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Chronic estradiol and progesterone treatment in conscious dogs: effects on insulin sensitivity and response to hypoglycemia

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Abstract

We evaluated the effect of chronic (3 wk) subcutaneous treatment with progesterone and estradiol (PE; producing serum levels observed in the 3rd trimester of pregnancy) or placebo (C) on hepatic and whole body insulin sensitivity and response to hypoglycemia in conscious, overnight-fasted nonpregnant female dogs, using tracer and arteriovenous difference techniques. Insulin was infused peripherally for 3 h at $1.8 \text{ mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$. Glucose was allowed to fall to 3 mM (Hypo) or maintained at 6 mM (Eugly) by peripheral glucose infusion. Insulin concentrations were significantly higher in Eugly-PE ($n = 7$) and Hypo-PE ($n = 7$) than in Eugly-C ($n = 6$) and Hypo-C groups ($n = 7$), but there were no significant differences in hepatic insulin extraction. Concentrations of glucagon, cortisol, epinephrine, and norepinephrine did not differ significantly between Eugly groups or between Hypo groups. Whole body glucose disposal, adjusted for the differences in insulin between groups, was 35% higher in Eugly-C vs. Eugly-PE groups ($P < 0.05$). Eugly-C and Eugly-PE groups exhibited similar rates of net hepatic glucose uptake, but the rate of glucose appearance was greater in Eugly-PE in the last hour ($P < 0.05$). Net hepatic glucose output was greater ($P < 0.05$) in Hypo-PE than in Hypo-C groups, and the glucose infusion rate required to maintain equivalent hypoglycemia was less ($P < 0.05$). The rate of gluconeogenic flux did not differ between Hypo groups. Chronic progesterone and estradiol exposure caused whole body (primarily skeletal muscle) insulin resistance and enhanced the liver's response to hypoglycemia without altering counterregulatory hormone concentrations.

Keywords

hepatic glucose production; hyperinsulinemia; hypoglycemic counter-regulation; insulin clearance

Pregnancy is associated with numerous changes in carbohydrate metabolism. In late pregnancy these changes are characterized by a reduction in insulin sensitivity, elevation of fasting and postprandial insulin concentrations, and enhancement of endogenous glucose production (EGP). In women with normal glucose tolerance before pregnancy, insulin sensitivity is reduced 33–70% during the third trimester compared with the nonpregnant state (8,12,13,58). The wide range of values for the percent reduction can be at least partly explained by the different sources of nonpregnant reference values, e.g., the subjects' own preconception values, their postpartum values, or data from a separate group of nonpregnant women. In longitudinal studies in the same subjects, the decrease from prepregnant values was 56% (13). The reduction

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in insulin sensitivity in normal pregnancy is physiological, designed to ensure that the fetal energy needs are met. However, ~7% of pregnancies are affected by gestational diabetes mellitus (GDM) (2).

Women with GDM have even less whole body insulin sensitivity than nondiabetic pregnant women, coupled with an impairment in suppression of EGP by insulin (11). Because maternal hyperglycemia during pregnancy is associated with a number of adverse outcomes, the current emphasis in diabetes care for women with GDM and those with preexisting diabetes who become pregnant is on maintaining maternal glucose concentrations normal or near normal at all times. In many instances, this necessitates intensive pharmacological therapy. Hypoglycemia is a potential side effect of both insulin and the oral hypoglycemic agents, but the care of pregnant women with diabetes is further complicated by the fact that the counterregulatory response to hypoglycemia is blunted during pregnancy (24,55), predisposing pregnant women to more frequent and severe hypoglycemic episodes than nonpregnant individuals. Glucagon (18,24,56), epinephrine (24,55,56), norepinephrine (18), and growth hormone (55) responses to hypoglycemia are impaired in normal pregnant women and/or animal models.

The placenta produces a number of hormones that have an impact on maternal glucose metabolism. Placental lactogen and placental growth hormone have been implicated in the insulin resistance and impaired glucose tolerance of pregnancy (4–6,35,57). However, hormones and cytokines other than these classic pregnancy hormones may be just as important or even more so in regulation of maternal glucose metabolism. Both tumor necrosis factor- α (TNF- α) and leptin are released by the placenta, and their levels are predictive of insulin sensitivity in pregnancy, with TNF- α being the stronger predictor (38). These placental hormones and cytokines might have an impact on the counterregulatory response to hypoglycemia, but so far there is little evidence for such a role. Infusion of leptin did not alter the catecholamine responses to insulin-induced hypoglycemia in normal nonpregnant rats (32). Individuals with malaria have elevated TNF- α levels, as well as a tendency to develop hypoglycemia, but this does not appear to be related to blunting of the counterregulatory hormone responses (53).

Estrogen and progesterone increase early in pregnancy and remain elevated throughout pregnancy. These hormones are generally recognized to have an impact on glucose homeostasis in nonpregnant women, but it has been difficult to identify specific effects of estrogen and progesterone in pregnancy (40). This is likely because of the impact of simultaneous changes in the many other hormones and cytokines during pregnancy. In nonpregnant women and animal models, estradiol is commonly regarded as improving insulin sensitivity (39,51), but this is not observed in all investigations (62,65). Moreover, combining estradiol and progesterone treatment has been reported by some (51,63), but not all (39), investigators to reverse any estradiol-induced improvement in insulin sensitivity. In regard to the response to hypoglycemia, recent data suggest that oral or subcutaneous doses of estradiol blunt the hypoglycemic counterregulatory response (1,61). Progesterone stimulates both α - and β -cell proliferation and has been suggested to be responsible for increasing insulin secretion in pregnancy (50). However, direct effects of progesterone on glucose metabolism remain undefined. The effects of progesterone treatment on glucose metabolism vary from study to study, possibly because of the use of different forms of progesterone (particularly synthetic vs. natural) and different periods of treatment, as well as differences in the levels of progesterone achieved. Glucose tolerance in women is variously reported to deteriorate during long-term progesterone treatment, to remain unchanged, and, in one report, to improve (reviewed in Ref. 34). On the other hand, treatment of ovariectomized rats with natural progesterone for 5–7 days did not change insulin-mediated glucose intake in peripheral tissue, but it reduced the ability

of insulin to suppress EGP (49). Thus progesterone therapy might be expected to alter the liver's response to insulin-induced hypoglycemia, but this has not been examined.

