HM. Niclosamide as an anti-obesity drug: an experimental study. *Eat Weight Disord*. 2017;22(2):339-344.
51. Litwak SA, Wilson JL, Chen W, *et al.* Estradiol prevents fat accumulation and overcomes leptin resistance in female high-fat diet mice. *Endocrinology*. 2014;155(11):4447-4460.
52. Fungfuang W, Terada M, Komatsu N, Moon C, Saito TR. Effects of estrogen on food intake, serum leptin levels and leptin mRNA expression in adipose tissue of female rats. *Lab Anim Res*. 2013;29(3):173.
53. Dissanayake WC, Sorrenson B, Cognard E, Hughes WE, Shepherd PR. β-Catenin is important for the development of an insulin responsive pool of GLUT4 glucose transporters in 3T3-L1 adipocytes. *Exp Cell Res*. 2018;366(1):49-54.
54. Song K, Wang S, Mani M, Mani A. Wnt signaling, de novo lipogenesis, adipogenesis and ectopic fat. *Oncotarget*. 2014;5(22):11000-11003.
55. Prather GR, MacLean JA, Shi M, Boadu DK, Paquet M, Hayashi K. Niclosamide as a potential nonsteroidal therapy for endometriosis that preserves reproductive function in an experimental mouse model. *Biol Reprod*. 2016;95(4):76.
56. Awad OM. Assessment of the developmental toxicity of in utero exposure of Wistar albino rats to ametryne and niclosamide. *Pestic Biochem Physiol*. 1995;53(1):1-9.
57. World Health Organization (WHO). *Data Sheet on Pesticides: Niclosamide*. WHO; 1988.