The divergent data regarding the effects of estradiol and progesterone treatment on glucose metabolism, and the intriguing data indicating that estradiol blunts the counterregulatory response to hypoglycemia, led us to conduct the present study. Our aim was to evaluate the effect of chronic (3 wk) subcutaneous treatment with a combination of natural progesterone and estradiol, to create concentrations similar to those during the third trimester of canine pregnancy, on insulin sensitivity and particularly the response to insulin-induced hypoglycemia. We used young, sexually mature, nonpregnant female dogs as a model of young, reproductive-aged women and examined both the whole body and hepatic responses to hyperinsulinemia during euglycemia and hypoglycemia. The use of nonpregnant animals enabled us to define the effects of the two sex steroids alone, in the absence of other pregnancy-related modifiers of glucose metabolism such as the placenta-derived cytokines and hormones.

METHODS

Animals and surgical procedures

Experiments were performed on 27 overnight (18 h)-fasted, conscious, purpose-bred adult female mongrel dogs ~1 yr of age weighing 22.0 ± 0.3 kg. Diet and criteria for study were as previously described (19). Dogs that had undergone estrus within the previous 12 wk were excluded from study, as were any animals found to have elevated estradiol or progesterone concentrations before treatment and any control animals found to have elevated concentrations at any time. The protocols were approved by the Vanderbilt University Institutional Animal Care and Use Committee, and the dogs were housed in a U.S. Department of Agriculture-approved facility. Twenty-three to 24 days before the experiment, blood sampling catheters were inserted into the hepatic portal vein, left common hepatic vein, and femoral artery, and their ends were placed in subcutaneous pockets as described in detail previously (19). Ultrasonic flow probes (Transonic Systems, Ithaca, NY) were placed around the hepatic artery and hepatic portal vein (26).

The animals were randomly assigned to hormone or placebo treatment, and at the time of surgery they began the appropriate treatment. Progesterone pellets [60-day time release, 700 mg (3 200-mg and 1 100-mg pellets); Innovative Research, Sarasota, FL] and an osmotic pump (Alzet model 2ML4, delivery rate 2.5 μ l/h; Durect, Cupertino, CA) filled with freshly prepared estradiol solution (14.75 mg estradiol/ml saline; Sigma, St. Louis, MO) were implanted subcutaneously on the backs of the progesterone-estradiol-treated (PE) dogs. The control (C) dogs received placebo pellets (inert matrix only; Innovative Research) and an osmotic pump filled with saline. The plasma estrogen and progesterone concentrations were measured before implantation, 7–10 days after the initial implantation, and on the day of study. In pilot studies we determined that progesterone concentrations rose, remained elevated 14–17 days, and then returned to pretreatment levels by the time of study in the PE dogs receiving implants on the day of surgery. Therefore, in the animals included in this report, 500- to 700-mg progesterone (depending on the plasma progesterone concentrations) or placebo pellets, as appropriate, were implanted subcutaneously under local anesthesia 10–12 days after the initial surgery. Estradiol and progesterone in the PE-treated dogs were maintained in the target range, normal values during the third trimester of canine pregnancy [estradiol, 20–30 pg/ml; progesterone, 4–16 ng/ml (16)], and measurements made 7–10 days after implantation did not differ from those on the day of study. Canine pregnancy is 9 wk in duration, and thus the hormone treatment period was approximately equivalent to a trimester of pregnancy. Weight gain between the day of surgery and the day of study did not differ significantly in PE and C dogs (1.1 ± 0.2 and 0.6 ± 0.4 kg, respectively).

Experimental design

On the morning of study, the sampling catheters and Transonic flow probes were exteriorized under local anesthesia, and peripheral venous access was established by insertion of angiocaths (Deseret Medical, Becton Dickinson, Sandy, UT) percutaneously into one saphenous vein and bilaterally into the cephalic veins. Each study was divided into three periods: equilibration (–150 to –30 min), basal (–30 to 0 min), and an experimental period during which hyperinsulinemia was created (0 to 180 min). At –150 min, a primed (3.4 $\mu\text{Ci/kg}$), constant (0.7 $\mu\text{Ci/min}$) infusion of [^3H]glucose (NEN, Boston, MA) was begun via the left cephalic vein to continue throughout the experiment. In addition, indocyanine green dye (0.08 mg/min; Sigma) was infused continuously via the same vein. After basal sampling was complete at 0 min, insulin was infused via the saphenous vein at a rate of $1.8 \text{ mU}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ and glucose either was allowed to fall to $\sim 3 \text{ mM}$ in two groups of dogs (Hypo-C, $n = 7$; Hypo-PE, $n = 7$) or was maintained at euglycemia ($\sim 6 \text{ mM}$) in two groups (Eugly-C, $n = 6$; Eugly-PE, $n = 7$) by glucose infusion into the right cephalic vein. At the end of study, the dog was anesthetized with pentobarbital sodium, samples from three liver lobes were immediately freeze-clamped with Wallenberg tongs prechilled in liquid nitrogen, and the dog was euthanized (19).

Analytical procedures

Parameters measured included hematocrit and plasma glucose, insulin, glucagon, cortisol, estrogen, progesterone, epinephrine, norepinephrine, C-peptide, and nonesterified fatty acids (NEFA), as well as blood concentrations of lactate, glycerol, β -hydroxybutyrate, acetoacetate, alanine, glutamine, glutamate, serine, threonine, and glycine, as previously described (19). Plasma pancreatic polypeptide was analyzed as described by Hagopian et al. (31). Serum estradiol and progesterone concentrations were analyzed by RIA (52) in the Animal Health Diagnostic Laboratory, Cornell University College of Veterinary Medicine (Ithaca, NY). Liver glycogen concentrations were analyzed using the method of Keppler and Decker (36).

Calculations

Total hepatic blood flow was assessed using two methods, hepatic extraction of indocyanine green and ultrasonic probes (see Ref. 19). All calculations reported in RESULTS utilize the ultrasonic flow data because they do not require any assumptions to be made about the distribution of flow. Calculations performed with the indocyanine green and ultrasonic flow data did not differ significantly.

Net hepatic substrate balance was the difference between hepatic load_{out} and load_{in}. Hepatic substrate load_{in} was calculated as $([A] \times AF) + ([P] \times PF)$, where $[A]$ and $[P]$ are arterial and portal vein substrate concentrations, respectively, and AF and PF are arterial and portal vein blood or plasma flow, as appropriate. Hepatic load_{out} was calculated as $[H] \times (AF \times PF)$, where $[H]$ is the hepatic vein substrate concentration. Net hepatic fractional extraction was the net hepatic balance divided by the hepatic load_{in}. The [^3H]hepatic glucose balance was calculated in the same manner as for cold net hepatic glucose balance and divided by the inflowing plasma [^3H]glucose specific activity (dpm/ μmol glucose) to yield the unidirectional hepatic glucose uptake. Unidirectional hepatic glucose release was the difference between net hepatic glucose balance and hepatic glucose uptake. The rates of glucose appearance (R_a) and disappearance (R_d) were calculated with a two-compartment model, using dog parameters (25,45). Endogenous glucose R_a (Endo R_a) was the difference between the total glucose R_a and the glucose infusion rate. Sampling was conducted under steady-state conditions ($<1\%$ change in the glucose infusion rate required to maintain the clamp over the preceding 10 min). During the final hour of study, changes in glucose infusion rates were minimal in all groups; the integrated glucose infusion rate over the 15-min time interval centered at each sampling time was used as the glucose infusion rate at that time point. The insulin sensitivity index during the hyperinsulinemic clamp (S_{ip}) was calculated exactly as described by Saad et al. (59) as the

steady-state ratio of the increment in glucose uptake (ΔR_d) to the increment in plasma insulin concentration (ΔI), normalized to the ambient plasma glucose concentration (G): $S_{ip} = \Delta R_d / (\Delta I \times G)$. Glucagon release was calculated using the formula: $PF \times \{[\text{glucagon}]_{\text{portal}} - (0.85 \times [\text{glucagon}]_{\text{artery}})\}$, because the gut fractional extraction of glucagon is 15% (26). The gluconeogenic rate from circulating precursors was estimated using the arteriovenous difference technique as described in detail previously (19).

Statistical comparisons were made using two-way ANOVA with a repeated-measures design, using Tukey's test for post hoc measurements (SigmaStat; SPSS, Chicago, IL). Unless otherwise indicated, the basal data reported in RESULTS are averages of the values obtained during the 30-min basal sampling period, and the experimental values are means of the values obtained during 120–180 min, when steady-state conditions existed. Area under the curve (AUC) was calculated with the trapezoidal rule. All data are expressed as means $\pm$ SE.

RESULTS

Hormone levels

Progesterone levels in the PE groups increased significantly after hormone implantation (from 1.2 ± 0.2 ng/ml pretreatment to 9.6 ± 1.8 ng/ml on day of study; $P < 0.05$) and were similar to those seen in the third trimester of pregnancy in the dog. In the C groups progesterone remained at pretreatment levels (from 0.8 ± 0.2 ng/ml pretreatment to 0.6 ± 0.1 ng/ml on day of study). Estradiol remained at pretreatment levels in the C groups (16.7 ± 3.1 ng/ml pretreatment and 18.4 ± 2.7 pg/ml posttreatment) and increased modestly but significantly in the PE groups (from 17.8 ± 1.7 ng/ml pretreatment to 31.8 ± 4.8 pg/ml posttreatment; $P < 0.05$), consistent with the small rise in estradiol occurring in the pregnant canine during the third trimester (16). The C and PE dogs were randomly assigned on the day of study to the euglycemic or hypoglycemic treatments, and there were no differences in estradiol or progesterone concentrations between Eugly-C and Hypo-C groups or between Eugly-PE and Hypo-PE groups.

Basal insulin concentrations did not differ among the four groups. In response to peripheral insulin infusion, insulin concentrations rose in all groups, but they plateaued at slightly higher levels in the PE groups (432 ± 21 and 384 ± 19 pmol/l in Eugly-PE and Hypo-PE, respectively) than in the C groups (332 ± 22 and 344 ± 34 pmol/l in Eugly-C and Hypo-C, respectively) (Fig. 1; $P < 0.05$ for Eugly-C vs. Eugly-PE and for Hypo-C vs. Hypo-PE). Hepatic insulin extraction (no units) during the insulin infusion period did not differ significantly between the two euglycemic groups (0.38 ± 0.06 and 0.38 ± 0.04 in Eugly-C and Eugly-PE, respectively) or the hypoglycemic groups (0.55 ± 0.07 and 0.42 ± 0.04 in Hypo-C and Hypo-PE, respectively; $P = 0.10$). Arterial plasma C-peptide concentrations fell similarly during hyperinsulinemia in all groups [from 0.51 ± 0.07 (basal) to 0.28 ± 0.11 ng/ml (final hour) in Eugly-C, 0.42 ± 0.08 to 0.25 ± 0.04 ng/ml in Eugly-PE, 0.50 ± 0.08 to 0.22 ± 0.03 ng/ml in Hypo-C, and 0.40 ± 0.07 to 0.16 ± 0.04 ng/ml in Hypo-PE].

Arterial plasma glucagon levels fell gradually in the Eugly-C and Eugly-PE groups (25 and 40% from basal, respectively; Fig. 1) and did not differ between groups at any time. Arterial plasma glucagon levels rose in the Hypo-C and Hypo-PE groups to peaks of 65 ± 7 and 73 ± 14 ng/l, respectively, at 60 min (significantly greater than basal in both groups) and then declined to levels not different from basal by the end of study (Fig. 1). Glucagon release did not differ significantly between the hypoglycemic groups at any time. Basal rates were 200 ± 32 and 209 ± 52 pg·kg⁻¹·min⁻¹ in Hypo-C and Hypo-PE, respectively. Peak rates of glucagon release (664 ± 164 and 853 ± 222 pg·kg⁻¹·min⁻¹, respectively) occurred in both groups at 90 min. By the end of study, glucagon release had returned to rates not different from basal in both groups (251 ± 83 and 246 ± 73 pg·kg⁻¹·min⁻¹, respectively).

There were no differences between the Eugly-C and Eugly-PE groups or between the Hypo-C and Hypo-PE groups in arterial plasma cortisol, norepinephrine, and epinephrine levels (Fig. 2). The arterial plasma cortisol, norepinephrine, and epinephrine levels rose significantly in the Hypo-C and Hypo-PE groups but did not change significantly in the Eugly-C and Eugly-PE groups.

Arterial plasma pancreatic polypeptide, an indicator of parasympathetic activity, tended to decline ($P = 0.10$ for change from basal within each group) during euglycemic hyperinsulinemia [from 216 ± 61 to 126 ± 23 pg/ml (Eugly-C) and 204 ± 45 to 146 ± 27 pg/ml (Eugly-PE)]. It increased significantly ($P < 0.05$) in both hypoglycemic groups [from 360 ± 122 to 770 ± 181 pg/ml (Hypo-C) and 200 ± 42 to $1,038 \pm 218$ pg/ml (Hypo-PE)], and the concentrations during the last hour were not different between groups.

Hepatic blood flow and glucose metabolism

Hepatic arterial blood flow increased significantly during hyperinsulinemia in all groups except Eugly-PE [from 7.4 ± 1.2 to 8.7 ± 0.8 ml·kg⁻¹·min⁻¹ (Eugly-C), 7.2 ± 1.3 to 8.1 ± 1.2 ml·kg⁻¹·min⁻¹ (Eugly-PE), 6.3 ± 0.5 to 8.4 ± 0.8 ml·kg⁻¹·min⁻¹ (Hypo-C), and 6.9 ± 0.4 to 9.1 ± 0.5 ml·kg⁻¹·min⁻¹ (Hypo-PE)]. On the other hand, portal vein flow increased significantly only in Hypo-PE [from 20.8 ± 2.3 to 22.0 ± 2.8 ml·kg⁻¹·min⁻¹ (Eugly-C), 23.2 ± 1.6 to 22.6 ± 1.3 ml·kg⁻¹·min⁻¹ (Eugly-PE), 24.1 ± 3.2 to 26.2 ± 2.9 ml·kg⁻¹·min⁻¹ (Hypo-C), and 21.2 ± 2.1 to 26.5 ± 2.9 ml·kg⁻¹·min⁻¹ (Hypo-PE)]. There were no significant differences among groups for either parameter.

The arterial glucose concentrations did not differ between the Eugly-C and Eugly-PE groups at any time (Fig. 1), with the mean value in the final hour being 6.2 ± 0.2 in the two groups. In the Hypo-C and Hypo-PE groups, mean plasma glucose concentrations were 6.0 ± 0.2 and 6.2 ± 0.2 mM in the basal period; in both groups, the concentrations declined to 3.0 ± 0.1 mM during 30–180 min, with no difference in the rate of fall between groups (Fig. 1). Basal rates of net hepatic glucose output (NHGO) were similar in all groups. In response to insulin, NHGO was completely suppressed by the last hour of study in the Eugly-C and Eugly-PE groups, and they exhibited net hepatic glucose uptake [-5.6 ± 1.6 and -6.7 ± 2.0 μmol·kg⁻¹·min⁻¹ respectively; $P =$ not significant (NS); Fig. 3]. NHGO values in the Hypo-C and Hypo-PE groups were 6.3 ± 1.6 and 14.5 ± 2.1 μmol·kg⁻¹·min⁻¹, respectively, during the same time period ($P < 0.05$; Fig. 3).

Exogenous glucose was required to clamp the glucose concentrations in all groups during insulin infusion. The mean rates of exogenous glucose infusion during 30–180 min did not differ significantly in the Eugly-C and Eugly-PE groups (66.6 ± 2.3 and 59.2 ± 5.8 μmol·kg⁻¹·min⁻¹, respectively; $P = 0.24$). However, the glucose infusion rate in the Hypo-C group was significantly greater than that required to maintain a comparable level of hypoglycemia in the Hypo-PE group (7.1 ± 1.6 vs. 3.6 ± 1.4 μmol·kg⁻¹·min⁻¹, respectively; $P < 0.05$; Fig. 3).

EndoR_a was significantly greater in the Eugly-PE group than in the Eugly-C group during the last hour of study, but there were no differences in EndoR_a between the two Hypo groups (Fig. 4). Unidirectional hepatic glucose production (HGP) was nil during the final hour of study in the Eugly groups, and low rates of unidirectional hepatic glucose uptake were exhibited ($P =$ NS between groups). The Hypo-C group exhibited a lower rate of HGP than the Hypo-PE group (8.0 ± 1.5 vs. 15.0 ± 2.4 μmol·kg⁻¹·min⁻¹, respectively; $P < 0.05$), in keeping with the greater rate of glucose infusion required in the Hypo-C group. There were no significant differences in R_d between the two Eugly groups or between the two Hypo groups (Fig. 4). However, because of the prevailing differences in insulin concentrations, we calculated S_{ip}, an index of peripheral insulin sensitivity that accounts for differences in plasma insulin

concentrations (7,59). S_{ip} was ~35% greater in the Eugly-C than in the Eugly-PE group, and it was greater in the Hypo-C than in the Hypo-PE group ($P < 0.05$ for both comparisons; Fig. 4).

Lactate and gluconeogenic amino acid metabolism

Neither arterial concentrations nor net hepatic balances of lactate, alanine, serine, threonine, glycine, glutamine, or glutamate changed significantly throughout the studies in the Eugly-C or Eugly-PE groups, and the data did not differ between these groups at any time (data not shown). Neither arterial concentrations of lactate nor net hepatic lactate balance differed between the Hypo groups at any time. Both Hypo groups exhibited an increase in net hepatic lactate uptake during hyperinsulinemia, but only in the Hypo-PE group did the change from basal reach statistical significance (Table 1).

There were no differences in arterial levels of alanine in the basal and experimental periods between the two Hypo groups, and the concentrations declined significantly during hyperinsulinemia in both groups (Table 1). Net hepatic alanine uptake remained statistically unchanged from basal in the Hypo-C group but rose significantly in the Hypo-PE group. Although the rates did not differ between groups in either period, the change from basal in the Hypo-PE group was greater than in the Hypo-C group ($P < 0.05$). Neither the arterial blood concentrations nor the net hepatic balances of serine, glycine, threonine, and glutamate changed significantly from basal in either Hypo group (data not shown). Glutamine concentrations declined ($P < 0.05$) in both groups during hyperinsulinemic hypoglycemia and did not differ between groups. Net hepatic glutamine output fell significantly from basal in the Hypo-C but not in the Hypo-PE group ($P < 0.05$ between groups).

Glycerol, NEFA, and ketone metabolism

Arterial blood glycerol concentrations fell ~50% in response to hyperinsulinemia in both Eugly groups, and net hepatic glycerol uptake declined by 30–45%, with no significant difference in rates between the groups at any time (Table 2). Arterial glycerol concentrations increased approximately twofold in both Hypo groups, and net hepatic glycerol uptake increased twofold or more ($P = \text{NS}$ between Hypo groups at any time for either parameter).

The plasma arterial levels of NEFA declined significantly in all four groups (Table 2). The mean decline was much greater in the Eugly groups (~90% of basal) than in the Hypo groups (~33% of basal). Net hepatic NEFA uptake was suppressed by hyperinsulinemia to ~15% of basal in the Eugly groups. Net hepatic NEFA uptake tended ($P = 0.09$) to decrease in the Hypo-C group but remained at basal level in the Hypo-PE group ($P < 0.05$ between Hypo groups).

Arterial blood β -hydroxybutyrate concentrations declined significantly during hyperinsulinemia in all four groups, but the magnitude of the decrease was greater in the Eugly groups ($\geq 60\%$) than in the Hypo groups (~30%) (Table 2). Net hepatic β -hydroxybutyrate output declined ~90% from basal ($P < 0.05$) in the Eugly groups but did not change significantly in the Hypo groups. Neither the β -hydroxybutyrate concentrations nor net hepatic β -hydroxybutyrate output differed at any time between Eugly-C and Eugly-PE groups or between Hypo-C and Hypo-PE groups. Acetoacetate concentrations did not change from basal in any of the four groups. Although net hepatic acetoacetate output tended to decline (40–50% in all groups) during hyperinsulinemia, this was significant only in the Hypo-C group.

Gluconeogenesis and glycogenolysis

The rate of gluconeogenic flux remained unchanged from basal in the Eugly-C and Eugly-PE groups, and there was no net contribution of gluconeogenesis to NHGO (see METHODS) in the euglycemic groups during hyperinsulinemia, because the gluconeogenic flux was directed

into glycogen synthesis (as evidenced by negative rates of net hepatic glycogenolysis). The rate of gluconeogenic flux remained basal throughout the studies in the Hypo-C group, whereas it was significantly higher than basal during the last hour of study in the Hypo-PE group (Table 3). In the Hypo-C group, the net contribution of gluconeogenesis to NHGO did not change from basal during hyperinsulinemia, but it tended to increase (~2-fold) in Hypo-PE ($P = \text{NS}$ between groups).

There was no significant change from basal in the rate of net hepatic glycogenolysis in either of the Hypo groups, although the rate tended to increase (2-fold) in Hypo-C and to fall (40%) in Hypo-PE. There were no significant differences between Hypo groups during the hyperinsulinemic period. There were no differences in hepatic glycogen content between the two Eugly groups or between the two Hypo groups in the biopsies taken at the end of study (data not shown).

DISCUSSION

The 3-wk treatment created progesterone and estradiol concentrations similar to those normal during the last 3 wk of gestation in the dog. Neither the glucose infusion rate required to maintain euglycemia nor the glucose R_d was significantly reduced in the Eugly-PE vs. the Eugly-C group. However, during the insulin infusion, insulin concentrations in the Eugly-PE group were nearly 30% higher than in the Eugly-C group, obscuring a difference in insulin sensitivity between groups. Thus the S_{ip} index (increment in glucose disposal during hyperinsulinemic euglycemia adjusted for the increment in insulin concentration) was reduced ~35% in the Eugly-PE group ($P < 0.05$ vs. Eugly-C). Interestingly, the magnitude of loss of whole body insulin sensitivity was within the range of the reduction in insulin sensitivity (33–70%) reported for normal women in late pregnancy (8,12,13,58) and was similar to the 24–35% reduction in insulin sensitivity we have previously observed in the pregnant (3rd trimester) dog (17). This does not imply that estradiol and progesterone changes are the primary factors altering insulin sensitivity during pregnancy but, instead, highlights the importance of the entire hormonal milieu of pregnancy in the regulation of carbohydrate metabolism.

The S_{ip} index primarily reflects insulin resistance in the skeletal muscle (7,59). In keeping with this, the liver responded similarly in the two Eugly groups, with a suppression of net hepatic glucose output and a shift to net hepatic glucose uptake during hyperinsulinemia. Similarly, tracer-determined hepatic glucose release did not differ between groups. The dosage of insulin used was not optimal for eliciting subtle differences in hepatic insulin responsiveness (15), but the data nevertheless indicate that the peripheral tissues (primarily skeletal muscle) are responsible for most of the loss of insulin sensitivity. Maintenance of hepatic insulin sensitivity during PE treatment is consistent with findings in nondiabetic normal-weight pregnant women, who exhibit insulin resistance in skeletal muscle but virtually complete suppression of glucose production by insulin (13). Nevertheless, EndoR_a during the last hour was significantly higher in the Eugly-PE group than in the Eugly-C group. This finding suggests that there was either sufficient variability in our measurements that we erroneously identified a difference between groups in R_a , which seems unlikely under these steady-state conditions, or that the kidney in the Eugly-PE group was resistant to the effects of insulin.

C-peptide concentrations among the groups in the current study were not different during the basal period, and they were similarly suppressed by hyperinsulinemia, indicating that the higher circulating insulin concentrations in the PE group during insulin infusion were due to reduced clearance, rather than enhanced secretion. Estrogen therapy in animal models and postmenopausal women increases insulin clearance (9,27,66), whereas use of progesterone concomitantly with estrogen has been reported to reduce insulin clearance relative to estrogen alone (41) or to have no effect on insulin concentrations (27). Thus the progesterone therapy

appears to be the most likely explanation for the discrepancy in insulin concentrations between the C and PE groups in the current studies. Hepatic insulin extraction did not differ significantly between the PE groups and their respective control groups, however, suggesting that another organ, e.g., the kidney (60) or adipose tissue (3), exhibited reduced clearance during PE treatment.

There were no significant differences in the epinephrine, norepinephrine, cortisol, or glucagon concentrations between the Hypo-C and Hypo-PE groups. In contrast, the glucagon response to hypoglycemia is severely blunted in late pregnancy in the dog (18), rat (56), and human (55), and the epinephrine response to hypoglycemia also is impaired in these species (10,55, 56). The islets of Langerhans contain estrogen receptors, and in vitro evidence from isolated mice islets indicates that physiological levels of estradiol (1 nM) suppress glucagon secretion (48,54). Consistent with this, premenopausal women display a blunted glucagon response (compared with men) to insulin-induced hypoglycemia (21,29,30). Also, postmenopausal women receiving estradiol have significantly smaller glucagon responses to insulin-induced hypoglycemia than those not receiving estradiol (61). Nevertheless, estradiol alone cannot account for the blunted glucagon response, because non-estradiol-treated postmenopausal women have a significantly lower response than men of a comparable age, who have similar serum estradiol concentrations (61).

As is true with glucagon, both in vitro and in vivo evidence indicates that estrogen can suppress catecholamine release. Short-term estrogen exposure of the perfused adrenal gland (44) or a medullary chromaffin cell line (37) suppresses catecholamine secretion. Moreover, both chronic and acute treatments of ovariectomized rats with physiological doses of estradiol are associated with an impaired epinephrine response to hypoglycemia (33). Similarly, estradiol-treated postmenopausal women have a blunted epinephrine response to insulin-induced hypoglycemia compared with nonestradiol-treated women or similarly aged men (61). Both young men and postmenopausal women treated with transdermal estradiol exhibit a blunted epinephrine response to laboratory-induced behavioral stress (22,42). Nevertheless, catecholamine responses to insulin-induced hypoglycemia are not affected by phase of menstrual cycle in young women (23,43), and the Hypo-C and Hypo-PE groups did not differ in their catecholamine release. The relatively short-term changes in estradiol during the menstrual cycle and the modest rise in estradiol with PE treatment likely explain the lack of impact on catecholamine levels under these conditions.

The primary difference observed in the response of the livers of the PE and C dogs occurred during hypoglycemia. The Hypo-PE dogs maintained a significantly higher rate of NHGO and consequently required a lower glucose infusion rate. The higher rate of NHGO in the Hypo-PE than in the Hypo-C group, as well as the higher rate of EndoR_a in the Eugly-PE than in the Eugly-C group, is consistent with findings of impaired hepatic insulin sensitivity in obese women during late pregnancy (11,64). Our findings are not consistent with those in lean women, in whom hepatic insulin sensitivity was maintained throughout pregnancy (14). This discrepancy in the response may be caused by differences in study design, e.g., different dosages of insulin. In the obese women, the defect in suppression of EGP was not apparent with an insulin infusion rate of 20 mU·m⁻²·min⁻¹, but it became evident when the infusion rate was doubled (11). Exactly how the PE treatment might have brought about changes in glucose production is not clear. Chronic treatment (1 mo) of mature and old rats with both estradiol and progesterone (but not with either hormone alone) suppressed glucose 6-phosphatase activity in the liver and kidney in the basal state (47), which would be expected to decrease HGP. However, no metabolic studies were performed on those animals. A progesterone receptor, different from the classic steroid receptor, has been identified in porcine hepatocytes and found to be localized to the endoplasmic reticulum, along with glucose 6-phosphatase (28), affording an avenue for progesterone to act directly on hepatic glucose

metabolism. Oophorectomized rats treated for 5–7 days with subcutaneous natural progesterone exhibited a 27% elevation of basal HGP compared with the rate in oophorectomized control rats (49). During a low-dose hyperinsulinemic euglycemic clamp (circulating insulin ~2.5 times basal), the residual HGP in the progesterone-treated rats was ~2.5 times greater than in the control animals, but in both groups HGP was virtually completely suppressed during high-dose insulin infusion (~20 times basal) (49). In contrast, premenopausal women taking oral doses of natural progesterone for 11–14 days exhibited no difference in HGP in the basal state or during a two-step hyperinsulinemic euglycemic clamp in studies carried out before and after treatment (65). HGP was similarly unaffected in women who took combined oral dosages of natural estradiol and progesterone (65). The route of progesterone administration (subcutaneous vs. oral), the progesterone levels achieved [>15 ng/ml in the women vs. 95 ng/ml (equivalent to levels in the last trimester of pregnancy) in rats], or the duration of use may explain the differences in the human and rat findings. However, few data are available regarding the effect of progesterone on hypoglycemic counterregulation, other than the observation that there seems to be little effect of menstrual cycle phase on the response to an insulin tolerance test (43). Despite the lack of effect of PE treatment on counterregulatory hormone release in response to hypoglycemia, we cannot rule out the possibility that PE treatment may have increased NHGO by amplifying the action of or increasing the sensitivity to one or more of the counterregulatory hormones.

There was little evidence for an effect of the PE treatment on gluconeogenic and glycogenolytic flux. Although there was a trend toward a greater stimulation of the gluconeogenic contribution to NHGO and a concomitant tendency toward suppression of glycogenolysis in the Hypo-PE compared with the Hypo-C group, these findings did not reach statistical significance. Moreover, they appeared to be related to basal (rather than hypoglycemia induced) differences in flux rates, with the Hypo-C group exhibiting a lower gluconeogenic and higher glycogenolytic contribution to NHGO in the basal state. Treatment of rats with either estradiol or estradiol plus progesterone results in a decrease in the rate of gluconeogenesis from alanine (46). Treatment with progesterone alone, on the other hand, results in a stimulation of phosphoenolpyruvate carboxykinase activity and glycogenesis (20), suggesting that the progesterone effect may have dominated that of estradiol in the Hypo-PE group.

Thus concomitant increases in estradiol and progesterone to levels observed during the last trimester of pregnancy alter both insulin clearance and glucose disposal. Moreover, there are intriguing effects of sex hormone treatment on glucose production that remain to be explored.

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Fig. 1.

Arterial plasma insulin, glucagon, and glucose concentrations. Insulin was infused continuously via peripheral vein from 0 to 180 min, and glucose was infused peripherally as needed to maintain euglycemia (Eugly; *left*) or match the rate of fall in plasma glucose in the 2 hypoglycemic groups (Hypo) and maintain glycemia at ~3 mM (*right*). C, control; PE, progesterone and estradiol treatment. * $P < 0.05$ vs. the corresponding C group.

Fig. 2. Arterial plasma epinephrine, norepinephrine, and cortisol concentrations in the Eugly and Hypo groups. Study conditions were as described in Fig. 1. There were no significant differences between Eugly-C and Eugly-PE groups or between Hypo-C and Hypo-PE groups.

Fig. 3. Net hepatic glucose output and glucose infusion rate required to maintain the clamp in the Eugly (*left*) and Hypo groups (*right*). Note that scale is different for glucose infusion rate in the Eugly and Hypo groups. Study conditions were as described in Fig. 1. * $P < 0.05$, Hypo-C vs. Hypo-PE.

Fig. 4. Endogenous rate of glucose appearance (R_a), rate of glucose disappearance (R_d), and change in glucose clearance per unit change in plasma insulin concentration (S_{ip}) in the Eugly (*left*) and Hypo groups (*right*). Study conditions were as described in Fig. 1. * $P < 0.05$ between groups.

Table 1
Lactate and amino acid data for Hypo groups

Parameter and Group	Basal Period	Hyperinsulinemic Period (120–180 min)
Lactate		
Arterial blood concentration, $\mu\text{mol/l}$		
Hypo-C	490 $\pm$ 47	842 $\pm$ 294
Hypo-PE	526 $\pm$ 81	881 $\pm$ 207
Net hepatic output, $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$		
Hypo-C	-3.4 $\pm$ 1.2	-5.7 $\pm$ 1.4
Hypo-PE	3.3 $\pm$ 3.7	-6.4 $\pm$ 2.9*
Alanine		
Arterial blood concentration, $\mu\text{mol/l}$		
Hypo-C	312 $\pm$ 42	195 $\pm$ 42*
Hypo-PE	332 $\pm$ 37	242 $\pm$ 21*
Net hepatic uptake, $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$		
Hypo-C	2.5 $\pm$ 0.5	3.6 $\pm$ 1.2
Hypo-PE	1.8 $\pm$ 0.2	3.5 $\pm$ 0.5*
Glutamine		
Arterial blood concentration, $\mu\text{mol/l}$		
Hypo-C	808 $\pm$ 33	625 $\pm$ 37*
Hypo-PE	938 $\pm$ 51	614 $\pm$ 37*
Net hepatic output, $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$		
Hypo-C	1.1 $\pm$ 0.3	-0.3 $\pm$ 0.6*
Hypo-PE	0.8 $\pm$ 0.4	1.6 $\pm$ 0.6 [†]

Data are means $\pm$ SE. Basal values are the mean of 2–3 samples during -30 to 0 min. Negative values indicate net uptake. Hypo, hypoglycemic dogs treated with progesterone and estradiol (PE) or control (C).

* $P < 0.05$ vs. basal values in the same group.

[†] $P < 0.05$ vs. Hypo-C.

Table 2
Glycerol, NEFA, and ketone data

Parameter and Group	Basal Period	Hyperinsulinemic Period (120–180 min)
Glycerol		
Arterial blood concentration, $\mu\text{mol/l}$		
Eugly-C	66 $\pm$ 10	33 $\pm$ 5 [*]
Eugly-PE	60 $\pm$ 7	24 $\pm$ 5 [*]
Hypo-C	86 $\pm$ 9	158 $\pm$ 23 [*]
Hypo-PE	69 $\pm$ 10	152 $\pm$ 16 [*]
Net hepatic uptake, $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$		
Eugly-C	1.3 $\pm$ 0.3	0.8 $\pm$ 0.2 [*]
Eugly-PE	1.1 $\pm$ 0.2	0.5 $\pm$ 0.1 [*]
Hypo-C	1.7 $\pm$ 0.3	3.4 $\pm$ 0.7 [*]
Hypo-PE	1.5 $\pm$ 0.2	3.6 $\pm$ 0.3 [*]
NEFA		
Arterial plasma concentration, $\mu\text{mol/l}$		
Eugly-C	858 $\pm$ 66	58 $\pm$ 14 [*]
Eugly-PE	765 $\pm$ 149	101 $\pm$ 22 [*]
Hypo-C	1,010 $\pm$ 109	690 $\pm$ 135 [*]
Hypo-PE	891 $\pm$ 133	594 $\pm$ 121 [*]
Net hepatic uptake, $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$		
Eugly-C	2.2 $\pm$ 0.2	0.3 $\pm$ 0.1 [*]
Eugly-PE	1.6 $\pm$ 0.2	0.2 $\pm$ 0.1 [*]
Hypo-C	2.0 $\pm$ 0.6	1.6 $\pm$ 0.3 [*]
Hypo-PE	3.0 $\pm$ 0.5	3.0 $\pm$ 0.6 [†]
β -Hydroxybutyrate		
Arterial blood concentration, $\mu\text{mol/l}$		
Eugly-C	38 $\pm$ 12	13 $\pm$ 2 [*]
Eugly-PE	25 $\pm$ 6	9 $\pm$ 1 [*]
Hypo-C	40 $\pm$ 7	25 $\pm$ 5 [*]
Hypo-PE	27 $\pm$ 5	17 $\pm$ 2 [*]
Net hepatic output, $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$		
Eugly-C	1.0 $\pm$ 0.5	0.1 $\pm$ 0.0 [*]
Eugly-PE	0.7 $\pm$ 0.2	0.1 $\pm$ 0.0 [*]
Hypo-C	0.8 $\pm$ 0.2	0.5 $\pm$ 0.2
Hypo-PE	1.1 $\pm$ 0.4	0.4 $\pm$ 0.1
Acetoacetate		
Arterial blood concentration, $\mu\text{mol/l}$		
Eugly-C	71 $\pm$ 19	80 $\pm$ 18
Eugly-PE	62 $\pm$ 7	85 $\pm$ 14
Hypo-C	99 $\pm$ 14	116 $\pm$ 18
Hypo-PE	68 $\pm$ 10	98 $\pm$ 24
Net hepatic output, $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$		
Eugly-C	0.7 $\pm$ 0.5	0.0 $\pm$ 0.3
Eugly-PE	0.8 $\pm$ 0.4	0.5 $\pm$ 0.5
Hypo-C	1.1 $\pm$ 0.3	0.4 $\pm$ 0.2 [*]
Hypo-PE	0.7 $\pm$ 0.3	0.7 $\pm$ 0.3

Data are means $\pm$ SE. Basal values are the mean of 2–3 samples during –30 to 0 min. Eugly, euglycemic dogs; NEFA, nonesterified fatty acids.

^{*} $P < 0.05$ vs. basal value in the same group.

[†] $P < 0.05$ vs. Hypo-C.

Table 3
Gluconeogenic and glycogenolytic indexes in Hypo groups

Parameter and Group	Basal Period	Hyperinsulinemic Period (120–180 min)
Gluconeogenic flux, $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$		
Hypo-C	6.1 $\pm$ 0.8	7.8 $\pm$ 1.5
Hypo-PE	4.7 $\pm$ 0.5	9.7 $\pm$ 1.7 [*]
Gluconeogenic contribution to NHGO, $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$		
Hypo-C	5.0 $\pm$ 0.7	4.4 $\pm$ 2.9
Hypo-PE	3.7 $\pm$ 0.6	8.9 $\pm$ 1.6
Glycogenolytic contribution to NHGO, $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$		
Hypo-C	2.2 $\pm$ 1.0	4.6 $\pm$ 3.1
Hypo-PE	7.8 $\pm$ 1.6 [†]	4.5 $\pm$ 2.0

Data are means $\pm$ SE. NHGO, net hepatic glucose output.

^{*} $P < 0.05$ vs. basal in the same group.

[†] $P < 0.05$ vs. Hypo-C.